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Research Report

Involvement of serotonin 2A receptors in the analgesic effect of tramadol in mono-arthritic rats

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ABSTRACT

The analgesic effects of tramadol are considered to be mediated by both the opioid system and the serotonergic system. This study investigated the involvement of a subtype of serotonin receptors, 5-hydroxytryptamine (5-HT)$_{2A}$ receptor, in the analgesic effect of tramadol. The intraperitoneal (i.p.) injection of tramadol reduced the paw withdrawal latency (PWL) to radiant heat testing in mono-arthritic rats. The antagonistic effect of i.p. ketanserin (a 5-HT_{2A} receptor antagonist) on tramadol analgesia was observed. The expression of the 5-HT_{2A} receptor mRNA in the nucleus of raphe magnus (NRM), ventrolateral periaqueductal gray (vlPAG) and spinal dorsal horn of mono-arthritic rats after a ten-day treatment with tramadol was measured with in situ hybridization. Either single injections or 10 days of tramadol treatment dose-dependently elevated PWL of arthritic rats while ketanserin could partially antagonize the tramadol analgesic effect. Expression of the 5-HT_{2A} receptor mRNA in NRM, ipsilateral vlPAG, and the ipsilateral spinal dorsal horn of arthritic rats was significantly increased after tramadol treatment. These results suggest that 5-HT_{2A} receptors are involved in the analgesic effect of tramadol. This study provides evidence for involvement of 5-HT_{2A} receptors in the tramadol analgesia of inflammatory pain. The increase in this receptor mRNA in the chronic study may contribute to the sustaining effect of tramadol long-term treatments in clinical practice.

1. Introduction

Tramadol is widely used in the clinical treatment of chronic pain. It has few cardiovascular and respiratory side-effects and a low incidence of abuse with long-term treatment (Grond and Sablotzki, 2004; McClellan and Scott, 2003; Shipton, 2000). Tramadol activates μ opioid receptors, and it also inhibits the reuptake of serotonin (5-hydroxytryptamine (5-HT)) (Grond and Sablotzki, 2004; Lee et al., 1993). The analgesic effect of tramadol is believed to be mediated by the μ opioid receptor. However, the analgesic action of tramadol is partially decreased, but not completely blocked, by naloxone, an opioid receptor antagonist, which suggests that the opioid receptors only mediate part of tramadol analgesia (Raber et al., 1999). In

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Abbreviations: 5-HT, 5-hydroxytryptamine; NRM, nucleus of raphe magnus; vlPAG, ventrolateral periaqueductal gray; CFA, complete Fruends' adjuvant; PWL, paw withdrawal latency

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addition, tramadol, unlike other opioids, has a low incidence of tolerance during long-term (months) treatments, indicating that some non-opioid mechanisms probably contribute to tramadol analgesia, especially in the long-term treatments (Colpaert, 2006; Mattia and Coluzzi, 2005).

Serotonin in the central nervous system (CNS) regulates pain transmission through a complex family of receptors (Eide and Hole, 1993; Furst, 1999; Mason, 1999; Millan, 2002; Stamford, 1995). Subtypes of receptors in 5-HT1, 2 and 3 families are involved in the pain transmission (Colpaert, 2006; Faerber et al., 2007; Millan, 2002). Some studies have reported that the 5-HT_{2A} receptors, a subtype of serotonin receptors, are involved in serotonergic pain regulation. The 5-HT_{2A} receptors exist in the brainstem descending pain modulation pathways, including nucleus raphe magnus (NRM), ventrolateral periaqueductal gray (vlPAG) and the spinal dorsal horn (Cornea-Hebert et al., 1999; Fonseca et al., 2001; Lopez-Gimenez et al., 2001). The expression of the 5-HT_{2A} receptor mRNA in vlPAG, NRM and spinal dorsal horn is increased in rat inflammatory pain models, suggesting that these 5-HT_{2A} receptors are involved in the inflammatory pain process (Xie et al., 2002; Zhang et al., 2001). Other studies reported that activation of 5-HT_{2A} receptors with 5-HT or 5-HT_{2A} receptor agonists suppressed inflammatory pain or neuropathic pain (Bardin et al., 2000; Radhakrishnan et al., 2003; Sasaki et al., 2003). These studies suggested that the 5-HT_{2A} receptors may mediate the antinoceceptive effects of serotonin.

Since tramadol increases serotonin by inhibiting reuptake, the serotonergic system has been suggested to be involved in tramadol analgesia (Grond and Sablotzki, 2004; Rojas-Corrales et al., 2005). It is possible that 5-HT_{2A} receptors may also be involved in the antinoceceptive effect of tramadol. The first experiment of the present study is designed to test this possibility by evaluating the effect of a 5-HT_{2A} receptor antagonist, ketanserin, on tramadol analgesia. The second experiment explores the mechanisms that contribute to the low incidence of tolerance during the long-term treatments of tramadol. It is possible that more 5-HT receptors mediate tramadol analgesia in the long-term treatments, which compensates the tolerance developed by opioid receptors mediated mechanisms. We observe the changes in the expression of 5-HT_{2A} receptor mRNA in the brainstem and spinal cord after a ten-day treatment with tramadol.

2. Results

2.1. *Analgesic effects of different doses of tramadol after single injection*

Mono-arthritis was induced in the rat by an injection of 0.05 ml of complete Fruends' adjuvant (CFA) into the left hind tibiotarsal joint. The paw withdrawal latency (PWL) from radiant heat was used to assess nociceptive responses to thermal stimuli.

The first part of the test evaluated the effects of different doses of tramadol on thermal hyperalgesia in arthritic rats. The arthritic rats were randomly assigned to 4 groups of 8 rats per group. One group received intraperitoneal (i.p.) 0.2 ml normal saline (NS-4hr group). Rats in the other three groups received either 5 mg/kg tramadol (Tra5-4hr group), or 10 mg/kg tramadol (Tra10-4hr group) or 20 mg/kg tramadol (Tra20-4hr group). PWLs were determined before and at 30, 60, 120, and 240 min after drug injection. PWLs were determined just once for each time point.

The PWLs of rats in all groups significantly decreased from baseline at 3 days after an injection of CFA into the joint. The time-dose curve was obtained for three different doses of tramadol with NS as a control group (Fig. 1). The effects of each dose of tramadol or NS on PWLs were significantly different over the test period ($F=2.77$, $p<0.01$). NS did not increase the PWL at any time point. Tra5-4hr group had similar results to the NS-4hr group ($p>0.05$). Compared to NS-4hr group, Tra10-4hr group had increased PWL at 30 and 60 min (both $p<0.05$), and Tra20-4hr group had increased PWL at 30, 60, 120 and 240 min (all $p<0.05$). This study suggests that a single injection of tramadol at 10 or 20 mg/kg dose-dependently elevated the PWL of arthritic rats, but not at the 5 mg/kg dose.

2.2. *Antagonistic effect of ketanserin on tramadol analgesia*

After selecting the optimal dose of tramadol, the effect of i.p. ketanserin on tramadol analgesia was tested. 32 other arthritic rats were randomly assigned to 4 groups of 8 rats per group. The NS-single group received i.p. 0.2 ml normal saline, the Ket-single group received i.p. 1 mg/kg ketanserin, the Tra-single group received i.p. 10 mg/kg tramadol, or lastly the T+K-single group received i.p. 1 mg/kg ketanserin and 10 mg/kg tramadol.

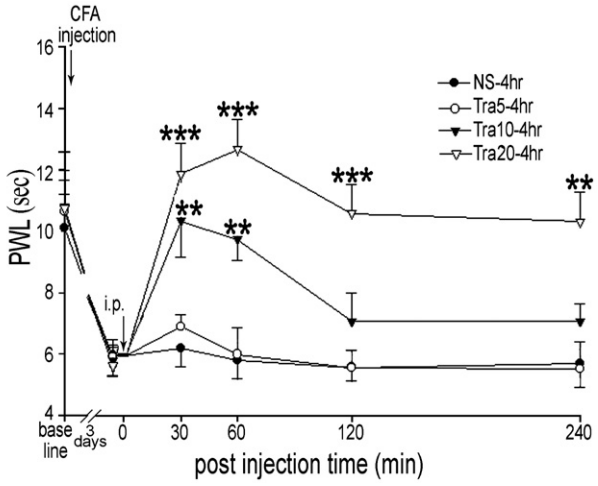

Fig. 1 – Effects of a single injection of tramadol or saline on paw withdraw latency (PWL) of CFA-induced arthritic rats in a 4 hour (4 h) observation. The arrows indicate the time of the injection of CFA and the i.p. injection of tramadol or saline. A CFA injection significantly decreased PWL from baseline at day 3 in all groups. Saline did not alter the PWLs. Tra5-4hr group had similar results as NS-4hr group. In contrast, Tra10-4hr group and Tra20-4hr group had significantly longer PWLs than NS-4hr group at 30 and 60 min after tramadol injection. Furthermore, PWLs of Tra20-4hr group were also longer than that of NS-4hr group at 120 and 240 min. The error bars indicate the S.E.M. (*: $p<0.05$, **: $p<0.01$, and ***: $p<0.005$ compared to NS-4hr group; $n=29$).

In the T+K-single group, ketanserin was injected 15 min prior to tramadol. PWLs were determined before drug administration and at 30, 60, 120 and 240 min after i.p. injection.

The effects of NS, tramadol, ketanserin, and tramadol+ketanserin on the PWLs of arthritic rats were significantly different over the test period ($F=7.26$, $p<0.01$, Fig. 2). An injection of NS did not alter the PWLs, but the injection of 10 mg/kg tramadol in Tra10-single group increased PWL at 30 and 60 min compared to that of the NS-single group (both $p<0.05$). The effect of tramadol was reduced when given after an injection of ketanserin in the T+K-single group. The reduction was significant at 60 min ($p<0.05$) when using Student–Newman–Keuls test to analyze the data at each time point. In addition, the PWLs of the T+K-single group were not significantly different from those of the NS-single group at all time points (all $p>0.05$). An injection of ketanserin alone had no effect on PWL in the Ket-single group at any time point (all $p>0.05$). This study suggested that ketanserin partially antagonized tramadol analgesia.

2.3. *Analgesic effects of different doses of tramadol during 10-day treatment*

32 other arthritic rats were randomly assigned to 4 groups of 8 rats per group, which received i.p. 0.2 ml per day normal saline (NS-10day group), 5 mg/kg per day tramadol (Tra5-10day group), 10 mg/kg per day tramadol (Tra10-10day group) or lastly 20 mg/kg per day tramadol (Tra20-10day group). Drugs were administered for 10 days. PWLs were determined immediately before the injection of tramadol on day 2, 4, 6, 8 and 10.

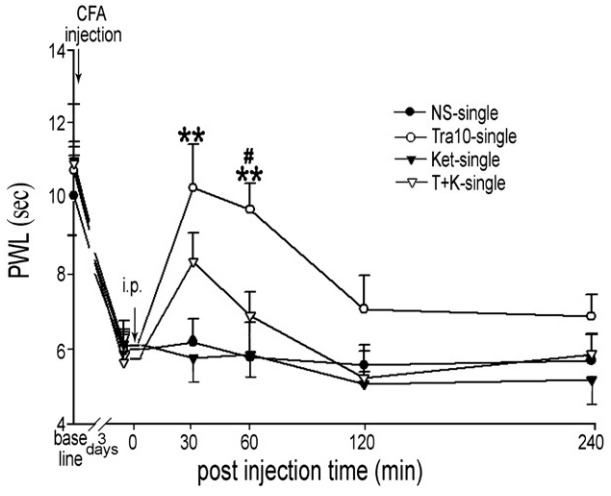

Fig. 2 – The effect of ketanserin on tramadol analgesia. The PWLs decreased in all groups in 3 days after the CFA injection. An injection of NS and 1 mg/kg ketanserin did not alter the PWLs of arthritic rats in NS-single group and Ket-single group, but 10 mg/kg tramadol significantly elevated the PWLs at 30 and 60 min after a single i.p. injection. This effect was not seen if tramadol was given after a ketanserin injection (*p<0.05, **: $p<0.01$, and ***: $p<0.005$ compared to NS-4 h group; #: $p<0.05$ comparing Tra10-single group and T+K-single group; other conventions are same as Fig. 1; $n=28$).

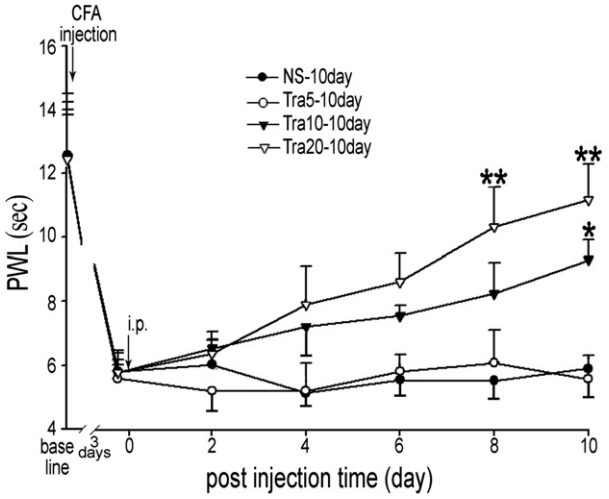

Fig. 3 – Effects of tramadol or saline on PWL of CFA-induced arthritic rats in 10-day treatments. Saline did not alter the PWL during 10 days in NS-10day group. Tra5-10day group had similar results as NS-10day group. In contrast, Tra10-10day group had a longer PWL at day 10, and Tra20-10day group had significantly longer PWLs than NS-10day group at days 8 and 10. (Conventions are same as Fig. 1; $n=29$).

PWLs were determined three times each day with an interval of 5 min. The mean of the three readings was taken as the PWL.

The time-response relationship was obtained for different doses of tramadol and NS during 10 days of treatment (Fig. 3). The effects of each dose of tramadol or NS were significantly different over the treatment period ($F=10.26$, $p<0.01$). NS did not increase the PWL on any day. The Tra5-10day group had similar results as the NS-10day group ($p>0.05$). Compared to NS-10 day group, Tra10-10day group had increased PWL at day10 ($p<0.05$), and Tra20-10day group had increased PWL from day 8 to 10 (all $p<0.05$). These results showed that 10 or 20 mg/kg chronic tramadol treatment dose-dependently relieved nociceptive responses to thermal stimuli in arthritic rats. The NS or 5 mg/kg tramadol did not increase the PWL over 10 days. In addition, the 10 mg/kg tramadol produced a detectable increase in PWL within a 10-day dosing period.

2.4. *Effect of 10-day tramadol treatment on the expression of 5-HT_{2A} receptor mRNA in some of the brain nuclei of arthritic rats*

After determining the dose-response relationship of tramadol, 24 other rats were divided into three groups to examine the expression of 5-HT_{2A} receptor mRNA with in situ hybridization. Eight normal rats were treated with i.p. 0.2 ml normal saline per day (normal-chronic group). Mono-arthritis was induced in remaining 16 rats. 8 arthritic rats were treated with i.p. 0.2 ml normal saline per day (NS-chronic group) and the other 8 arthritic rats were treated with i.p. 10 mg/kg tramadol per day (Tra-chronic group). All rats were treated for 10 days and then they were sacrificed on the next day for in situ hybridization.

The 5-HT_{2A} receptor mRNA-positive cells were stained with the hybridized reactive products in the cell cytoplasm. No hybridized stain was observed in nuclei and axons. Sections

were incubated in hybridization solution with or without sense probe showed no or very faint signals. When unlabeled probe was added in excess (30×) to hybridization solution or the sections were pre-incubated in RNase (200 μg/ml), the signal was not observed (not shown).

Expression of 5-HT_{2A} receptor mRNA of normal rats in the normal-chronic group was low in NRM and vlPAG (Fig. 4A, B). In the NS-chronic group and in the Tra-chronic group, distributions of 5-HT_{2A} receptor mRNA-positive cells in NRM and ipsilateral vlPAG of arthritic rats were similar to normal rats. However, the number of positive cells in NRM and ipsilateral vlPAG of arthritic rats in the NS-chronic group was significantly more than that of rats in the normal-chronic group (both $p<0.05$; Figs. 4C, D and 5). After 10 mg/kg tramadol treatment for 10 days, the number of 5-HT_{2A} receptor mRNA-positive cells in NRM and ipsilateral vlPAG were more than the positive cells in the same nuclei of normal-chronic group (both $p<0.05$) and of NS-chronic group (both $p<0.05$; Figs. 4E, F and 5). In summary, the arthritic rats had more 5-HT_{2A} receptor mRNA-positive cells in NRM and ipsilateral vlPAG than the normal rats, and a ten-day treatment of tramadol enhanced this increase in the number of positive cells (NRM: $F=33.25$, $p<0.001$; vlPAG: $F=28.27$, $p<0.001$).

2.5. *Effect of 10-day tramadol treatment on the expression of 5-HT_{2A} receptor mRNA in the lumbar spinal dorsal horn of arthritic rats*

In normal rats, the expression of 5-HT_{2A} receptor mRNA was low to moderate in laminae I–VI of lumbar spinal dorsal horn with an increasing gradient from the superficial laminae to the deeper ones (Fig. 6A).

The 5-HT_{2A} receptor mRNA-positive cells in all layers of lumbar spinal dorsal horn ipsilateral to the arthritic joint in

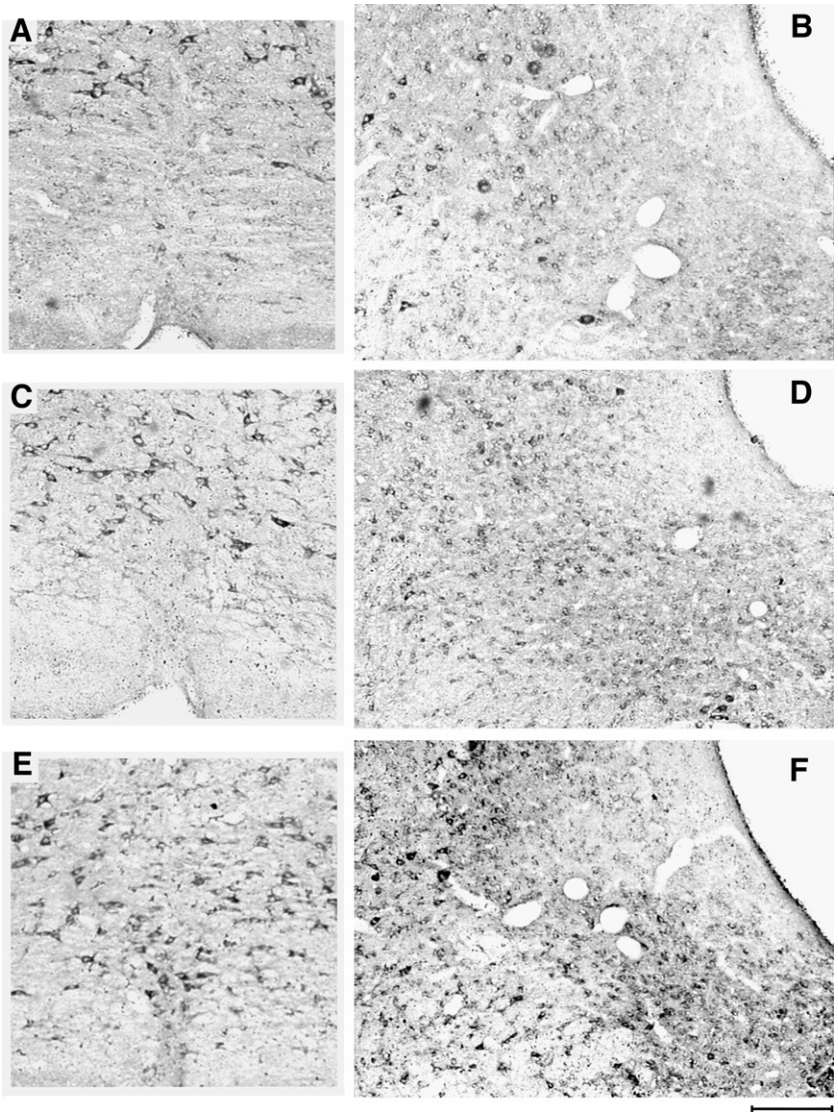

Fig. 4 – Examples of expression of 5-HT_{2A} receptor mRNA in NRM and in vlPAG of normal rats, and of the arthritic rats treated with tramadol or saline for 10 days (chronic). NRM (A) and ipsilateral side vlPAG (B) of normal-chronic group; NRM (C) and ipsilateral side vlPAG (D) of NS-chronic group; and NRM (E) and ipsilateral side vlPAG (F) of Tra-chronic group. Calibration: 200 μm.

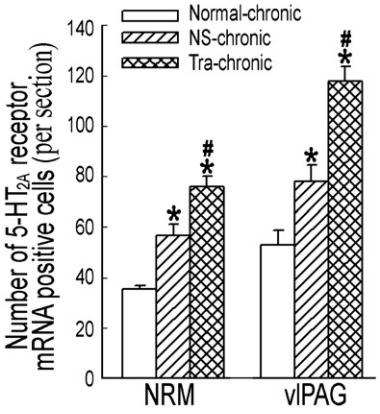

Fig. 5 – The effect of tramadol on the number of 5-HT$_{2A}$ receptor mRNA-positive cells in rat NRM and vlPAG after a chronic treatment (10 days). The bars indicate the average of cell counts crossing the sections from different animals (n=24) and the error bars indicate the SEM. The CFA-induced arthritic rats in the NS-chronic group and Tra-chronic group had significantly more 5-HT$_{2A}$ receptor mRNA-positive cells in NRM and ipsilateral vlPAG than the normal rats in the normal-chronic group (*: $p<0.05$). Tra-chronic group had more positive cells that NS-chronic group (#: $p<0.05$).

the NS-chronic group was significantly more than that of rats in normal-chronic group (laminae I–VI: all $p<0.05$; Figs. 6B and 7). After treatment of 10 mg/kg tramadol for 10 days, the positive cells in the injury side of lumber spinal dorsal horn were markedly increased compared to the positive cells in the same layers of normal-chronic group and of NS-chronic group (all $p<0.05$; Figs. 6C and 7). Over all, a ten-day treatment of tramadol further increased the number of 5-HT$_{2A}$ receptor mRNA-positive cells in all superficial, middle and deep layers of ipsilateral spinal dorsal horn of arthritic rats (SPI–II: $F=31.08$, $p<0.001$; SPIII–IV: $F=33.15$, $p<0.001$; SPV–VI: $F=21.37$, $p<0.001$).

3. Discussion

The present study suggests that the analgesic effect of tramadol in mono-arthritic rats was partially blocked by ketanserin, a 5-HT$_{2A}$ receptor antagonist. This result is consistent with the idea that the analgesic effect of tramadol is, in part, mediated by 5-HT$_{2A}$ receptors. The second experiment showed that tramadol produced significant analgesia in the mono-arthritic rats during a ten-day treatment and 5-HT$_{2A}$ receptor mRNA in NRM, vlPAG and lumbar spinal dorsal horn further increases after this ten-day treatment. These findings imply that more 5-HT$_{2A}$ receptors are available to contribute to tramadol analgesia after the long-term treatments.

The involvement of serotonin in the descending control of pain pathway is widely recognized. Abundant 5-HT neurons in NRM integrate input from the PAG and forebrain regions and then project diffusely to the spinal dorsal horn to modulate pain transmission (Mason, 1999). The effect of 5-HT is partly mediated by 5-HT$_{2A}$ receptors which are widely distributed in this system (Millan, 2002). The 5-HT$_{2A}$ receptors are expressed in vlPAG, NRM and spinal dorsal horn. In spinal dorsal horn, the 5-HT$_{2A}$ receptors are distributed throughout deep (V–VI), and superficial laminae (I–II), both of which receive noxious information from C and Aδ afferent fibers (Cornea-Hebert et al., 1999; Zhang et al., 2001). Behavioral studies further suggest the involvement of the 5-HT$_{2A}$ receptors in pain modulation (Bardin et al., 2000; Sasaki et al., 2003). Microinjection of 5-HT into

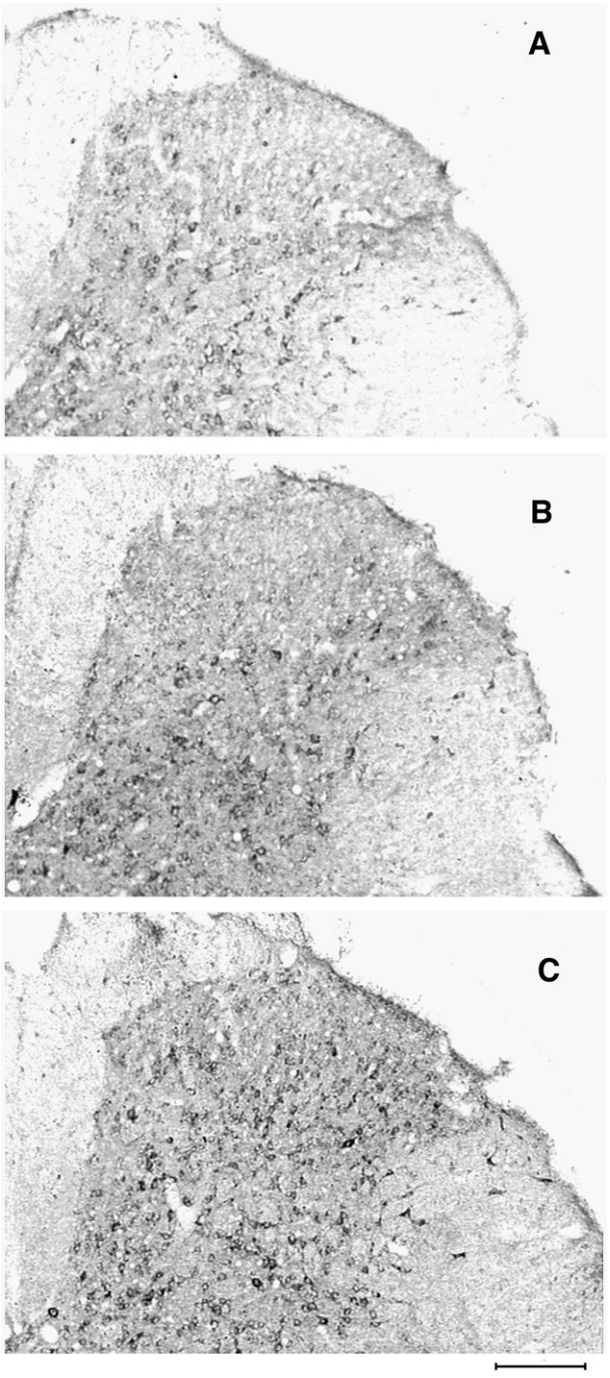

Fig. 6 – Examples of expression of 5-HT$_{2A}$ receptor mRNA in the lumbar spinal dorsal horn of normal rats, and of ipsilateral side of the arthritic rats treated with tramadol or saline for 10 days (chronic). A: Normal-chronic group; B: NS-chronic group; and C: Tra-chronic group. Calibration: 200 μm.

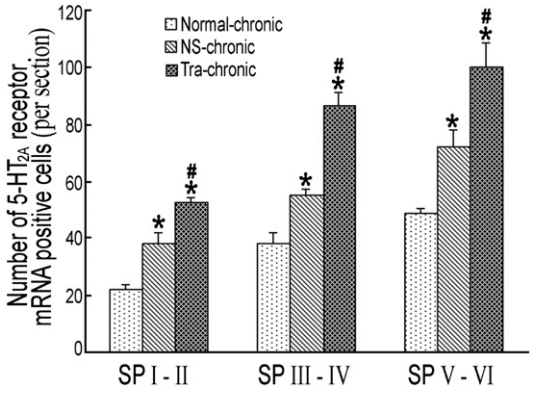

Fig. 7 – The effect of tramadol on the number of 5-HT$_{2A}$ receptor mRNA-positive cells in rat lumbar spinal dorsal horn after a chronic treatment (10 days). The CFA-induced arthritic rats in the NS-chronic group and Tra-chronic group had significantly more 5-HT$_{2A}$ receptor mRNA-positive cells in the lumbar spinal dorsal horn ipsilateral to the arthritic joint than the normal rats in the normal-chronic group. Furthermore, Tra-chronic group had more positive cells than NS-chronic group. (Conventions are same as Fig. 5; $n=24$).

NRM and PAG raises the threshold of pain sensation, which can be abolished or attenuated by an injection of ketanserin into PAG (Lin et al., 1987). Moreover, the 5-HT$_{2A}$ receptors in NRM may mediate morphine analgesia because microinjection of 5-HT$_{2A}$ receptor antagonists into NRM significantly reduces morphine analgesia (Paul and Phillips, 1986; Paul et al., 1988). These results suggest that supraspinal 5-HT$_{2A}$ receptors may contribute to the descending serotonergic down-regulation of pain.

Although the role of spinal 5-HT$_{2A}$ receptors in pain regulation is still uncertain (Hori et al., 1996), several studies suggest that activation of spinal 5-HT$_{2A}$ receptors may also down-regulate pain since intrathecal injections of 5-HT or 5-HT$_{2A}$ receptor agonists produce analgesia that can be reduced by 5-HT$_{2A}$ receptor antagonists (Bardin et al., 2000; Danzebrink and Gebhart, 1991; Sasaki et al., 2003). The down-regulation of pain of 5-HT$_{2A}$ receptors may be attributed to the fact that 5-HT$_{2A}$ receptors are mainly located on inhibitory interneurons. These 5-HT$_{2A}$ receptors trigger glycenergic/GABAergic inhibition to suppress nociceptive inputs (Barnes and Sharp, 1999). The 5-HT$_{2A}$ receptors appear to be involved in pain regulation, therefore, activating this type of receptors may down-regulate pain.

Tramadol likely affects the 5-HT$_{2A}$ receptors because tramadol can inhibit the neuronal 5-HT reuptake and increase serotonin efflux (Bamigbade et al., 1997; Giusti et al., 1997). Behavioral studies show that the analgesic effect of tramadol in formalin tests is mediated by 5-HT$_{2A}$ receptors (Oliva et al., 2002). In the present study, tramadol reduced thermal hyperalgesia in mono-arthritic rats. This effect of tramadol was partially blocked by a 5-HT$_{2A}$ receptor antagonist. These findings suggest that the analgesic effect of tramadol on the thermal pain model is partially mediated by 5-HT$_{2A}$ receptors.

In our study, 10 days of tramadol treatment enhanced the increase in 5-HT$_{2A}$ receptor mRNA in NRM, vlPAG and spinal dorsal horn. We have looked at mRNA as an indicator of protein expression and acknowledging the caveats that protein expression does not always coincide with the changes in the level of mRNA. Under normal physiological conditions, the 5-HT$_{2A}$ receptors are expressed with low to moderate levels in vlPAG, NRM and spinal dorsal horn (Cornea-Hebert et al., 1999; Zhang et al., 2001). The expression of 5-HT$_{2A}$ receptor mRNA in vlPAG, NRM and spinal dorsal horn was significantly increased during CFA-induced mono-arthritis (Xie et al., 2002). The increase in 5-HT$_{2A}$ receptor mRNA may produce more receptors. More 5-HT$_{2A}$ receptors could enhance the descending analgesic system because of the analgesic effect of 5-HT$_{2A}$ receptors in these nuclei according to the behavioral studies. The further increase in 5-HT$_{2A}$ receptor mRNA after tramadol treatments in the present study may augment the down-regulation of pain. Based on this finding, we propose that the 5-HT$_{2A}$ receptor may play an increasing role in the long-term analgesic effect of tramadol. In order to prove our proposal, the examination of 5-HT$_{2A}$ receptor protein level is necessary to rule out the possibility that the protein synthesis may not change even though the expression of mRNA increases.

The mechanisms underlying the 5-HT$_{2A}$ receptor mRNA increase by chronic tramadol treatment remain to be investigated, but again it could be related to the findings that tramadol can inhibit the neuronal 5-HT reuptake and increase serotonin efflux. Available studies suggest that increases in 5-HT levels induce the expression of 5-HT$_{2A}$ receptor mRNA through multiple pathways, including (1) activation of transcriptional promoter in the primary cell culture of cerebellar cells (Akiyoshi et al., 1993) or myometrial smooth muscle cells (Rydelek-Fitzgerald et al., 1993); and (2) a post-transcriptional, protein kinase C-dependent mechanism in P11 cell culture (Ferry and Molinoff, 1996).

Clinical studies have reported that tramadol, particularly when used with other analgesics, has sustaining efficacy in long-term (several months or longer) pain treatment (Petzke et al., 2001; Ruoff et al., 2003; Schug, 2006). There is a low risk of developing tolerance to tramadol, unlike many other opioids (Mattia and Coluzzi, 2005). It has been speculated that this effect is due to serotonergic analgesia (Colpaert, 2006). The present finding of increased 5-HT$_{2A}$ receptor mRNA after a 10-day tramadol treatment, suggests an increase in serotonergic analgesia. If a similar increase in 5-HT$_{2A}$ receptor mRNA happens after several months of treatments of tramadol, the increase in serotonergic analgesia may account for a significant part of the sustaining effect of tramadol long-term treatment.

In conclusion, the present study suggests that tramadol treatment alters the 5-HT$_{2A}$ receptor mRNA in the brainstem nuclei and spinal dorsal horn, which may partially mediate the analgesic effect of tramadol. Future studies to explore the effect of acute or chronic tramadol treatments on the various subtypes of 5-HT receptors are important to understand the mechanisms of tramadol analgesia.

4. Experimental procedures

4.1. *Experimental animals*

Adult male Sprague–Dawley rats (Experimental Animal Center, Shanghai Medical College, Fudan University, China), weighing

200–220 g, were used. After arrival at the laboratory, the rats were allowed to acclimate for 1 week in groups of six rats per cage and maintained under a regular 12 h light/dark schedule with free access to food and water. The experiment was approved by the Animal Care and Use Committee of Shanghai and conformed to NIH guidelines.

Mono-arthritis was induced by the injection of 0.05 ml of complete Fruends' adjuvant (CFA) into the left hind tibiotarsal joint of the rat under anesthesia (Butler et al., 1992). After CFA injection, the rats were housed in cages with the floor of the cage covered with sawdust to minimize the possibility of painful mechanical stimulation. Rats were able to eat and drink unaided.

4.2. *Nociceptive testing*

All nociceptive experiments were performed between 9:00AM and 2:00PM. The paw withdrawal from radiant heat was used to assess nociceptive responses to thermal stimuli. Prior to testing, the rats were placed in a clear plexiglass box on an elevated plexiglass platform for at least 30 min for acclimatization. A model 33 Tail Flick Analgesia Meter (IITC INC. Life Science Instrument, U.S.A.), with a constant intensity radiant heat source and a built-in timer, was directed toward the hind tibiotarsal joints. When the rats withdrew the paw from the heat stimulus, the heat source and timer were stopped. The time, in seconds, from initial heat source activation until paw withdrawal was defined as the paw withdrawal latency (PWL). A cut-off time was set at 20 s to avoid excessive animal nociception. The intensity of the heat source was adjusted to obtain a baseline of PWL between 8 and 15 s. The entire baseline PWL to heat was determined bilaterally before mono-arthritis was induced. The rats were observed 3 days after CFA injection before the nociceptive testing was performed.

4.3. *In situ hybridization*

Twenty-four hours after the last treatment, they were perfused with normal saline followed by 4% paraformaldehyde under i.p. 75 mg/kg of sodium pentobarbital anesthesia. After being post-fixed in the fixative solution and immersed in 30% sucrose, frozen sections (30 μm) were cut and placed in cryoprotectant solution and then stored at −20 °C until further study.

The method of in situ hybridization was previously described (Xie et al., 2002). A 48 mer oligonucleotide probe for rat 5-HT_{2A} receptor mRNA containing the sequence 5′-AGTGTTAAGCATCTCTGGAGTTGAAGTCATTATGGTAGAGCCTCCTCGGGC-3′ (nucleotides 3–51) was synthesized and purified by Beijing Bioneer Corporation (Nebigil et al., 1995). The probe was 3′ end-labeled with DIG-11-UTP by using the oligonucleotide-tailing kit (Boehringer Mannheim Biochemical, Germany). The specificity of the DIG-labeled antisense oligonucleotide probe was examined by hybridizing with a labeled sense probe, a saturation of unlabeled probe or omitting the probe in the hybridization solution as well as RNase pretreatment.

Tissue sections were rewarmed for 10 min in 4% paraformaldehyde at room temperature. Every other section was used for in situ hybridization and the adjacent sections were stained with cresyl violet to aid in anatomic localization. Sections were rinsed in 0.1 M PBS (pH 7.4, 4×15 min), proteinase K (1 μg/ml in 0.1 M Tris–HCl with 1 mM EDTA; pH 8.0; 20 min at 37 °C), 0.1 M PBS (2×15 min), 0.1 M triethanolamine in 0.25% acetic anhydride (pH 8.0; 10 min) and 50% formamide in 4×SSC (saline sodium citrate; pH 7.2; 20 min). Sections were then incubated in the hybridization solution (50% formamide, 5×SSC, 0.02% SDS, 2% blocking reagent and 1 mg/L of the DIG-labeled oligonucleotide probe) for 20 h at 37 °C. After hybridization, sections were subsequently rinsed in 2×SSC (2×15 min at 37 °C), 0.1×SSC (2×15 min at 42 °C) and buffer II (0.1 M Tris–HCl with 0.15 M NaCl, 0.3% Triton X-100 and 2% normal goat serum, 30 min). Sections were treated with alkaline phosphatase-labeled anti-DIG antibody (diluted 1:2500) for 2 h and rinsed in buffer I (0.1 M Tris–HCl with 0.15 M NaCl, 2×15 min) and buffer III (0.1 M Tris–HCl with 0.15 M NaCl and 0.05 M $MgCl_2$, 3 min). Finally, sections were visualized with NBT/BCIP in buffer III for over 6 h in the dark, and were mounted on gelatin-coated slides. The 5-HT_{2A} receptor mRNA-positive cells were counted using a computerized image analysis system (Leica Q500IW, Germany). The number of positive cells was evaluated in eight sections of NRM (10.3–11.3 mm caudal to bregma), ipsilateral vlPAG (7.5–8.0 mm caudal to bregma) and ipsilateral lumbar spinal dorsal horn in each animal, respectively. The investigator counting the labeled cells was blind to the treatment of the animals.

4.4. *Drugs*

Tramadol hydrochloride (Grünenthal GmbH, Germany) and ketanserin (Sigma, U.S.A) were diluted into various concentrations and used in experiments with normal saline. Complete Freund's adjuvant and alkaline phosphatase-labeled anti-DIG antibody were purchased from Sigma (U.S.A).

4.5. *Statistical analysis*

Data are presented as mean±SEM. Statistical analysis for the analgesic effects of tramadol was performed by using a two-way ANOVA and Bonferroni post tests. Statistical analysis for the expression of 5-HT_{2A} receptor mRNA was performed by one-way ANOVA followed by Newman–Keuls Multiple Comparison Test. $p<0.05$ was considered statistically significant.

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