0022-3565/07/3213-1046–1053$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 321, No. 3
115204/3204835
Printed in U.S.A.

JPET 321:1046–1053, 2007

Roles of 5-Hydroxytryptamine (5-HT) Receptor Subtypes in the Inhibitory Effects of 5-HT on C-Fiber Responses of Spinal Wide Dynamic Range Neurons in Rats

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Received October 18, 2006; accepted February 27, 2007

ABSTRACT

5-Hydroxytryptamine (5-HT; serotonin) plays an important role in the descending control of nociception. 5-HT and its receptors have been extensively studied in the modulation of nociceptive transmission at the spinal level using behavioral tests that may be affected by the effects of 5-HT on motor performance and skin temperature. Using electrophysiological methods, the present study aimed to systematically investigate the roles of 5-HT receptor subtypes on the inhibitory effects of 5-HT on responses of the spinal wide dynamic range (WDR) neurons to C-fiber inputs in rats. Under basal conditions, topical application of 5-HT to the spinal cord inhibited the C-fiber responses of WDR neurons dose-dependently, whereas antagonists of 5-HT_{1A} [WAY 100635 [*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexanecarboxamide maleate salt]], 5-HT_{1B} [GR 55562 [3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyrid-dinyl)phenyl]benzamide dihydrochloride]], 5-HT_{2A} [ketanserin [3-[2-[4-(fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1*H*,3*H*]-quinazolinedione tartrate]], 5-HT_{2C} [RS 102221 [8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulfonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride]], 5-HT_3 [MDL 72222 [3-tropanyl-3,5-dichlorobenzoate]], and 5-HT_4 [GR 113808 ([1-[2-[(methylsulfonyl)-amino]ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole-3-carboxylate)] had no effect on their own. The inhibitory effects of 5-HT were reversed by antagonists of 5-HT_{1B} (GR 55562), 5-HT_{2A} (ketanserin), 5-HT_{2C} (RS 102221), 5-HT_3 (MDL 72222), and 5-HT_4 (GR 113808) but not by 5-HT_{1A} (WAY 100635) receptor antagonists. Topical administration of agonists of 5-HT_{1A} [(2*R*)-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide], 5-HT_{1B} [CGS 12066 [7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo-[1,2-*a*]quinoxaline maleate salt]], 5-HT_{2A} (α-methyl-5-hydroxytryptamine maleate), 5-HT_{2C} [MK 212 [6-chloro-2-(1-piperazinyl)pyrazine hydrochloride]], 5-HT_3 [1-(3-chlorophenyl)biguanide hydrochloride], and 5-HT_4 [2-[1-(4-piperonyl)piperazinyl]benzothiazole] also inhibited the C-responses. These results suggest that, under basal conditions, there is no tonic serotonergic inhibition on the C-responses of dorsal horn neurons, and multiple 5-HT receptor subtypes including 1B, 2A, 2C, 3, and 4 may be involved in mediating the inhibitory effects of 5-HT.

The dorsal horn of the spinal cord is critical for nociceptive transmission. Nociceptive information impinging upon the dorsal horn from the skin, viscera, and other tissues is subjected to segmental, extrasegmental, and descending inhibitory controls (Melzack and Wall, 1965). It has been established that the descending control system from the brain exerts an inhibitory influence upon the spinal processing of nociceptive information. 5-Hydroxytryptamine (5-HT; serotonin) is a major neurotransmitter in the descending control system. In the spinal cord, at least four subtypes of 5-HT receptors (5-HT_1-5-HT_4) have been identified, which are involved in spinal pain modulation. However, the roles of some of these receptor subtypes are not well defined, and previous

This work was supported by the National Natural Science Foundation of China (Grants 30370470, 30470559, 30570566, 30330230, and 30600173) and by the Beijing Natural Science Foundation (Grant 7052039).
F.-Y.L. and G.-G.X. contributed equally to this work.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.106.115204.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); 8-OH-DPAT, (2*R*)-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide; WDR, wide dynamic range; WAY 100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexanecarboxamide maleate salt; GR 55562, 3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyrid-dinyl)phenyl]benzamide dihydrochloride; ketanserin, 3-[2-[4-(fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1*H*,3*H*]-quinazolinedione tartrate; RS 102221, 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulfonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride; MDL 72222, 3-tropanyl-3,5-dichlorobenzoate; GR 113808, [1-[2-[(methylsulfonyl)-amino]-ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole-3-carboxylate; CGS 12066, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo-[1,2-*a*]quinoxaline maleate salt; α-m-5-HT, α-methyl-5-hydroxytryptamine maleate; MK 212, 6-chloro-2-(1-piperazinyl)pyrazine hydrochloride; mCPBG, 1-(3-chlorophenyl)biguanide hydrochloride; BZTZ, 2-[1-(4-piperonyl)piperazinyl]benzothiazole; DMSO, dimethyl sulfoxide; NS, normal saline; ANOVA, analysis of variance.

experimental investigations have often had contradictory results. Thus, further studies are needed to clarify the role of 5-HT receptor subtypes in spinal cord in pain modulation, both under normal physiological conditions and after 5-HT activation (Millan, 2002).

The uncertainty about the roles played by 5-HT receptors in spinal nociception may result to some extent from the complexity of the serotonergic system itself. Furthermore, the appropriateness of experimental methodology that has been used may need to be reevaluated. When addressing the roles of spinal 5-HT in nociception, most studies have used behavioral tests with noxious heat, mechanical, or chemical stimulation. However, these results could be misinterpreted for several reasons (Bardin et al., 2000). First, the vascular effects of 5-HT influence skin temperature, which in turn affects the measurement of the heat response latency. For example, it has been shown that the effects of i.t. applied serotonergic agents or manipulation of the descending serotonergic pathway on the tail-flick test may be explained by changes in the tail temperature (Minfeng and Jisheng, 1979; Eide and Tjolsen, 1988; Eide and Rosland, 1989; Han and Ren, 1991). Second, spinal 5-HT is also known to be involved in the control of movement. Changes in the 5-HT system may affect behavioral performance of animals and thus interfere with many commonly used behavioral nociceptive tests using mechanical (paw pressure), thermal (tail immersion, tail-flick, and hot-plate) or chemical (formalin) stimulation. For example, i.t. administration of the 5-HT_{1A} agonist (2*R*)-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT) can elicit spontaneous tail-flicking in rats (Millan et al., 1991).

Projection neurons in the spinal cord can be classified into three groups based on their responses to afferent inputs. Nociceptive-specific neurons are activated exclusively by noxious stimuli mediated by A_δ- and C-fibers. Non-nociceptive neurons are driven most effectively by the innocuous mechanical stimuli mediated primarily by A_β- and A_δ-fibers. Wide dynamic range (WDR) neurons respond to both noxious and innocuous stimuli of different modalities. With converging noxious and innocuous inputs, WDR neurons have a fundamental role in the segmental suppression of pain according to "gate control theory." In contrast to the nociceptive-specific neurons, WDR neurons are more accurate in encoding stimulus intensity and in signaling the spatial and qualitative aspects of nociception (Almeida et al., 2004).

In the present study, instead of behavioral tests with noxious stimulation, electrophysiological recordings of the responses of WDR neurons to electrical stimulation were used to systematically evaluate the roles of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, and 5-HT_4 receptors in spinal nociceptive modulation both under basal conditions and with 5-HT administration in rats.

Materials and Methods

Animals and Surgery. Male Sprague-Dawley rats weighing 250 to 320 g were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. The treatment of the animals was in compliance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983), and all experimental protocols were approved by the University Research Ethics Committee. Rats were initially anesthetized by i.p. injection of urethane (1.2~1.5 g/kg). After cannulation of the trachea and the left jugular vein, the rat was positioned in an SN-3 stereotaxic frame (Narishige, Tokyo, Japan), and the lumbar enlargement of the spinal cord was exposed by a laminectomy at vertebrae T_{13} and L_1. The vertebral column was tightly fixed in the frame with clamps. A small well was built with 3% agar on the dorsal spinal cord at the recording segment to allow application of drugs or vehicles (Kelly and Chapman, 2002). A bipolar silver hook electrode was placed under the sciatic nerve immediately proximal to the trifurcation. During recording, the animals were paralyzed with i.v. injection of curare (2.0 mg/kg) and artificially ventilated. During the experiment, continuous anesthesia and paralysis were maintained with urethane (0.10~0.17 g/kg/h) and curare (0.21 mg/kg/h). The depth of anesthesia was monitored by examination of pupillary size and reflexes, heart rate, and stability of expired CO_2 concentration. The animal was allowed to recover from paralysis transiently to judge the depth of anesthesia before a supplemental anesthetic dose was given. The physiological condition of the animal was monitored by recording the electrocardiogram, end-expiratory CO_2, and rectal temperature. These physiological parameters were maintained within 330 to 460 beats/min, 3.5 to 4.5%, and 36.5 to 37.5°C, respectively (Zhang et al., 2001).

Extracellular Recording. Single-unit extracellular recordings were made from the lumbar dorsal horn neurons within 1300 μm of the dorsal surface of the spinal cord with 4-MΩ parylene-coated tungsten microelectrodes (FHC Inc., Bowdoinham, ME). The microelectrode was inserted perpendicularly into the dorsal horn from a point about midway between the midline and the medial edge of the dorsal root entry zone (Rygh et al., 2000). During electrode advancement, electrical pulses were applied to the ipsilateral sciatic nerve as search stimuli so that a neuron with no spontaneous firing could be identified. Once a single unit was identified, the receptive field and response characteristics were determined by a range of mechanical stimuli of varying intensities, including brushing or touching the skin with a cotton brush, light pressure with a probe, and pinching a fold of skin with a toothed forceps. A neuron responding to innocuous tactile stimuli, light pressure, and noxious pinch in a graded manner was identified as a WDR neuron and was selected for further investigation (Zhang et al., 2001; Almeida et al., 2004). A train of 10 stimuli (0.5 Hz, 0.5-ms pulse width, 0.5~5.0 mA, about twice the C-fiber response threshold) was applied repeatedly to the sciatic nerve at a 5-min intervals, and poststimulus histograms were constructed. Data were captured and analyzed by a CED 1401 interface (Cambridge Electronic Design, Cambridge, UK) coupled to a Pentium computer with Spike 2 software.

Mapping of the Receptive Field. The nociceptive receptive field (high-threshold receptive field) was mapped with a pair of fine-toothed forceps, moving from outside loci to within the receptive field. Based on the pinch response of each WDR neuron, the area of the receptive field was mapped on paper. Field size was measured with a planimeter.

Experimental Procedure. The first part of the experiment was designed to determine the roles of 5-HT receptor subtypes in the C-responses of WDR neurons under basal conditions. After three stable control responses were recorded, various antagonists of 5-HT receptor subtypes were applied topically. Drugs were administered in a cumulative fashion, and the effects of each application were measured at 5-min intervals, for up to 60 min. The second part of the experiment was designed to determine the involvement of 5-HT receptor subtypes in the effect of 5-HT on the C-responses of WDR neurons. 5-HT at different doses (0.5, 1.5, and 5.0 μg in a volume of 50 μl) was topically applied 5 min before 5-HT application with or without prior application of 5-HT receptor antagonists. It should be noted that the dose of 5-HT used in the present study was kept relatively low in an attempt to mimic physiologic conditions. Finally, various kinds of 5-HT receptor agonists were also administered.

Drugs. 5-Hydroxy-3-(2-aminoethyl)indole hydrochloride (5-HT) (Sigma-Aldrich, Saint Louis, MO) was used as a nonselective 5-HT receptor agonist. All other drugs were purchased from Tocris Cook-

son (Bristol, UK), unless otherwise stated. Antagonists employed included 5-HT_{1A} antagonist WAY 100635 (Sigma-Aldrich), 5-HT_{1B} antagonist GR 55562, 5-HT_{2A} antagonist ketanserin, 5-HT_{2C} antagonist RS 102221, 5-HT_3 antagonist MDL 72222, and 5-HT_4 antagonist GR 113808 (Sigma-Aldrich). Agonists used included 5-HT_{1A} receptor agonist 8-OH-DPAT, 5-HT_{1B} receptor agonist CGS 12066 (Sigma-Aldrich), 5-HT_{2A} receptor agonist α-methyl-5-hydroxytryptamine maleate (α-m-5-HT), 5-HT_{2C} receptor agonist MK 212, 5-HT_3 receptor agonist 1-(3-chlorophenyl)biguanide hydrochloride (mCPBG), and 5-HT_4 receptor agonist 2-[1-(4-piperonyl)piperazinyl]-benzothiazole (BZTZ). The doses of the antagonists and agonists were chosen according to our preliminary data and to previous reports (Obata et al., 2001, 2002; Hurley et al., 2003; Jeong et al., 2004). RS 102221, MDL 72222, GR 113808, and CGS 12066 were dissolved in 2.0% dimethyl sulfoxide (DMSO), BZTZ in 8.0% DMSO, and other drugs were dissolved in normal saline (NS).

Recording Sites. Direct current (20.0 μA for 20 s) was passed through the recording electrode to mark the recording sites at the end of the experiment. The animal was then perfused transcardially with NS followed by 10% paraformaldehyde under deep anesthesia. The spinal cord was removed, fixed in 10% paraformaldehyde for 12 h at 4°C, and cryoprotected overnight in 30% sucrose in 10 mM phosphate-buffered saline before cryosection. Slide sections were 20 μm thick, and the recording site was confirmed under light microscope.

Statistical Analysis. According to the response threshold and latency, the electrically evoked response of a WDR neuron was arbitrarily divided into four categories: A_β-response (0~20 ms), A_δ-response (20~45 ms), C-response (45~300 ms), and postdischarge (300~800 ms) (Rygh et al., 2000). In general, because the A_δ- and C-responses and the postdischarge are all nociception related, the effects of drugs on each of these responses are usually analyzed in experiments of this type. However, in the present study, only the C-response was examined and analyzed (see below). The C-responses values were expressed as percentages of the mean response value of three consecutive trains of stimuli. Neurons showing variation of less than 20% were selected for further experiments. For data analysis, all C-response values after drug treatment were also expressed as a percentage of this mean C-response value. Data were expressed as mean ± S.E.M. and were analyzed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison. $p < 0.05$ was considered to be a significant difference.

Results

Electrophysiological Characteristics of WDR Neurons. A total of 175 WDR neurons were recorded from 133 rats. Most (136/175, 77.7%) of the neurons were located at a depth of 550 to 1250 μm (849.7 ± 22.5 μm) below the dorsal surface of the cord, corresponding to laminae IV to VI of the dorsal horn. The nociceptive receptive fields of most WDR neurons were on the ipsilateral hindlimb and included areas ranging from two toes to nearly the whole hindpaw or hindquarter (168.8 ± 14.6 mm^2, $n = 175$). Some neurons (21.7%, 38/175) exhibited background activity in the absence of stimulation at a frequency that ranged from 0.03 to 48.9 impulses/s (9.0 ± 1.8 impulses/s, $n = 38$).

Responding to electrical stimulation of the sciatic nerve, almost all units had discharges with two phases: the early discharges (A-responses) and the late train discharges (C-responses). The threshold and the latency of the C-responses were 1.2 ± 0.1 mA and 84.1 ± 1.6 ms ($n = 175$), respectively. In the majority of WDR neurons (159/175, 90.9%), a separation was observed between A- and C-responses (Fig. 1).

The stability of the electrical stimulation-evoked responses of the WDR neurons is shown in Fig. 2. The variation in A_δ- and C-responses did not exceed 20% during the 3-h observation period; however, changes in the postdischarges exceeded 20%. The 95% confidence limits for normal fluctuation of nociceptive responses were within ±20% under the experimental conditions of the present study, like those previously reported (Zhang et al., 2001). Thus, the basal A_δ- and C-responses were rather stable, but the postdischarges were not. The average discharge numbers of the C-responses were 88.6 ± 3.6 impulses/10 stimuli ($n = 175$), whereas the average discharge numbers of the A_δ-responses were very low (6.0 ± 0.7 impulses/10 stimuli, $n = 175$). As such, only the C-fiber responses of WDR neurons were selected and analyzed in the present study.

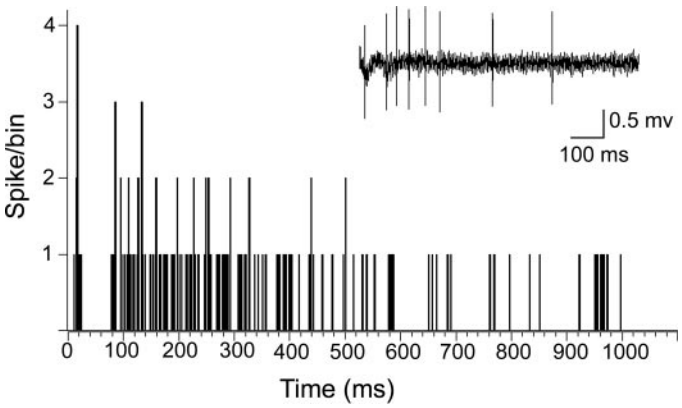

Fig. 1. Poststimulus histograms (10 stimuli) showing the typical response of a WDR neuron to noxious electrical stimuli delivered to the sciatic nerve (bin width, 2 ms). Oscilloscope record shows a single sweep (inset).

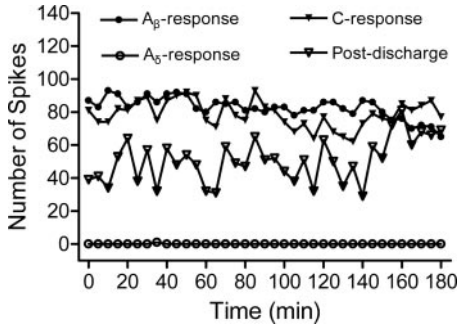

Fig. 2. An example illustrating the stability of base lines. The A_β-, A_δ-, and C-responses of a WDR neuron evoked by electrical stimulation on the sciatic nerve were stable over the observation period of 3 h. The postdischarge of WDR neurons was unstable.

Effects of 5-HT Receptor Subtype Antagonists on the C-Fiber Responses of WDR Neurons under Basal Conditions. As stated above, the basal C-fiber responses of WDR neurons without drug administration were stable. Topical application of NS to the surface of the dorsal spinal cord had no effect on the C-fiber responses of WDR neurons over the 60-min period of observation (Fig. 3A), and no significant difference was observed between responses before and after spinal application of NS ($p > 0.05$, ANOVA, $n = 8$).

Spinal application of 5-HT_{1A} antagonist WAY 100635 at 10.0 μg, 5-HT_{1B} antagonist GR 55562 at 30.0 μg, and 5-HT_{2A} antagonist ketanserin at 15.0 μg did not affect the C-responses of WDR neurons compared with the responses of the NS-treated group ($p > 0.05$, ANOVA, $n = 6 \sim 8$) (Fig. 3A). Likewise, 5-HT_{2C} antagonist RS 102221 at 30.0 μg, 5-HT_3

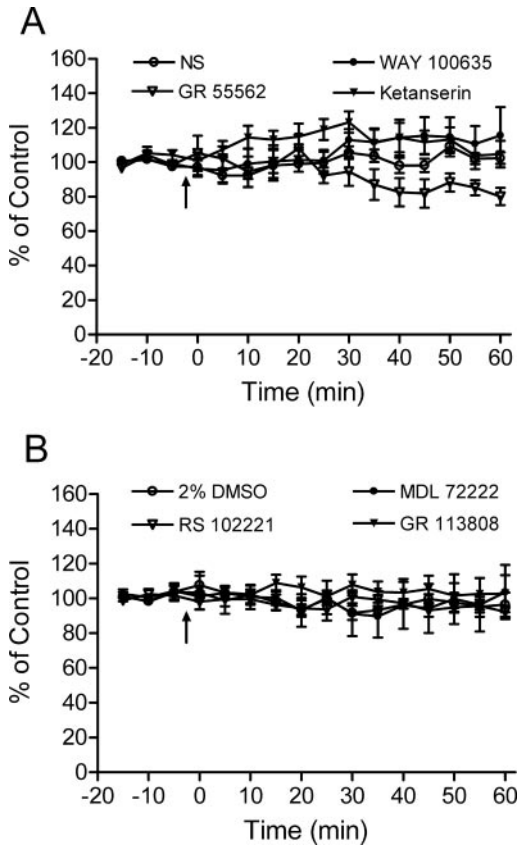

Fig. 3. Effect of spinal application of 5-HT antagonists on C-fiber responses of WDR neurons under basal conditions. A, 5-HT_{1A} antagonist WAY 100635 (10.0 μg), 5-HT_{1B} antagonist GR 55562 (30.0 μg), or 5-HT_{2A} antagonist ketanserin (15.0 μg) did not affect the C-responses. $n = 6–8$. B, 5-HT_{2C} antagonist RS 102221 (30.0 μg), 5-HT_3 antagonist MDL 72222 (15.0 μg), or 5-HT_4 antagonist GR 113808 (15.0 μg) did not affect the C-responses. $n = 5–8$. All data are expressed as mean ± S.E.M. Arrow, application of NS, 2.0% DMSO, or the antagonists.

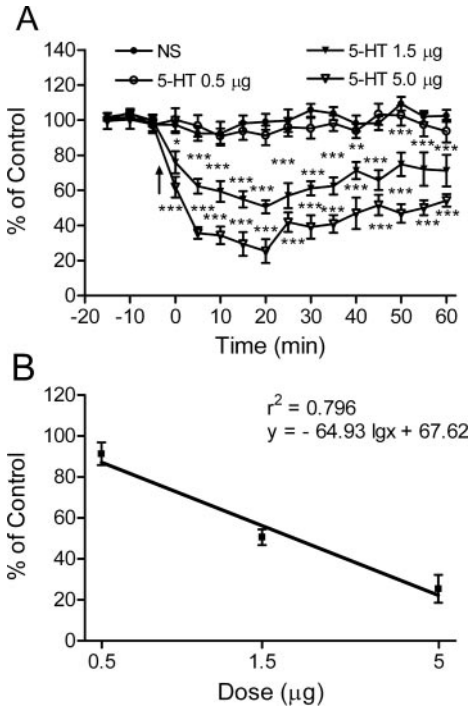

Fig. 4. Effects of 5-HT on C-fiber responses of WDR neurons in normal rats. A, 5-HT at 0.5, 1.5, and 5.0 μg dose dependently inhibited the C-responses. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the NS-treated group (two-way ANOVA followed by Dunnett's multiple comparison). All data are expressed as mean ± S.E.M. $n = 5–8$. Arrow, application of NS or 5-HT. B, dose-response curve of 5-HT (0.5, 1.5, and 5.0 μg) on the C-responses.

antagonist MDL 72222 at 15.0 μg, and 5-HT_4 antagonist GR 113808 at 15.0 μg showed no significant effects compared with the vehicle (2.0% DMSO) ($p > 0.05$, ANOVA, $n = 5\sim7$) (Fig. 3B).

Effects of 5-HT on the C-Fiber Responses of WDR Neurons and Involved 5-HT Receptor Subtypes. 5-HT was applied spinally at three dose levels (0.5, 1.5, and 5.0 μg). As shown in Fig. 4, at a dose of 0.5 μg, 5-HT did not significantly change the C-responses of WDR neurons compared with NS ($p > 0.05$, ANOVA, $n = 5$), whereas 5-HT at 1.5 and 5.0 μg significantly inhibited the C-responses ($p < 0.001$, ANOVA, $n = 7$ for group 1.5 μg; $n = 6$ for group 5.0 μg) (Fig. 4A). Maximal inhibition was observed at 10 to 25 min after 5-HT administration (Fig. 4A), and the ID_{50} of 5-HT was 1.9 μg (95% confidence intervals, 1.1~3.1 μg) (Fig. 4B).

Antagonists were given topically 5 min before spinal application of 5-HT (1.5 μg). When the 5-HT_{1A} antagonist WAY 100635 was given at 10.0 μg, the inhibitory effect of 5-HT on the C-responses was not changed ($p > 0.05$, ANOVA, $n = 5$) (Fig. 5A). However, when the 5-HT_{1B} antagonist GR 55562 was given at 30.0 μg, the inhibitory effects of 5-HT were significantly reduced ($p < 0.001$, ANOVA, $n = 5$) (Fig. 5B). Similar inhibitory results were obtained for 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, and 5-HT_4 receptor antagonists. As shown in Fig. 5, 5-HT-induced inhibition on the C-responses was significantly reversed by 5-HT_{2A} antagonist ketanserin at 15.0 μg ($p < 0.001$, ANOVA, $n = 7$) (Fig. 5C), 5-HT_{2C} antagonist RS 102221 at 30.0 μg ($p < 0.001$, ANOVA, $n = 5$) (Fig. 5D), 5-HT_3 antagonist MDL 72222 at 15.0 μg ($p < 0.001$, ANOVA, $n = 5$) (Fig. 5E), and 5-HT_4 antagonist GR 113808 at 15.0 μg ($p < 0.001$, ANOVA, $n = 6$) (Fig. 5F).

Effects of 5-HT Receptor Agonists on the C-Fiber Responses of WDR Neurons. All 5-HT receptor agonists inhibited the C-fiber responses of WDR neurons (Fig. 6). Agonists and their inhibitory effects were as follows: 5-HT_{1A} receptor agonist 8-OH-DPAT at 5.0 and 50.0 μg ($p < 0.001$, ANOVA, $n = 6$ for 5.0 μg; $n = 7$ for 50.0 μg) (Fig. 6A), 5-HT_{1B} receptor agonist CGS 12066 at 50.0 μg ($p < 0.01$, ANOVA, $n = 6$) (Fig. 6B), 5-HT_{2A} receptor agonist α-m-5-HT at 3.0 μg ($p < 0.001$, ANOVA, $n = 7$) (Fig. 6C), 5-HT_{2C} receptor agonist MK 212 at 10.0 μg ($p < 0.01$, ANOVA, $n = 8$) (Fig. 6D), 5-HT_3 receptor agonist mCPBG at 10.0 and 100.0 μg ($p < 0.001$, ANOVA, $n = 5$ for 10.0 μg; $n = 8$ for 100.0 μg) (Fig. 6E), and 5-HT_4 receptor agonist BZTZ at 30.0 μg ($p < 0.05$, ANOVA, $n = 6$ for 8.0% DMSO; $n = 8$ for 30.0 μg) (Fig. 6F). Spinal application of CGS 12066 at 5.0 μg, α-m-5-HT at 0.3 μg, MK 212 at 100.0 μg, and BZTZ at 3.0 μg did not affect the C-responses of WDR neurons ($p > 0.05$, ANOVA, $n = 5\sim8$) (Fig. 6).

Discussion

Characteristics of WDR Neuron Responses. The characteristics of WDR neurons recorded in the present study were in agreement with those recorded in previous studies (Zhang et al., 2001; Kelly and Chapman, 2002). The dis-

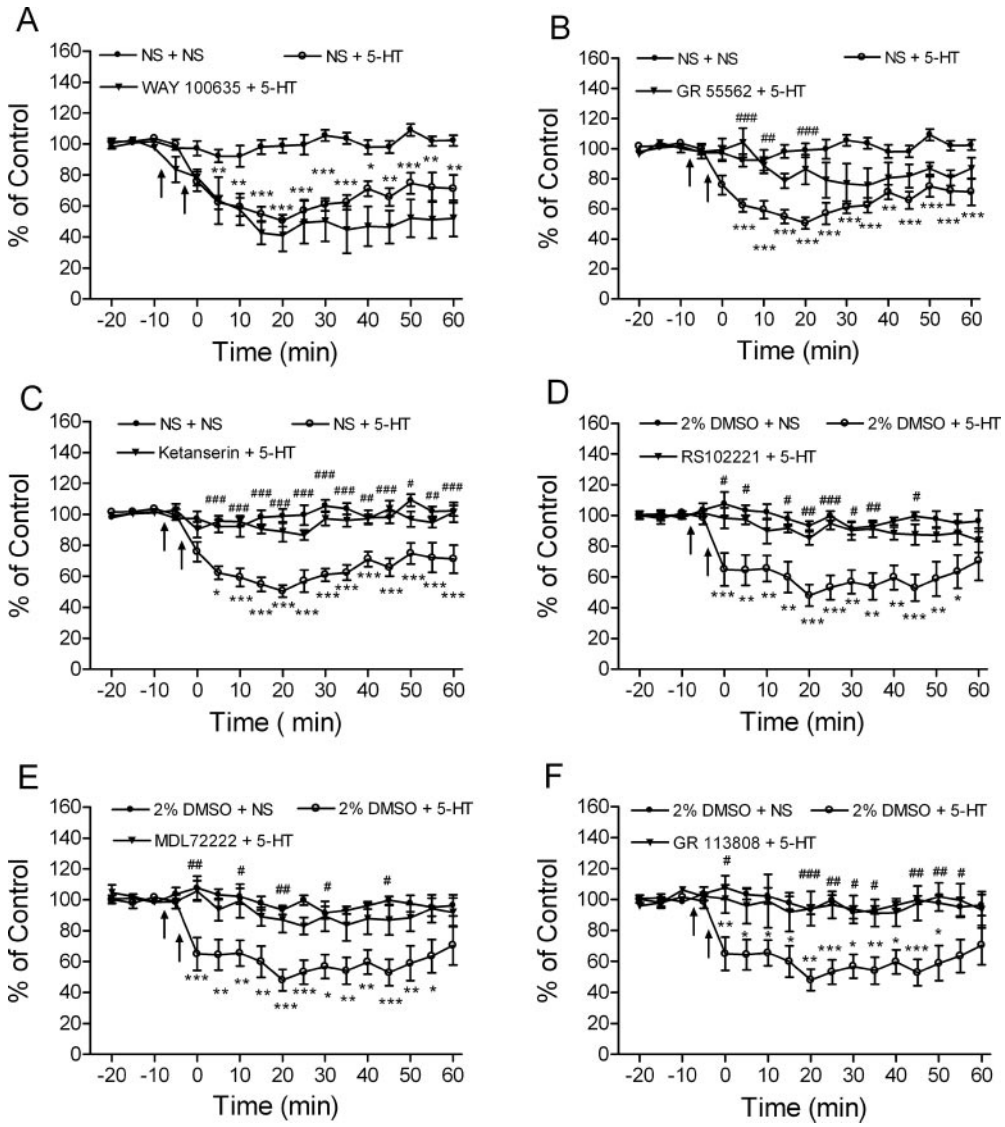

Fig. 5. Effects of 5-HT antagonists on the 5-HT-induced inhibition of the C-fiber responses of WDR neurons. A, 5-HT_{1A} antagonist WAY 100635 (10.0 μg). B, 5-HT_{1B} antagonist GR 55562 (30.0 μg). C, 5-HT_{2A} antagonist ketanserin (15.0 μg). D, 5-HT_{2C} antagonist RS 102221 (30.0 μg). E, 5-HT_3 antagonist MDL 72222 (15.0 μg). F, 5-HT_4 antagonist GR 113808 (15.0 μg). Antagonists were spinally applied 5 min before 5-HT (1.5 μg) application. All data are expressed as mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the vehicle (NS or 2.0% DMSO + NS)-treated group (two-way ANOVA followed by Dunnett's multiple comparison); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with 5-HT (NS + 5-HT or 2.0% DMSO + 5-HT)-treated group (two-way ANOVA followed by Dunnett's multiple comparison). $n = 5–8$. First arrow, application of NS, 2.0% DMSO, or antagonists; second arrow, application of NS or 5-HT.

charges of WDR neurons could be divided into A_β-, A_δ-, and C-responses and postdischarges according to their threshold and latency. Only the C-fiber responses were selected and analyzed, whereas the A_δ-responses and the postdischarges were not, because the number of A_δ-responses was very low (Fig. 2), and the number of postdischarges was unstable over the 3-h observation period (Fig. 2).

Receptor Subtypes of 5-HT Involved in C-Responses of WDR Neurons under Basal Conditions. To study the 5-HT receptor subtypes involved in pain modulation under normal basal conditions, antagonists to 5-HT receptor subtypes were applied topically to the spinal cord. None of the antagonists produced any changes in the C-responses of WDR neurons (Fig. 3), indicating that in these experiments, the spinal serotonergic system did not have a tonic inhibitory effect on the activities of WDR neurons under basal conditions. In Fig. 3A, GR 55562 (30.0 μg) seemed to show inhibition at later time points, but compared with the NS control, it was not statistically significant. It is possible that at a larger dose or over a longer time period, GR 55562 would exhibit more obvious inhibition on the C-fiber responses. As such, further experiments are needed to investigate the effects of GR 55562.

There have been conflicting reports concerning possible tonic effects of 5-HT on spinal nociception transmission. Some studies have reported that administration of 5-HT receptor antagonists or an experimentally induced lesion of the raphe-spinal serotonergic system could produce hyperalgesia with tail-flick and hot-plate tests and increase responses of the dorsal horn neurons to noxious and non-noxious stimulation (Fasmer et al., 1985; Liu et al., 1988; Saito et al., 1990). However, other investigators did not find any such tonic effects (Xu et al., 1994; Bardin et al., 2000). Such a discrepancy may be due to methodology since most previous studies have used behavioral tests that are prone to confounding factors such as changes in skin temperature or motor performance. This is especially important when the capacity of the serotonergic system to regulate vasomotor tone and motor neuron activities is taken into consideration (Millan, 2002).

The Inhibitory Effects of 5-HT on C-Responses of WDR Neurons. In the present study, exogenously applied 5-HT was used to mimic the 5-HT released from the activated descending terminals. Spinal application of 5-HT (0.5, 1.5, and 5.0 μg per rat) dose-dependently inhibited the C-responses of WDR neurons (Fig. 4). These results were consis-

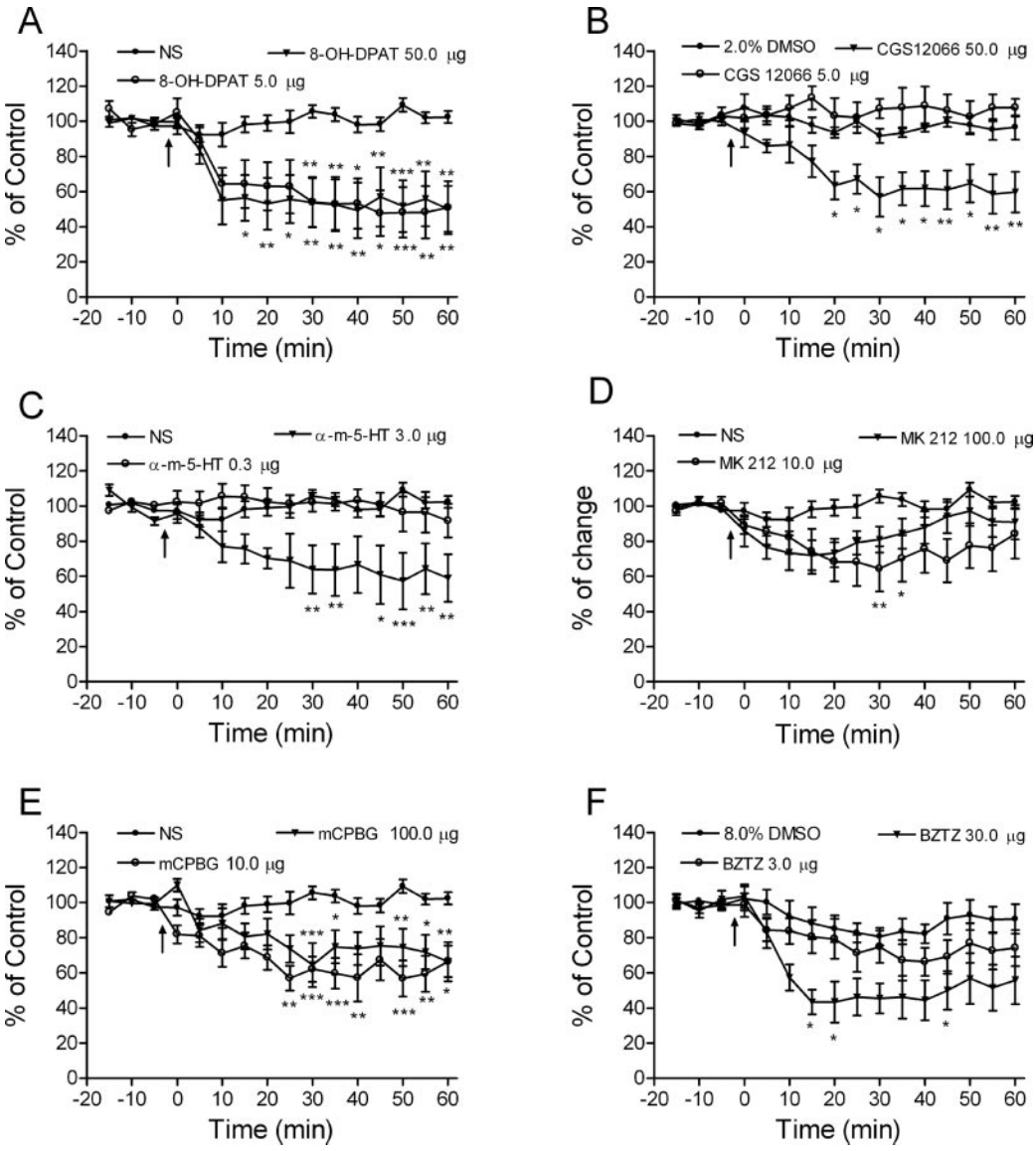

Fig. 6. Effects of 5-HT agonists on the C-fiber responses of WDR neurons. A, 5-HT_{1A} agonist 8-OH-DPAT (5.0, 50.0 μg). B, 5-HT_{1B} agonist CGS 12066 (5.0, 50.0 μg). C, 5-HT_{2A} agonist α-m-5-HT (0.3, 3.0 μg). D, 5-HT_{2C} agonist MK 212 (10.0, 100.0 μg). E, 5-HT_3 agonist mCPBG (10.0, 100.0 μg). F, 5-HT_4 agonist BZTZ (3.0, 30.0 μg). $n = 5–8$. All data are expressed as mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the vehicle (NS, 2.0% DMSO, or 8.0% DMSO)-treated group (two-way ANOVA followed by Dunnett's multiple comparison). Arrow, spinal application of NS, 2.0% DMSO, or 8.0% DMSO.

tent with the findings of other groups (Ali et al., 1994; Bardin et al., 1997) and confirmed our previous results (Xu et al., 1994). It should be noted that the dose of 5-HT used in the present study was kept relatively low, in an attempt to mimic physiologic conditions. In earlier studies, different dose levels of 5-HT were found to either inhibit or facilitate nociceptive responses, depending on the dosages (Ali et al., 1994; Bardin et al., 1997).

Involvement of 5-HT_{1B} Receptor Subtypes in the 5-HT Inhibition of C-Responses of WDR Neurons. In the present study, it was found that the 5-HT_{1B} receptor antagonist GR 55562 reversed the inhibitory effects of 5-HT on the C-responses (Fig. 5B), and 5-HT_{1B} receptor agonist inhibited the C-fiber responses of WDR neurons (Fig. 6B). These results strongly suggest that 5-HT_{1B} receptor is involved in the 5-HT-induced inhibition of the C-responses. Our results were consistent with the previous report in which 5-HT_{1B} receptor agonists mimicked the antinociceptive effects of 5-HT and inhibited the responses of WDR neurons (Ali et al., 1994). 5-HT_{1B} receptors exist throughout the dorsal horn and are especially prevalent in lamina I of the dorsal horn. 5-HT and CGS 12066 directly activated the 5-HT_{1B} receptors in the WDR neurons, resulting in membrane hyperpolarization and inhibition of the C-responses (Thor et al., 1993).

Involvement of 5-HT Receptor Subtypes 2A, 2C, 3, and 4 in the 5-HT Inhibition of C-Responses of WDR Neurons. It was found in the present study that the spinally applied 5-HT receptor antagonists ketanserin, RS 102221, MDL 72222, and GR 113808 reduced the inhibitory effects of 5-HT on the C-responses. Coincidentally, 5-HT receptor agonists α-m-5-HT, MK 212, mCPBG, and BZTZ inhibited the C-fiber responses. These results strongly suggest that 5-HT receptor subtypes 2A, 2C, 3, and 4 are also involved in the 5-HT-induced inhibition of C-responses.

Four subtypes of 5-HT receptors have been identified in the spinal dorsal horn (Helton et al., 1994; Fonseca et al., 2001; Millan, 2002). Activation of 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 receptors inhibits K^+-currents. The ionotropic 5-HT_3 receptor is a receptor-gated cation channel, activation of which increases the conductance of Na^+ and K^+ ions (Barnes and Sharp, 1999).

The direct neuronal effect of activation of 5-HT_{2A}, 5-HT_{2C},

5-HT_3, and 5-HT_4 is excitation. Thus, it is unlikely that these receptors mediate a direct inhibitory effect in the spinal cord, and it is possible that the observed 5-HT induced inhibition was mediated by excitation of inhibitory interneurons. Most WDR neurons recorded in the present study were located within 550–1250 μm (laminae IV~VI) of the dorsal spinal cord. When administered topically, 5-HT may interact simultaneously with different types of neurons, including the inhibitory interneurons (such as GABAergic, glycinergic, and cholinergic interneurons) that express 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, or 5-HT_4 receptors (for example, see Abi-Saab et al., 1999). Behavioral and electrophysiological studies have also shown that the inhibitory effects of 5-HT_3 receptor agonists on nociceptive transmission could be blocked by 5-HT_3- and GABA-receptor antagonists (Alhaider et al., 1991). Activation of 5-HT_3 receptors increased GABA concentration in the spinal dorsal horn (Kawamata et al., 2003). Low concentrations of a 5-HT_4 receptor agonist could also increase the release of GABA (Bianchi et al., 2002). Spinally injected $GABA_A$ and $GABA_B$ receptor antagonists may reduce the inhibitory effects of 5-HT (unpublished data). All these data strongly support the involvement of the spinal GABAergic system in 5-HT-induced inhibition.

It is widely accepted that 5-HT_{2A}, 5-HT_{2C}, or 5-HT_3 receptors participate in 5-HT-induced antinociception (Banks et al., 1988; Alhaider et al., 1991; Bardin et al., 2000; Jeong et al., 2004). Systemic or i.c.v. administration of 5-HT_4 agonists produced antinociception via a central cholinergic mechanism (Ghelardini et al., 1996). Contrary to Bardin et al. (2000), who excluded 5-HT_4 receptors as a component of 5-HT-induced antinociception by using a mechanical nociceptive test, our results support the concept of 5-HT_4 receptor participation in the inhibitory effects of 5-HT. This discrepancy may be related to differences in experimental methodology (electrophysiological versus behavioral), routes of drug application, and influence of 5-HT on motor activity. For example, in Bardin's study, drugs were injected i.t. via a subdural catheter, whereas in our experiment, drugs were applied directly onto the exposed dorsum of the spinal cord. In addition, GR 113808 was dissolved in NS in the studies of Bardin et al. (2000) but was dissolved in 2.0% DMSO in the present work.

Controversy over the Involvement of 5-HT_{1A} in the 5-HT Inhibition of C-Responses of WDR Neurons. The involvement of 5-HT_{1A} in 5-HT inhibition was in question in this study due to the discrepancies observed between the effects of its agonist and antagonist. Although the 5-HT_{1A} agonist 8-OH-DPAT inhibited C-responses, the 5-HT_{1A} antagonist WAY 100635 did not decrease the inhibitory effects of 5-HT. This discrepancy may be related to the receptor selectivity of these two drugs. Compared with WAY 100635, which is a highly selective antagonist for 5-HT_{1A} receptor, 8-OH-DPAT has only a moderate affinity for 5-HT_7 receptors (Harte et al., 2005). It is possible that the inhibitory effects of 8-OH-DPAT on C-responses were mediated through the 5-HT_7 rather than the 5-HT_{1A} receptor or by interaction of these two receptors.

In addition to receptors 5-HT_1 through 5-HT_4, receptors 5-HT_5 through 5-HT_7 have also been found in the central nervous system (Barnes and Sharp, 1999). The possible roles of 5-HT_5 through 5-HT_7 receptor subtypes in the mediation of spinal pain modulation need further investigation.

Conclusion

Using electrophysiological recording of discharges from WDR neurons as the endpoint, we report here in conclusion that: 1) 5-HT does not produce any tonic inhibition in the spinal cord under basal conditions; 2) direct application of 5-HT itself, as well as agonists of 5-HT receptor subtypes 1B, 2A, 2C, 3, and 4 produced inhibitory effects on the C-fiber-induced responses of WDR neurons, and thus the effects of 5-HT may be mediated by these receptors; and 3) the role of 5-HT_{1A} receptor in spinal nociceptive modulation needs further investigation.

Acknowledgments

We thank Xiao-Jun Xu (Karolinska University Hospital-Huddinge, Karolinska Institutet, Stockholm, Sweden) for critical review and discussion of the manuscript. We also thank Michael A. McNutt (Department of Pathology, Peking University Health Science Center) for help in editing the manuscript.

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