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Pronociceptive Effect of 5-HT_{1A} Receptor Agonist on Visceral Pain Involves Spinal NMDA Receptor

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Abstract

The functional role of serotonergic 5-HT_{1A} receptors in the modulation of visceral pain is controversial. The objective of this study was to systematically examine the mechanism and site of action of a selective 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (DPAT) on visceral pain. In the behavioral model of visceral pain, systemic injection (5 to 250µg/kg) of DPAT produced a significant increase in the visceromotor response (VMR) to colorectal distension (CRD) and this effect was blocked by the selective 5-HT_{1A} receptor antagonist WAY-100135 (5mg/kg, s.c.). Similarly, intrathecal (i.t.) injection (5µmol) of DPAT into the lumbo-sacral (L6-S1) spinal cord produced a significant increase in VMR. The administration of N-methyl D-aspartate (NMDA) receptor antagonist AP5 (50µg/kg) prior to DPAT injection completely blocked the pronociceptive effect of DPAT. Similarly, DPAT failed to increase VMR in rats chronically treated with NR1 subunit targeted antisense oligonucleotide (ON), whereas the drug increased VMR in rats treated with mismatched-ON. Chronic i.t. injection of allylglycine (AG), a γ-aminobutyrate (GABA) enzyme inhibitor, produced significant increase in VMRs, suggesting that the inhibition of GABA synthesis produces pronociception. In AG-treated rats, i.t. injection of DPAT failed to further increase in VMR, suggesting that the DPAT action is linked to GABA release. Similarly, WAY-100135 failed to attenuate VMR in AG-treated rats, suggesting that unlike DPAT, AG action is not via the activation of 5-HT_{1A} receptors. In electrophysiology experiments, DPAT (50µg/kg) significantly increased the responses of spinal neurons to CRD, but did not influence the mechanotransduction property of CRD-sensitive pelvic nerve afferent fibers. The effect of DPAT on spinal neurons remained unaffected when tested in spinal transected (C1–C2) rats. These results indicate that the 5-HT_{1A} receptor agonist DPAT produces pronociceptive effects, primarily via the activation of presynaptic 5-HT_{1A} receptors in GABAergic neuron to restrict GABA release and thereby disinhibits the excitatory glutamatergic neurons in the spinal cord.

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Introduction

5-HT₁, a negative G-protein (Gi/Go)-coupled serotonin (5-HT) receptor, plays diverse and complex functions including cognition, emotion (fear and rage) and pain modulation (Millan and Colpaert 1990, Millan et al., 1996, Kayser et al., 2007). Of five subclasses (5-HT_{1A}, B, D, E, F), 5-HT_{1A} is the most prevalent receptor in CNS. The specific binding of [³H]-8-hydroxy-2-(di-n-propylamino) tetralin ([³H]-DPAT), a 5-HT_{1A} receptor agonist, was detected in several regions of the rat brain. The major binding sites were found in the cortex, hippocampus, striatum, periaqueductal gray (PAG), rostroventral medulla (RVM) and spinal cord (Gozlan et al., 1983, Hall et al., 1985, Viisanen and Pertovaara 2010). Although 5-HT_{1A} receptors are present both at pre- and post-synaptic sites, in some areas including the spinal cord the expression is predominantly at pre-synaptic sites. This pre-synaptic location of the receptor has complicated autoreceptor functions that regulate the release of specific neurotransmitter to modulate neuronal functions. For example, studies have documented that 5-HT_{1A} expressing neurons in the midbrain and spinal cord play a significant role in pain modulation by regulating the opioid release (Millan and Colpaert 1990, 1991, Millan et al., 1996, Song et al., 2007). In the rat spinal cord, 5-HT_{1A} receptors are the major subclass accounting for 30–50% of the total population of 5-HT₁ (i.e., 5-HT_{1A–F}) and is primarily concentrated in the superficial laminae (I, III and IV) (Huang and Peroutka 1987, Marlier et al., 1991, Thor et al., 1990, Laporte et al., 1996). The receptor exhibits a rapid increase in the expression under pathological conditions including peripheral inflammation and spinal cord injury (Zhang et al., 2002, Otoshi and Walwyn 2009). Some studies indicate that the activation of 5-HT_{1A} receptors in the spinal cord and midbrain results in somatic antinociception (Gillet et al., 1985, Eide et al., 1988, El-Yassir et al., 1988, 1990, Giordano and Rogers 1989, 1992, Eide and Hole 1991, 1993, Xu et al., 1994, Oyama et al., 1996, Galeotti et al., 1997, Gjerstad et al., 1997, Xiao et al., 2005, Wei and Pertovaara 2006), whereas other studies report pronociceptive effect (Solomon and Gebhart 1988, Zemlan et al., 1988, Crisp et al., 1991a,b, Alhaider and Wilcox 1993, Ali et al., 1994, Zhang et al., 2001). Similarly, in the rodent model of visceral pain while one study indicates that the partial 5-HT_{1A} receptor agonist buspirone produces analgesia to noxious colon distension (Sivarao et al., 2004), a recent report indicates that 5-HT_{1A} antagonists (WAY-100635 and AZD7371) produce visceral analgesia in the same species (Lindström et al., 2009). Although these conflicting results are difficult to explain, it could be related to the subjective nature (visual estimate of abdominal contraction) of pain scoring; the strain of rats used and most importantly the physiological condition of the animal.

The objective of the present study was to examine the mechanism and the site of action of a selective 5-HT_{1A} receptor agonist DPAT in the rat model of visceral pain. We hypothesize that the pronociceptive effect of DPAT is due to activation of negative G-protein (Gi/Go)-coupled 5-HT_{1A} receptor located at the presynaptic terminals of GABAergic interneurons that results in hyperpolarization of cells and the reduction of γ -amino butyric acid (GABA) release. Since GABA primarily acts as an inhibitory neurotransmitter in the spinal cord and modulates the functions of excitatory glutamatergic neurons, the reduction of GABA release will disinhibit these neurons to produce hyperexcitation of spinal dorsal horn neurons resulting in visceral hyperalgesia.

Methods

Animals

The study was carried out in male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing about 400g (range: 350–450g). All experimental procedures were performed according to approved guidelines of The Institutional Animal Care and Use committee at the

Medical College of Wisconsin (approval # AUA0000356) and International Association for the Study of Pain (IASP).

General surgical procedures

Surgery for recording visceromotor response (VMR)—All surgical procedures were performed in adult rats anesthetized with sodium pentobarbital (45–50 mg/kg, i.p., Ovation Pharmaceuticals Inc. Brown Deer, IL, USA). A pair of teflon-coated electrode was implanted into the external oblique muscle of the abdomen and externalized through the neck for electromyography (EMG) recordings as previously described (Miranda et al., 2004). All rats received analgesic (Carprofen, 5mg/kg/day, i.m. for 3 days) and antibiotic (Enrofloxacin, 2.5mg/kg/day, i.m. for 3 days) post-operatively. Following the surgery rats were housed separately and allowed to recover for at least three days prior to further interventions.

Intrathecal (i.t.) catheterization and drug delivery—In addition to electrodes in the external abdominal muscle, in a group of rats a catheter was chronically implanted into the intrathecal (i.t.) space for the drug administration. A catheter (PE-10, length: 6–6.5cm) was inserted through the dura overlying the atlanto-occipital junction into the spinal subarachnoid space and guided until the tip of the catheter lay in the lumbo-sacral (LS) segment of the spinal cord. These rats were observed closely for 24 hours to check for hind limb paralysis. Rats that exhibited hind limb paralysis were excluded from the study. The drug was delivered intrathecally (i.t) in volume ranging from 5 to 10µl followed by 10µl saline injection.

Drugs

All drugs including the 5-HT_{1A} receptor selective agonist, 8-hydroxy-2-(di-n-propylamino)-tetralin (DPAT, Tocris Bioscience Ellisville, USA), 5-HT_{1A} receptor selective antagonist WAY-100135 (Tocris Bioscience Ellisville, USA), and the competitive NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5, Sigma Aldrich Chemicals, USA) were dissolved in saline. The NR1 antisense oligonucleotide (ON) or mismatch ON was dissolved in 0.9% sterile saline and 5µl (30nM) was injected twice daily (9.00AM and 5.00 PM) for 4 days. Each injection was followed by 10µl of saline injection. Since all drugs were dissolved in saline, a separate vehicle control was not performed for every experimental protocol.

VMR recording

Seventy-two hours following the surgery, rats were put inside the plexiglass restraining tubes two times a day for two hours (1 hour/session) in order to acclimatize them to experimental conditions. The VMR to colorectal distension (CRD) was used as an objective measure of visceral sensation as previously described (Miranda et al., 2004, Mickle et al., 2010). A graded isobaric distention (10, 20, 30, 40, and 60 mmHg) was delivered for duration of 30 seconds with 180 seconds inter-stimulus time interval.

Electrophysiology

Recording from sacral S1 dorsal root—The surgical procedure and recordings from the S1 dorsal root were carried out as previously described (Sengupta and Gebhart 1994). Briefly, following anesthesia the lower abdomen was exposed by a 3 – 4 cm long incision laterally at the left flank. The prostate lobe was reflected laterally to access the major pelvic ganglion (MPG) and pelvic nerve. A pair of teflon-coated stainless steel wires stripped (1–1.5cm) at the tips was wrapped around the pelvic nerve. The exposed parts of the wires were

covered with peritoneal fat tissue. The abdomen was closed in layers (muscle followed by skin) using 3-0 silk sutures.

The lumbo-sacral (LS) spinal cord was exposed by laminectomy (T₁₃-S₂) and the spinal cord was stabilized by clamping thoracic and ischial vertebrae. The dorsal skin was reflected laterally and tied to spinal posts to make a pool for mineral oil. The dura was carefully removed and the spinal cord was covered with warm (37°C) mineral oil. The pelvic nerve afferent (PNA) fibers in the sacral S1 dorsal root were identified by recording the evoked response to electrical stimulation (5–10mv, 0.5–1ms square wave pulse) of the pelvic nerve. The electrically-evoked afferent fiber was then tested to brief colon distension (40mmHg, 10sa) and once confirmed as CRD-sensitive fiber, a stimulus-response function (SRF) to graded CRD (10–60mmHg, 30s) was recorded.

Recording from lumbo-sacral (LS) spinal neurons—The surgical procedure for spinal neurons recordings was similar as described in the previous section. Stainless steel microelectrodes (8–10MΩ, FHC, Bowdoinham, ME) were used to record neurons 0.1–0.5mm lateral from the spinal midline and 0.6–1.3mm ventral from the dorsal surface. The action potentials were amplified through a low-noise AC differential amplifier (model 3000; A-M Systems) and continuously monitored and displayed on an oscilloscope. Once a CRD-sensitive neuron was identified, a SRF to graded CRD (10–60mmHg, 30s) was constructed.

In a separate set of experiments, responses of spinal neurons to CRD were recorded after cervical spinal transection to eliminate the supraspinal influence. For spinal cord transection, a laminectomy was performed to expose the cervical (C₁–C₂) spinal cord. The dura membrane was removed and 10–15μl of 2% lidocaine was applied on the dorsal surface of the exposed spinal cord. After 10 minutes, the spinal cord was completely transected by using a scalpel blade. The transected area was covered with a small piece of gelfoam soaked in warm saline. Animals exhibited a vasodepressor response immediately after spinal transection that recovered in 15–30 min. Recording from spinal neuron was performed two hours after the spinal transection.

Experimental protocols

VMR—The effect of DPAT on VMR was tested in naïve rats. The drug was given either (1) intraperitoneally (i.p.) to test the systemic effect or (2) i.t. to test the effect in the spinal cord. SRF to graded CRD (10–60mmHg) was recorded before injecting DPAT and 10 minutes following injection. To test the 5-HT_{1A} receptor specific action of DPAT, we tested the effect of the drug in the presence of a selective 5-HT_{1A} receptor antagonist WAY-100135. The dose of WAY-100135 was carefully selected so that it had no effect on VMR. In these experiments, a baseline SRF was constructed before injection of WAY-100135 (5mg/kg, s.c.) and the SRF was repeated 10 minutes following the injection of WAY-100135 to test the effect of the antagonist on VMR. After the completion of second SRF, DPAT (50μg/kg, i.p.) was injected and the third SRF was constructed 15 minutes after the injection of DPAT. A similar protocol was followed to test the effect of competitive NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5). Unlike WAY-100135, AP5 (50μg/kg) was injected intravenously (i.v.).

To investigate whether DPAT directly activates glutamate release or acts via the inhibition of GABA release from the GABAergic interneurons, in a set of rats we injected allylglycine (AG, 10μg in 10μl i.t.), a potent inhibitor of γ-amino decarboxylase isoform 65 (GAD 65), twice a day (9am and 5pm) for three days to block GABA synthesis. A baseline SRF to graded CRD was recorded before the beginning of AG injection and on the 4th day following three days of drug treatment SRF was repeated. A third SRF was constructed after injecting DPAT. To further investigate whether AG treatment influences the post-synaptic

GABA_A receptors, in another set of AG-treated rats we tested the effect of selective GABA_A receptor agonist muscimol (1.5μmol, i.t) on SRF and repeated after injecting DPAT (5μmol, i.t) in presence of muscimol. Finally, in another batch of AG-treated rats, we tested the effect of WAY-100135.

NR1 antisense injection—NR1 antisense is an 18-mer phosphodiester oligonucleotide (ON) (5'CAGCAGGTGCATGGTGCT 3') targeted to the nucleotide sequence 4 through 21 that directly follows the initiation codon of rat NR1. The mismatch-ON (5' GAGCAGCTGCGTGATGCT 3') contains the same C:G nucleotide content as the antisense but in a different sequence. A database search using BLAST program (National Center for Biotechnology Information, Bethesda, MD) indicated that the anti-sense sequence was specific for rodent NMDAR1, and the search did not identify a corresponding rodent sequence for the mismatch. The ONs were obtained from Integrated DNA Technologies (Coralville, IA). Before the beginning of ON injection we constructed a baseline SRF to graded CRD (10–60mmHg, 30s). The NR1 anti-sense ON or mismatch ON was injected (30nmol in 5μl of saline) into the LS segment of the spinal cord via the intrathecal catheter twice a day (9am and 5pm) for four days. On the 5th day, ON was injected at 9am and the SRF (post-ON) was constructed one hour following the injection. This was followed by i.t. injection of DPAT and another SRF.

Electrophysiology experimental protocols—After identification of CRD-sensitive PNA fibers or LS spinal neurons a baseline SRF to graded CRD was constructed prior to drug injection and SRF was repeated 10 minutes after injection of DPAT (50μg/kg, i.p.). In spinal recording experiments, a set of rats underwent cervical (C1-C2) spinal cord transection prior to recording. A baseline SRF was recorded about two hours after spinal transection and repeated 10 minutes after administration of DPAT. To test the 5-HT_{1A} receptor specific effect of DPAT, WAY-100135 (5mg/kg, s.c.) was injected 10 minutes prior to DPAT. A SRF was constructed following the injection of WAY-100135 and repeated following DPAT injection. Effect of AP5 was tested in similar fashion. All spinal neurons were recorded between the depths of 750 to 1100μm from the dorsal surface of the spinal cord.

Data analysis

The statistical analysis was performed using SigmaStat (V2.03, SPSS Inc, Chicago, IL). All results are expressed as mean ± SEM. Values with $p < 0.05$ are considered to be significant. For behavioral experiments a baseline VMR was recorded before acute or chronic administration of drug and response was calculated as area under the curve. The effect of the drug was statistically compared and represented by applying Student 't'-test.

In electrophysiology experiments, the total number of action potentials before (60 seconds) and during the distension period (30 seconds) were counted and represented as impulses/sec (imp/s). The firing frequency during the resting period was subtracted from the firing frequency during distension to determine actual response during the distension. In each experiment, a baseline SRF was constructed for each neuron prior to drug injection and repeated following the drug injection. Since each spinal neuron had different spontaneous firing and response to CRD, we normalized the value against its maximum response to distending pressure of 60mmHg. The data were analyzed at each distending pressure and the effect of drug was statistically represented using Student 't'-test.

Results

Effect of DPAT on VMR to CRD

A systemic injection of DPAT increased the VMR to CRD (fig 1). Low dose (5µg/kg, i.p., n=6) of DPAT increased the VMR significantly ($p<0.05$ vs baseline) at distending pressure 30mmHg (fig 1A, n=6). However, at higher doses (50 and 250µg/kg, i.p., n=6 and 7, respectively) the VMR increased significantly ($p<0.05$ vs baseline) at much lower distension pressure (~10mmHg) (fig 1B and 1C). There were no dose-dependent differences in VMRs between 50 and 250µg/kg. Therefore, in subsequent experiments, we used 50µg/kg of DPAT to test the mechanism of action of the drug.

To test the receptor-specific action of DPAT, a 5-HT_{1A} receptor selective antagonist WAY-100135 (5mg/kg s.c.) was injected 10 minutes before DPAT injection. The VMR was not attenuated by WAY-100135 alone (fig 2A, n=3), but DPAT-induced increase in the VMR was completely blocked in the presence of WAY-100135 (fig 2B, n=5).

To test whether DPAT produces a pronociceptive effect by acting at the spinal level, we injected DPAT (5µmol, i.t.) into the lumbo-sacral (LS) segment of the spinal cord 10 minutes prior to CRD. The drug significantly ($p<0.05$ vs baseline) increased VMR at all intensities (10 – 60mmHg) of CRD (fig 3, n=7).

The DPAT-induced increase in the VMR was also blocked when rats were pretreated with the competitive NMDA receptor antagonist AP5 (50µg/kg, i.v.), suggesting that the increase in VMR by DPAT is linked with the activation of NMDA receptors (fig 4, n=5). To further investigate the involvement of NMDA receptors, a set of rats were injected with NR1 anti-sense ON (n=5) or mismatch-ON (n=5) into the LS segment of the spinal cord. A baseline VMR was recorded prior to injection and repeated five days later. In the NR1 anti-sense ON-treated group, there was no change in VMR and DPAT failed to increase the VMR (fig 5A). In contrast, DPAT significantly ($p<0.05$ vs baseline) increased the VMR at all intensities of distending pressure in NR1 mismatch ON-treated rats (fig 5B).

The DPAT-induced increase in VMR could be either by (1) inhibition of GABAergic transmission through activation of pre-synaptic 5-HT_{1A} receptors in GABAergic interneurons. The activation of 5-HT_{1A} receptor would reduce the release of GABA and remove the inhibitory influence over the excitatory glutamatergic system or (2) by direct activation of 5-HT_{1A} receptors located at the presynaptic terminals of the excitatory glutamatergic neurons in the spinal cord to release glutamate. To test this hypothesis, we designed experiments to block GABA synthesis by injecting γ -amino decarboxylase (GAD) inhibitor allylglycine (AG, 10µg, 2 times for 3 days) and then tested the effects of DPAT (5µmol, i.t.) (fig 6A, n=4) or its effect following the inhibition of VMR by injecting GABA_A receptor agonist muscimol (1.5µmol, i.t.) (fig 6B, n=5). The AG treatment significantly ($p<0.05$ vs pre-AG baseline) increased the VMRs in these rats suggesting a lack of inhibitory GABAergic tone in the spinal cord to modulate visceral nociception (fig 6A, B and C). Although DPAT injection in these rats slightly increased the VMR, the enhanced response was not statistically significant (fig 6A, $p>0.05$). In second set of experiment, i.t. injection of muscimol (1.5µmol, i.t.) in AG-treated rats significantly ($p<0.05$ vs post-AG) inhibited the VMR and DPAT (5µmol, i.t.) injection slightly reversed the inhibition (fig 6B). In order to confirm that the pronociceptive effect of AG is by inhibiting GABA synthesis in the spinal cord and not by modulating GABA release via the activation of pre-synaptic 5-HT_{1A} receptor, we tested the effect of i.t. injection of 5-HT_{1A} receptor antagonist WAY-100135 (10µmol). The drug did not attenuate VMRs of AG-treated rats (fig 6C).

Effect of DPAT on responses of CRD-sensitive pelvic nerve afferent (PNA) fibers and lumbosacral (LS) spinal neurons

To test whether the increase in VMR to systemic DPAT injection is due to excitation of CRD-sensitive PNA fibers, we tested responses of PNA fibers ($n=7$) to colon distension before and after the systemic injection of DPAT ($50\mu\text{g/kg}$, i.p.). The mean baseline spontaneous firing of these fibers before and after DPAT injection were 0.75 ± 0.3 impulses/s and 0.63 ± 0.2 impulses/s, respectively. Figure 7A illustrates a typical increase in firing of a CRD-sensitive PNA fiber to graded CRD ($10\text{--}60\text{mmHg}$, 30s). The DPAT injection did not affect responses of these fibers to CRD, suggesting that the increase in VMR by DPAT is not due to hyperexcitation of CRD-sensitive PNA (figs 7B and 7C).

We then tested the effect of DPAT on responses of CRD-sensitive LS spinal neurons. These experiments were performed in - (1) spinal intact ($n=4$) and (2) cervical (C1-C2) spinal cord transected rats ($n=6$). The rationale for recording responses of CRD-sensitive spinal neurons in spinal transected rats was to remove supraspinal influence that may contribute to pronociceptive effect of DPAT. Figures 8A and 8C illustrate typical responses of CRD-sensitive LS neurons before and after systemic DPAT ($50\mu\text{g/kg}$, i.p.) injection. The mean baseline spontaneous firings of these spinal neurons in intact rats before and after DPAT injection were 3.17 ± 1.80 impulses/s and 7.25 ± 4.2 impulses/s, respectively. In spinal transected rats, the mean baseline spontaneous firing significantly increased following DPAT injection (3.15 ± 0.9 impulses/s pre-DPAT vs 11.58 ± 3.76 impulses/s post-DPAT, $p<0.05$). In both spinal intact and spinal transected rats the drug significantly ($p<0.05$ vs baseline) increased responses of these neurons to CRD (figs 8B and 8D). Since DPAT increased responses of these neurons in spinal transected rats, it suggests that the excitatory effect of the drug occurs at the spinal cord.

In subsequent experiment, we tested the effect DPAT in rats pretreated with WAY-100135 (5mg/kg , s.c.). The mean baseline spontaneous firings of these spinal neurons before and after WAY-100135 injection were 3.80 ± 1.60 impulses/s and 3.10 ± 1.5 impulses/s, respectively. Following DPAT the mean baseline spontaneous firing of spinal neurons was 4.07 ± 1.40 impulses/s. WAY-100135 alone did not affect the responses of LS neurons to graded CRD, but blocked the excitatory effect of DPAT (fig 9, $n=3$).

In another set of experiments, we injected the competitive NMDA receptor antagonist AP5 ($50\mu\text{g/kg}$, i.v.) prior to testing the effect of DPAT on responses of CRD-sensitive spinal neurons ($n=5$) in spinal transected rats. The mean baseline spontaneous firings of these neurons before and after AP5 injection were 1.21 ± 0.7 impulses/s and 1.03 ± 0.6 impulses/s, respectively. Following DPAT administration the mean spontaneous firing was 1.64 ± 0.70 impulses/s. While AP5 did not affect responses of CRD-sensitive neurons, preemptive administration of AP5 completely blocked the excitatory effect of DPAT (fig 10).

Discussion

In the present study, we have systematically examined the mechanism of action of the 5-HT_{1A} receptor agonist DPAT and the site of action of the drug. The results indicate that the drug produces visceral pronociceptive effects by unmasking the action of excitatory glutamatergic neurons at the spinal level. The effect of the drug is most likely due to activation of 5-HT_{1A}, a negative G-protein (Gi/Go)-coupled receptors located at presynaptic terminals of inhibitory GABAergic interneurons. This would result in hyperpolarization of these neurons and restrict the release inhibitory neurotransmitters. Our result clearly indicates that glutamate is associated with DPAT-induced visceral hyperalgesia, since the competitive NMDA receptor antagonist AP5 as well as knocking down of NR1 subunit by chronic treatment with NR1 targeting anti-sense ON prevented the effect of DPAT. The

inactivation of inhibitory GABAergic interneurons disinhibits the excitatory function of glutamatergic neurons by enhancing the release of glutamate. The result also suggests that the effect of DPAT is not mediated via the excitation of mechanosensitive PNA fibers innervating the colon, since the drug fails to affect the responses of these fibers to colon distension. On the other hand, DPAT significantly increases responses of CRD-sensitive LS spinal neurons to colon distension by directly acting at the spinal level, since this effect was observed in cervical spinal transected rats. This excitatory effect of DPAT can be blocked by the 5-HT_{1A} receptor antagonist WAY-100135 as well as by NMDA receptor antagonist AP5. Thus, our electrophysiology experiments confirm the findings of behavioral studies that the pronociceptive effect of 5-HT_{1A} receptor agonist DPAT is partly due to disinhibition of excitatory glutamatergic neurons that activates CRD-sensitive spinal neurons via the activation of NMDA receptors.

5-HT_{1A} receptors in the spinal cord and diverse actions

In the rat spinal cord, there are primarily four types (5-HT₁, 5-HT₂, 5-HT₃ and 5-HT₄) of serotonin (5-HT) receptors (Peroutka 1988, Marlier et al., 1991, Laporte et al., 1996, Coggeshall and Carlton 1997, Bardin et al., 2000). Among these receptors, 5-HT₁ subclass accounts for 50–60% of binding with 5-HT and it is predominantly (30–50%) 5-HT_{1A} subtypes that expresses in the superficial laminae (Huang and Peroutka 1987, Marlier et al., 1991). Interestingly, there is a gradient in 5-HT_{1A} receptor densities along the rostro-caudal axis of the spinal cord with the highest expression observed in the lumbo-sacral (LS) segment of the spinal cord (Thor et al., 1990, Marlier et al., 1991). The LS spinal cord receives the major projection from the pelvic organs including descending colon, urinary bladder and other pelvic reproductive structures. In the spinal cord, a subset of 5-HT_{1A} receptors is possibly located at the pre-synaptic terminals of the primary afferent fibers, since dorsal rhizotomy or capsaicin-induced degeneration of C-fibers results in 20–25% reduction in the density of receptor within the superficial laminae (Daval et al., 1987, Laporte et al., 1996). It is well recognized that 5-HT_{1A} plays an important role in pain modulation. However, the existing reports are conflicting as in some studies 5-HT_{1A} receptor agonist produced antinociception (Eide et al., 1988, El-Yassir et al., 1988, Eide and Hole 1991, 1993, Xu et al., 1994, Gjerstad et al., 1996, Millan et al., 1996, Oyama et al., 1996), pronociception (Solomon and Gebhart 1988, Zemlan et al., 1988, Murphy and Zemlan 1990, Crisp et al., 1991a, b, Alhaider and Wilcox 1993, Ali et al., 1994, Zhang et al., 2001) or no effect (Millan and Colpaert 1990, Mjelle et al., 1992, Millan et al., 1996, Bardin et al., 2000). Although the reason for such a diverse effect of 5-HT_{1A} agonist is not clearly known, it is speculated that the effect of 5-HT_{1A} agonist can be variable depending on the type of pain model, pathological conditions, route of administration, and species. Several studies including our current study have documented that i.t. injection of 5-HT_{1A} agonist produces hyperalgesia. In *in vivo* electrophysiology recordings from the lumbar spinal cord, i.t. injection of DPAT dose-dependently increased responses of wide-dynamic range (WDR) neurons to mechanical and thermal stimuli (Ali et al., 1994, Zhang et al., 2001). Similarly, our present result also shows that the injection of DPAT significantly increases the responses of LS spinal neurons to colon distension. Interestingly, these findings are very different from the effect of DPAT that was examined in *in vitro* electrophysiology recordings in tissue slices from the spinal cord, raphe nucleus and periaqueductal gray (Murase and Randi 1983, Soltesz et al., 1991, Behbehani et al., 1993, Pan et al., 1993, Kishimoto et al., 2001, Jeong et al., 2008, 2011). In these studies 5-HT_{1A} receptor activation resulted in an increase in inwardly rectified K⁺-conductance and membrane hyperpolarization. This change in membrane conductance and hyperpolarization is possibly associated with 5-HT_{1A}-activated inhibition of the cyclic AMP pathway, since the receptor is a negative G-protein ($\alpha_{i/o}$ G-protein)-coupled receptor and therefore can inactivate voltage-gated Ca⁺⁺ channels (Aghazanian 1995, Albert et al., 1996). The

conflicting results of in vivo and in vitro electrophysiology studies can be reconciled on the fact that the hyperpolarization of inhibitory neurons following the activation of 5-HT_{1A} receptors may decrease the release of inhibitory neurotransmitters resulting in disinhibition of excitatory neurons (Koyama et al., 2002, Gronier 2008).

The regulation of nociceptive processing by 5-HT_{1A} receptor can be differentially influenced depending on the nature of noxious stimulus. For example, in formalin-induced paw biting and licking experiment, i.t. injection of 5-HT produced a significant anti-nociception and this effect was reversed by the selective 5-HT_{1A} receptor antagonist WAY-100635. In the paw pressure test, 5-HT injection did not produce any anti-nociception, but in combination with WAY-100635 it produced anti-nociception. It has been proposed that such complex action of 5-HT_{1A} receptors is possibly dependent on the localization of the receptor in the neural circuitry and how it is activated under different physiological conditions. For example, it is thought that the antinociceptive effect of 5-HT_{1A} agonist is primarily due to activation of inhibitory GABAergic interneurons in the spinal cord, since the effect can be blocked by GABA_A receptor antagonist bicuculline (Millan and Colpaert 1990, Bonnefont et al., 2005). On the other hand, in the paraventricular nucleus (PVN) activation of presynaptic 5-HT_{1A} receptors prevents GABA synaptic transmission (Lee et al., 2008). The pronociceptive effect of 5-HT_{1A} agonist at the spinal level can occur either by (1) inhibition of GABA synaptic transmission resulting in an unmasking of excitatory system or (2) by direct activation of excitatory transmission including glutamate release. Electrophysiology experiments have shown that i.t. injection of 5-HT_{1A} receptor agonist DPAT increases responses of wide-dynamic range (WDR) projection neurons in the spinal cord, which can be blocked by 5-HT_{1A} receptor antagonist (Ali et al., 1994, Zhang 2001). Although these results indicate the excitatory effect of 5-HT_{1A} receptor mediated in the spinal cord, it does not address whether this excitation is direct action of 5-HT_{1A} agonist on post-synaptic 5-HT_{1A} receptor or indirectly via presynaptic release of excitatory neurotransmitters.

Expression of 5-HT_{1A} receptors in GABAergic and enkephalinergic neurons in the spinal cord

It has been shown that the receptors are located in the second order projection neurons as well as in the GABAergic and enkephalinergic inhibitory interneurons (Hamon and Bourgoin 1999). Using fluorescent in situ hybridization (FISH) and double-staining immunohistochemical techniques, it has been documented that 5-HT_{1A} receptor mRNA is widely distributed in the spinal cord with highest density in laminae III and IV and about 49% of GABAergic neurons are 5-HT_{1A} receptor mRNA positive (Zhang et al., 2002, Wang et al., 2009). Since 5-HT_{1A} receptors are negatively coupled receptors, their activation attenuates the release of GABA from these interneurons leading to a reduction in the spontaneous miniature inhibitory postsynaptic currents (mIPSCs) that indirectly modulate the excitatory function in the spinal cord (Lee et al., 2008). However, it is not known whether the excitatory response of 5-HT_{1A} receptors is exclusively associated with the GABAergic pathway or through some other inhibitory or excitatory mechanisms. Our results indicate that the excitatory response is largely through the glutamate-induced NMDA receptor activation as DPAT failed to produce hyperalgesia in spinally NMDA-NR1 knock down rats. Further, our allylglycine (AG)-treated experiments document that the GABAergic system plays a major role in DPAT-induced hyperalgesia. However, it may not be exclusively through this mechanism.

5-HT_{1A} receptors also coexpress in enkephalinergic neurons in the spinal cord (Zhang et al., 2002). In our behavioral study, we therefore tested the effect of DPAT after removing the influence of GABAergic neurons by disrupting the GABA synthesis following repeated i.t. injection of AG, a potent GAD blocker (Castellano and Pavone 1984, Chapman et al., 1984,

Meldrum et al., 1987). DPAT injection in AG-treated rats failed to produce a significant increase in the VMR. Similarly, DPAT injection slightly, but not significantly, reversed the inhibitory effect of GABA_A receptor agonist muscimol in AG-treated rats. These results suggest that there could be additional mechanisms involved in DPAT-induced excitation. One of the possible mechanisms could be the inhibition of opioid release from the enkephalinergic neurons coexpressing 5-HT_{1A} receptors. In recent *in vitro* spinal slice experiments, it has been shown that the electrical stimulation of the dorsal horn induces opioid release and internalization of μ -opioid receptor following the binding with the ligand. The addition of DPAT into the perfusing chamber significantly blocks the μ -opioid receptor internalization, suggesting a reduction in opioid release (Song et al., 2007). Taking all this into account, it can be concluded that the activation of 5-HT_{1A} receptors attenuates inhibitory synaptic transmission in the spinal cord to produce pronociceptive effect to distension of the colon. Since the receptor is negatively-coupled receptor and generally produces hyperpolarization of the cell, it is unlikely that it directly excites the presynaptic terminals of the excitatory neurons to release glutamate. Therefore, selective blocking of spinal 5-HT_{1A} receptors could alleviate the visceral pain as it has been documented in the recent study (Lindström et al. 2009).

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Highlights

- 5-HT_{1A} receptor agonist DPAT produces pronociceptive effects in visceral pain.
- DPAT activates of presynaptic 5-HT_{1A} receptors to inhibit GABA release.
- Inhibition of GABAergic neurons unmasks the excitatory glutamatergic system.
- This effect results in visceral hyperalgesia in behavioral studies.
- The electrophysiology experiments also confirm the findings of behavioral studies.

Figure 1.

Summary data of visceromotor responses (VMRs) to colorectal distension (CRD) before and after intra-peritoneal (i.p.) injections of 5µg/kg (**A**), 50µg/kg (**B**) and 250µg/kg (**C**) of selective 5-HT_{1A} receptor agonist DPAT. At low dose (5µg/kg), the VMR is significantly higher at distending pressure > 20mmHg. However, at high doses (50µg and 250µg/kg) of DPAT VMRs were significantly higher at all intensities of distension. There was no dose-dependent increase in response between the doses of 50µg and 250µg/kg.

Figure 2.

(A) VMRs to colon distension before and after subcutaneous (s.c.) injection of selective 5-HT_{1A} receptor antagonist WAY-100135 (5mg/kg). This dose of WAY-10035 did not attenuate VMR. (B) DPAT (50µg/kg, i.p.) failed to increase the VMR in rats that were pretreated with WAY-100135 (5mg/kg, s.c.), suggesting a 5-HT_{1A} receptor selective effect of DPAT.

Figure 3.

Intrathecal (i.t.) injection of DPAT (5μmol) into the lumbo-sacral (LS) segment of the spinal cord significantly (* $p < 0.05$ vs baseline) enhanced VMR at all intensities of distension pressure (10 – 60mmHg).

Figure 4.

Increase in the VMR by DPAT is associated with NMDA receptor mediated visceral hyperalgesia. An injection of competitive NMDA receptor antagonist AP5 (50 μ g/kg, i.v.) 10 minutes prior to DPAT injection completely blocked the DPAT-induced increase in VMR. This dose of AP5 did not attenuate VMR.

Figure 5.

Illustrates the involvement of NMDA receptor in DPAT-induced increase in VMR. **A:** DPAT (5 μ mol, i.t.) failed to increase VMR in NMDA-NR1 knock down rats that received repeated injection of NR1 subunit targeted antisense oligoneucleotide (ON). The repeated i.t. injection of antisense ON did not attenuate VMR response. **B:** in mismatch ON-treated rats DPAT (5 μ mol, i.t.) produced significant (* p <0.05 vs pre-mis-ON) increase in VMR.

Figure 6.

Effects of DPAT (50μg/kg, i.p.) and WAY-100135 (10μmol, i.t.) in chronically allylglycine (AG)-treated rats. Repeated i.t. injection of AG, a GABA synthesis inhibitor, produced significant (* $p < 0.05$ vs baseline) increase in VMRs to colon distension. **A:** DPAT (5μmol, i.t.) injection produced a slight, but not significant, increase in VMR in AG-treated rats. **B:** GABA_A receptor agonist muscimol (1.5μmol, i.t.) significantly ($\#p < 0.05$ vs post-AG) decreased the VMR in AG-treated rats. DPAT partially, but not significantly, reversed the muscimol-induced inhibition of VMRs. ($^+p < 0.05$ vs muscimol). **C:** i.t. injection of WAY-100135 did not attenuate the AG-induced hyperalgesia suggesting that the effect of AG is independent of 5-HT_{1A} receptor.

Figure 7.

DPAT did not change the mechanotransduction properties of CRD-sensitive pelvic nerve afferent (PNA) fibers. **A:** illustrates responses of a non-spontaneous CRD-sensitive PNA fiber to graded intensities (20, 40, and 60mmHg) of colon distension before and 10 minutes after injection of DPAT (50µg/kg, i.p.). It should be noted that this dose of drug produced significant increase in VMR, but failed to sensitize responses of CRD-sensitive PNA fibers to colon distension. **B** and **C:** the mean stimulus-response functions (SRFs) before and after DPAT injection represented as the total number of firing and the percentage of maximum response of the fibers to 60mmHg before DPAT (pre-DPAT) injection, respectively.

Figure 8.

DPAT (50 μ g/kg, i.p.) significantly increased the responses of CRD-sensitive lumbo-sacral (LS) spinal neurons to colon distension in both spinal cord intact (**A & B**) and transected (**C & D**) conditions. Under both conditions the baseline spontaneous firing of the neurons increased following DPAT injection. **A**: an example of spontaneously firing of CRD-sensitive spinal neuron from spinal intact rat before and after DPAT (50 μ g/kg, i.p.) injection. Following drug injection neuron exhibited greater spontaneous firing and response to colon distension. **B**: the mean SRFs of LS spinal neurons (n=3) from spinal cord intact rats before and after DPAT injection. The neurons exhibited significantly ($p<0.05$ vs baseline) greater response after DPAT injection. **C**: an example of spontaneously firing CRD-sensitive spinal neuron from a spinal cord transected rat before and after DPAT injection. Following drug injection neuron exhibited greater spontaneous firing and response to colon distension. **D**: the mean SRFs of LS spinal neurons (n=6) from spinal cord transected rats before and after DPAT injection. These neurons exhibited significantly ($p<0.05$ vs baseline) greater response after DPAT injection suggesting that the drug produces excitatory effect at the spinal level without the supra-spinal influence.

Figure 9.

The mean SRFs of lumbo-sacral (LS) spinal neurons (n=3) to graded CRD and effects of DPAT (50µg/kg, i.p.) in presence of selective 5-HT_{1A} receptor antagonist WAY-100135 (5mg/kg, s.c.). The excitatory effect of DPAT on responses of CRD-sensitive LS spinal neurons was blocked in presence of WAY-100135.

Figure 10.

Pretreatment with the competitive NMDA receptor antagonist AP5 (50μg/kg, i.v.) blocked the DPAT-induced excitation of CRD-sensitive lumbo-sacral (LS) spinal neurons to colon distension. **A:** illustrates an example of CRD-sensitive LS spinal neuron following the injection of AP5 (50μg/kg, i.v.) and DPAT (50μg/kg, i.p.) in presence of AP5. **B:** The mean SRFs of LS spinal neurons (n=5) to graded CRD and effects of DPAT in presence of NMDA receptor antagonist AP5. DPAT failed to produce excitation of LS neurons to CRD in presence of AP5.