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## Spinal 5-HT<sub>2</sub> and 5-HT<sub>3</sub> receptors mediate low, but not high, frequency TENS-induced antihyperalgesia in rats

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### Abstract

Transcutaneous electrical nerve stimulation (TENS) is a form of non-pharmacological treatment for pain. Involvement of descending inhibitory systems is implicated in TENS-induced analgesia. In the present study, the roles of spinal 5-HT and  $\alpha_2$ -adrenoceptors in TENS analgesia were investigated in rats. Hyperalgesia was induced by inflaming the knee joint with 3% kaolin—carrageenan mixture and assessed by measuring paw withdrawal latency (PWL) to heat before and 4 h after injection. The (1)  $\alpha_2$ -adrenergic antagonist yohimbine (30  $\mu$ g), (2) 5-HT antagonist methysergide (5-HT<sub>1</sub> and 5-HT<sub>2</sub>, 30  $\mu$ g), one of the 5-HT receptor subtype antagonists, (3) NAN-190 (5-HT<sub>1A</sub>, 15  $\mu$ g), (4) ketanserin (5-HT<sub>2A</sub>, 30  $\mu$ g), (5) MDL-72222 (5-HT<sub>3</sub>, 12  $\mu$ g), or (6) vehicle was administered intrathecally prior to TENS treatment. Low (4 Hz) or high (100 Hz) frequency TENS at sensory intensity was then applied to the inflamed knee for 20 min and PWL was determined. Selectivity of the antagonists used was confirmed using respective agonists administered intrathecally. Yohimbine had no effect on the antihyperalgesia produced by low or high frequency TENS. Methysergide and MDL-72222 prevented the antihyperalgesia produced by low, but not high, frequency TENS. Ketanserin attenuated the antihyperalgesic effects of low frequency TENS whereas NAN-190 had no effect. The results from the present study show that spinal 5-HT receptors mediate low, but not high, frequency TENS-induced antihyperalgesia through activation of 5-HT<sub>2A</sub> and 5-HT<sub>3</sub> receptors in rats. Furthermore, spinal noradrenergic receptors are not involved in either low or high frequency TENS antihyperalgesia.

### Keywords

Inflammation; Serotonin; Adrenergic; Pain; Carrageenan; Spinal cord

### 1. Introduction

Transcutaneous electrical nerve stimulation (TENS) is a form of non-pharmacological therapy, clinically used to relieve acute and chronic pain (Johnson et al., 1992; Chesterton et al., 2002). This treatment modality is easy to use and devoid of major side effects. Although the use of TENS is very common, its analgesic mechanism is not fully understood. Conclusions

regarding the efficacy of TENS from systematic reviews have been contradictory (for review, see Sluka and Walsh, 2003). Cochrane database reviews conclude that TENS has significant analgesic effects above placebo in people with osteoarthritis (Osiri et al., 2000) but not for people with low back pain (Milne et al., 2001). Carroll et al. (2001) state that the results of a systematic review on TENS efficacy are inconclusive for people with chronic pain. Lack of randomized, controlled, blinded clinical trials are seen as the major reason for contradictory and inconclusive findings. Two types of TENS are used clinically, low frequency TENS (frequency of stimulation < 10 Hz) and high frequency TENS (frequency > 50 Hz). Different theories have been proposed for the mechanism of action of TENS, the popular one being the gate control theory proposed by Melzack and Wall (1965). According to the gate control theory, nociceptive information from small diameter afferents is overridden by stimulation of large diameter fibres and the pain stimulus is prevented from reaching supraspinal centers. Endogenous opioids released in the central nervous system are also implicated in the analgesic mechanism of TENS by various investigators (Woolf et al., 1977; Sjölund et al., 1977; Shimizu et al., 1981; Hughes et al., 1984; Sluka et al., 1999; Kalra et al., 2001). Data from our laboratory show that low frequency TENS produces antihyperalgesia by activating central  $\mu$ -opioid receptors while high frequency TENS activates  $\delta$ -opioid receptors both spinally and supraspinally in the rostral ventromedial medulla (RVM) (Sluka et al., 1999; Kalra et al., 2001).

The antihyperalgesic effect of TENS predominantly involves central (spinal and supraspinal) mechanisms (Sluka et al., 1999; Kalra et al., 2001) rather than peripheral mechanisms (Janko and Trontelj, 1980). Descending inhibition from the periaqueductal gray (PAG) and RVM is mediated by serotonergic and noradrenergic receptors spinally (Aimone et al., 1987; Fields and Basbaum, 1999) and the effector neurotransmitters are serotonin and norepinephrine. Norepinephrine is antinociceptive in the spinal dorsal horn and produces analgesic effects mainly through activating  $\alpha_2$ -adrenoceptors (Reddy et al., 1980; for review, see Yaksh, 1985). Although  $\alpha_1$ -adrenergic receptors are found in the spinal cord, they may not be involved in pain inhibition (Miller and Proudfit, 1990). Serotonin also has antinociceptive effects spinally, depending on the receptor type activated and dose used (Yaksh and Wilson, 1979; Schmauss et al., 1983). Seven types of serotonin receptors have been identified (5-HT<sub>1-7</sub>) with a total of 14 subtypes. Serotonin receptor types commonly implicated in spinal pain processing are 5-HT<sub>1</sub>, 5-HT<sub>2</sub> or 5-HT<sub>3</sub> (Danzebrink and Gebhart, 1991a; Giordano, 1991; Zhuo and Gebhart, 1991; Eide and Hole, 1993). Spinal blockade of serotonin receptors with methysergide reduces low frequency (20 Hz) peripheral electrical stimulation (PES) analgesia and systemic depletion of serotonin with *p*-chlorophenylalanine (PCPA) reduces high frequency PES analgesia (Woolf et al., 1980; Chen, 1992; Scherder and Bouma, 1993; Liss and Liss, 1996).

Since serotonin and norepinephrine receptors in spinal cord mediate descending inhibition of pain from supraspinal structures, we investigated the roles of spinal serotonergic and noradrenergic receptors in the antihyperalgesic mechanism of TENS. Preliminary results from this study were presented in abstract form (Radhakrishnan et al., 2002).

## 2. Materials and methods

### 2.1. Animals

Male Sprague—Dawley rats ( $n = 171$ , 225–300 g, Harlan, St Louis, MO, USA) were housed in a 12 h dark—light cycle with free access to food and water. All experiments were approved by University of Iowa Animal Care and Use Committee and were carried out according to the guidelines of the International Association for the Study of Pain and National Institute of Health.

## 2.2. Behavior testing

Animals were acclimated overnight in the behavioral testing room and all behavioral tests were done between 9 a.m. and 5 p.m. Prior to testing, rats were placed in plexi-glass restrainers on an elevated glass platform for at least 30 min for acclimatization. A radiant heat source, connected to a built-in timer, was shone on the plantar skin of the hind limb. When the animal withdrew the paw abruptly to heat stimulus, the heat source and timer were stopped. The duration in seconds from the start of heat application to the paw withdrawal was taken as the paw withdrawal latency (PWL). PWLs were determined five times bilaterally, with an interval of 5 min between each test, and the mean of five readings was taken as the PWL for each time. The intensity of the heat source was set at optimum level with an adjustable voltage power supply, to obtain a baseline response time between 12 and 16 s. Cut-off time was set to 30 s to minimize heat damage to the skin.

## 2.3. Intrathecal catheter placement

A 32 G polyethylene catheter was placed intrathecally as described before (Pogatzki et al., 2000). Briefly, animals were anesthetized with 2% halothane and the dorsal surface shaved and cleaned with Betadine® solution. A 2 cm incision was made at the iliac crest. A 32 G polyethylene catheter was introduced into the lumbar space between L4 and L5 with the help of a 23 G guide needle and advanced to a length of 3.5–4 cm rostrally. The catheter was fixed in place and the tip connected to a saline filled PE10 tube, which was externalized dorsally between the scapulas. The tip of the catheter was sealed and the animal was allowed to recover for 5–7 days.

## 2.4. Intra-articular injection

After baseline PWL measurements, the left knee joint was injected with 0.1 ml suspension of 3% kaolin and 3% carrageenan in normal saline (pH 7.0) while the rat was anesthetized with halothane (2–4% in oxygen).

## 2.5. Drugs

Clonidine hydrochloride ( $\alpha_2$ -adrenoceptor agonist, 5  $\mu$ g, Danzebrink and Gebhart, 1991b), serotonin hydrochloride (30  $\mu$ g, Bardin et al., 1997), ( $\pm$ )-8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT, 5-HT<sub>1</sub> agonist, 50  $\mu$ g, Obata et al., 2001), ( $\pm$ )-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI, 5-HT<sub>2</sub> agonist, 30  $\mu$ g, Sasaki et al., 2001), yohimbine hydrochloride ( $\alpha_2$ -adrenoceptor antagonist, 30  $\mu$ g; Solomon et al., 1989), methysergide maleate (5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptor antagonist, 30  $\mu$ g; Hammond et al., 1998) and ketanserin tartrate (5-HT<sub>2A</sub> receptor antagonist, 30  $\mu$ g; Sasaki et al., 2001) were dissolved in normal saline (pH = 7.0). 2-Methyl-serotonin maleate (5-HT agonist, 50  $\mu$ g, Giordano, 1991), 1-(2-methoxyphenyl)-4-(4-[2-phthalimido]butyl)piperazine hydrobromide (NAN-190, 5-HT<sub>1A</sub> receptor antagonist, 15  $\mu$ g; Mjelle et al., 1993) and 3-tropanyl-3,5-dichlorobenzoate (MDL-72222, 5-HT<sub>3</sub> receptor antagonist, 12  $\mu$ g; Sasaki et al., 2001) were dissolved in 20% dimethyl sulfoxide (DMSO). 8-OH-DPAT was obtained from Tocris (Ellisville, MO, USA) and others from Sigma Chemical Co. (St Louis, MO, USA). The doses of antagonists utilized were selected based on previously published studies that show selectivity for the individual receptor types/subtypes and confirmed in the current study against their agonists.

## 2.6. Intrathecal drug administration

Drugs or vehicles were injected into the intrathecal (i.t.) space in a volume of 5 or 10  $\mu$ l, using a Hamilton syringe connected to the i.t. catheter via a PE10 tubing followed by 10  $\mu$ l vehicle to flush the catheter. After the experiment, proper placement of the catheter was confirmed by injection of 10  $\mu$ l of 2% lidocaine i.t. and observing animals for hind limb paralysis. Methylene blue dye solution (10  $\mu$ l., i.t.) was then injected. Animals were sacrificed, spinal cord dissected

and the dye spread assessed. The data from those animals, which did not show hind limb paralysis with lidocaine and in which the dye was not found in L4—L6 levels of spinal cord, were not included in the analysis.

## 2.7. TENS application

Commercially available TENS units and electrodes (EMPI Inc., Minnesota, USA) were used in this study. After drug or vehicle was administered intrathecally, the inflamed hind limb of the rat was shaved and cleaned with alcohol. Two circular, pregelled electrodes of 2.5 cm diameter were placed, one to the medial and the other to the lateral aspect, on the inflamed knee joint. This electrode placement is similar to placement of larger electrodes on the knee joint of human subjects. Either high frequency (100 Hz) or low frequency (4 Hz) TENS, at sensory intensity, was then applied through the electrodes for 20 min under light halothane anesthesia (1–2% in oxygen). Sensory intensity was defined as just below threshold for motor contraction. The pulse duration was kept constant at 100  $\mu$ s. A complete reversal of kaolin—carrageenan-induced hyperalgesia occurs with this protocol by both low and high frequency TENS (Sluka et al., 1999). In this study, the effects of various antagonists were studied only on different frequencies of TENS and not different intensities or pulse durations, since a previous study shows that TENS effects depend on modulation of frequency, but not on intensity or pulse duration (Gopalkrishnan and Sluka, 2000).

## 2.8. Protocol

Five to 7 days after intrathecal catheter placement, baseline PWLs to heat for both hind paws were determined and knee joint inflammation was induced by intra-articular injection of kaolin—carrageenan unilaterally. Four hours after injection, PWLs were determined again. Animals were then intrathecally injected with the one of the following drugs: yohimbine (30  $\mu$ g; no TENS,  $n = 5$ ; low frequency TENS,  $n = 5$ ; high frequency TENS,  $n = 5$ ), methysergide (30  $\mu$ g, no TENS,  $n = 6$ ; low frequency TENS,  $n = 11$ ; high frequency TENS,  $n = 12$ ), NAN-190 (15  $\mu$ g, no TENS,  $n = 7$ ; low frequency TENS,  $n = 8$ ), ketanserin (30  $\mu$ g, no TENS,  $n = 7$ ; low frequency TENS,  $n = 8$ ), MDL-72222 (12  $\mu$ g; no TENS,  $n = 8$ ; low frequency TENS,  $n = 8$ ) or the vehicles, normal saline (no TENS,  $n = 8$ ; low frequency TENS,  $n = 8$ ; high frequency TENS,  $n = 8$ ) or 20% DMSO (no TENS,  $n = 7$ ; low frequency TENS,  $n = 8$ ). After 15 min, either high or low frequency TENS was applied to the ipsilateral knee. PWLs were determined again, 30 min after TENS/no TENS treatment was completed, when the animals were fully awake from halothane anesthesia. Another group of animals did not receive TENS, ‘no TENS’ (control) group, were also anesthetized with halothane. Prior experiments show that shaving and placement of electrodes to the knee joint does not reduce hyperalgesia (Sluka, 2000). Since methysergide, a non-selective antagonist at 5-HT receptors, had effects only on low frequency, but not on high frequency TENS antihyperalgesia, selective 5-HT antagonists were studied for effects on low frequency TENS antihyperalgesia. Since methysergide does not block 5-HT<sub>3</sub> receptors, MDL-72222 was tested in an additional group ( $n = 4$ ) that received high frequency TENS.

The selectivity of various agonists against their respective antagonists was studied in separate groups of animals. All drugs were tested in animals with knee joint inflammation at the same 4 h time point that TENS was applied in the previous experiment. Specifically (1) yohimbine was tested for its ability to antagonize the effects of clonidine (yohimbine + clonidine,  $n = 4$ ; vehicle + clonidine,  $n = 3$ ); (2) methysergide was tested against serotonin (methysergide + serotonin,  $n = 4$ ; vehicle + serotonin,  $n = 3$ ); (3) NAN-190 was tested against 8-OH-DPAT (NAN-190 + 8-OH-DPAT,  $n = 4$ ; vehicle + 8-OH-DPAT,  $n = 4$ ); (4) ketanserin was tested against DOI (ketanserin + DOI,  $n = 4$ ; vehicle + DOI,  $n = 4$ ); and (5) MDL-72222 was tested against 2-methyl-5-HT (MDL-72222 + 2-methyl-5-HT,  $n = 4$ ; vehicle + 2-methyl-5-HT,  $n = 4$ ). The antagonist was given 15 min prior to agonist, as done in the TENS experiment. All

groups were compared against a control group that received saline or 20% DMSO (instead of antagonist) 15 min prior to the agonist.

## 2.9. Statistical analysis

All data are presented as mean  $\pm$  SEM. Data were compared with a multivariate analysis of variance for TENS treatment (no TENS, high frequency TENS, low frequency TENS) and drug treatment (two vehicles, five drugs) for time (baseline, hour 4, and post TENS). Since only post TENS showed significant effects for TENS treatment, drug and the interaction of TENS treatment with drug, Tukey's post hoc test compared differences between the appropriate vehicle control group treated with TENS and the drug group treated with TENS. Paired *t*-tests compared differences between baseline and hour 4 to establish hyperalgesia. The level of significance was set at  $p < 0.05$ . Statistical analysis was performed with SPSS versions 10.1.

## 3. Results

### 3.1. Carrageenan—kaolin-induced hyperalgesia and effect of TENS

Four hours after injection of kaolin and carrageenan into the knee joint of rats, there was a significant reduction in the ipsilateral PWL when compared to baseline ( $p = 0.0001$ ). There was an overall significant effect for PWL following TENS treatment ( $F_{1,128} = 53.6, p = 0.0001$ ) such that both high ( $p = 0.0001$ ) or low frequency ( $p = 0.0001$ ) TENS significantly reverse their hyperalgesia (Fig. 1). There were no significant differences between groups for the PWL ipsilaterally before or after induction of inflammation, or for the contralateral side. In saline controls, the ipsilateral mean PWLs (s) were: baseline =  $17.2 \pm 1.3$ , 4 h after carrageenan =  $11.1 \pm 0.78$ , after low frequency TENS =  $14.8 \pm 0.87$ , after high frequency TENS =  $14.98 \pm 0.96$ ; and contralateral mean PWLs (s) were: baseline =  $16.24 \pm 0.75$ , 4 h after carrageenan =  $17.3 \pm 0.91$ , after low frequency TENS =  $15.96 \pm 1.41$ , after high frequency TENS =  $17.3 \pm 1.48$ .

There was a significant effect for drug ( $F_{6,128} = 19.1, p = 0.001$ ) such that saline was significantly different from methysergide ( $p = 0.001$ ) and ketanserin ( $p = 0.006$ ), and DMSO was significantly different from MDL-72222 ( $p = 0.0001$ ). Importantly, there was an interaction between drug and TENS treatment ( $F_{8,128} = 5.4, p = 0.0001$ ). Post hoc results are listed below in appropriate sections.

### 3.2. Effect of $\alpha_2$ -adrenoceptor antagonist

Intrathecal (i.t.) injection of the  $\alpha_2$ -adrenoceptor antagonist yohimbine (30  $\mu\text{g}$ ) had no effect on the reversal of the ipsilateral PWL produced by low or high frequency TENS when compared to vehicle controls (i.t. saline). Yohimbine alone had no effects on PWL when given intrathecally at the same dose in animals with knee joint inflammation (Fig. 1).

### 3.3. Effect of non-selective 5-HT antagonist

Methysergide (30  $\mu\text{g}$ , i.t.), a non-selective antagonist at 5-HT receptors acting on 5-HT<sub>1</sub> and 5-HT<sub>2</sub>, significantly inhibited the reversal of the ipsilateral PWL produced by low frequency TENS ( $p = 0.04$ ), but not that produced by high frequency TENS, when compared to vehicle controls (i.t. saline, Fig. 1). The same dose of intrathecal methysergide alone had no effect on PWL in animals with knee joint inflammation (Fig. 1). Since methysergide blocked only low frequency TENS-induced antihyperalgesia, the effects of selective 5-HT receptor antagonists were tested on the antihyperalgesia produced by low frequency TENS.



### 3.4. Effects of selective 5-HT antagonists

The selective antagonist of the 5-HT<sub>1A</sub> receptor subtype, NAN-190 (15 µg, i.t.), had no effect on the antihyperalgesia produced by low frequency TENS when compared to vehicle control animals (i.t. 20% DMSO, Fig. 2). The same dose of NAN-190 had no effect on the decreased PWL produced by knee joint inflammation (Fig. 2).

The 5-HT<sub>2A</sub> subtype selective antagonist, ketanserin (30 µg, i.t.), significantly attenuated the antihyperalgesia produced by low frequency TENS, compared to vehicle control (i.t. saline,  $p = 0.03$ ). The same dose of ketanserin had no effect on the reduction in PWL produced by knee joint inflammation (Fig. 2).

The 5-HT<sub>3</sub> receptor selective antagonist, MDL-72222 (12 µg, i.t.), significantly attenuated the antihyperalgesia produced by low frequency TENS, compared to vehicle control (i.t. 20% DMSO,  $p = 0.0001$ ). The same dose of MDL-72222 had no effect on the decreased PWL produced by knee joint inflammation (Fig. 2). MDL-72222 had no effect on antihyperalgesia produced by high frequency TENS ( $16.32 \pm 0.96$  s) when compared to vehicle controls ( $15.85 \pm 0.83$  s).

### 3.5. Selectivity of antagonists

Intrathecal administration of all adrenergic and 5-HT agonists studied, significantly reversed the decreased PWL to heat produced kaolin—carrageenan injection. The reversal was blocked by intrathecal pre-administration of selective antagonists used in this study. Pre-treatment with (1) the  $\alpha_2$ -antagonist yohimbine (30 µg,  $p = 0.0008$ ), or (2) the non-selective serotonin antagonist methysergide (30 µg,  $p = 0.004$ ), significantly prevented the antihyperalgesia produced by clonidine ( $p = 0.02$ ) or serotonin ( $p = 0.03$ ), respectively (Fig. 3). Similarly, in kaolin—carrageenan inflamed rats, antihyperalgesia produced by intrathecal administration of selective serotonin receptor agonists to 5-HT<sub>1A</sub> (8-OH-DPAT, 50 µg,  $p = 0.008$ ), 5-HT<sub>2A</sub> (DOI, 30 µg,  $p = 0.03$ ) and 5-HT<sub>3</sub> (2-methyl 5-HT, 50 µg,  $p = 0.02$ ), administered intrathecally, were blocked by their selective antagonists, NAN-190 (15 µg,  $p = 0.002$ ), ketanserin (30 µg,  $p = 0.02$ ), and MDL-72222 (12 µg,  $p = 0.0002$ ), respectively (Fig. 3).

## 4. Discussion

The data from the present study show that spinal serotonin (5-HT) receptors mediate, at least in part, the antihyperalgesia produced by low frequency TENS in rats, since the non-selective 5-HT antagonist methysergide attenuated the low frequency TENS-induced hyperalgesia when administered intrathecally. Further, ketanserin and MDL-72222, antagonists at 5-HT<sub>2A</sub> and 5-HT<sub>3</sub> receptor subtypes, respectively, also attenuated the effects of low frequency TENS, indicating involvement of these receptor subtypes in low frequency TENS antihyperalgesia. NAN-190 had no effect on low frequency TENS antihyperalgesia indicating that 5-HT<sub>1A</sub> receptors are not involved. The results also show that antihyperalgesia produced by high frequency TENS does not depend on 5-HT mediated mechanisms in the spinal cord. Another major finding from this study is that the spinal  $\alpha_2$ -adrenergic receptors are not involved in the antihyperalgesia produced by either low or high frequency TENS.

### 4.1. Descending inhibition and TENS effects

Descending inhibitory systems, originating in the midbrain and terminating in the spinal dorsal horn, consists of two major pathways—noradrenergic and serotonergic. Electrical or chemical stimulation of PAG induces spinal nociception by the activation of descending adrenergic and serotonergic pathways that arise from pontine noradrenergic nuclei and RVM, respectively (Odeh and Antal, 2001). Opioid, glutamate (Jensen and Yaksh, 1989; Fields and Basbaum, 1999) and possibly serotonin (Fields and Basbaum, 1999; Kishimoto et al., 2001)

receptors in PAG or RVM are believed to initiate descending inhibition in the midbrain or brainstem. Both norepinephrine (Reddy et al., 1980; for review, see Yaksh, 1985; Aran and Proudfit, 1990; Fairbanks et al., 2002) and serotonin (Yaksh and Wilson, 1979; Schmauss et al., 1983; Peng et al., 1996; Paul et al., 2001) are antinociceptive in the spinal cord. Descending serotonergic pathways are implicated in the analgesia produced by peripheral stimulation in the current study and in previous investigations (Chen-yu et al., 1979; Woolf et al., 1980; Shimizu et al., 1981; Chen, 1992; Scherder and Bouma, 1993). Shimizu et al. (1981) showed that PES increased the turnover rate of 5-HT in the cortex and brainstem, systemic administration of a 5-HT precursor, L-5-hydroxytryptophan, enhanced the antinociception produced by peripheral stimulation and spinal methysergide abolished peripheral stimulation-induced antinociception. Serotonin concentrations increase in brain homogenate after peripheral stimulation (Wang et al., 1985) and depletion of serotonin systemically prevents peripheral stimulation analgesia (Woolf et al., 1980). Furthermore,  $\mu$ -opioid antagonists injected into RVM block the effects of TENS in rats (Kalra et al., 2001). Results from the current study extend previous studies showing that spinal 5-HT<sub>2A</sub> and 5-HT<sub>3</sub> receptor subtypes are involved in the low, but not high, frequency TENS-induced antihyperalgesia. Taken together, these findings strongly suggest a pivotal role for central serotonergic system in mediating antinociception produced by low frequency PES.

#### 4.2. Lack of effect of yohimbine

Spinal antinociceptive effect of noradrenaline is mainly mediated through  $\alpha_2$ -adrenoceptors. Selective  $\alpha_2$ -adrenoceptor agonists are antinociceptive when administered intrathecally and selective antagonists of  $\alpha_2$ -adrenergic receptors block these antinociceptive effects (Howe et al., 1983; Fairbanks and Wilcox, 1999). In the current study, the  $\alpha_2$ -adrenoceptor agonist clonidine reversed the hyperalgesia produced by knee joint inflammation. This reversal was prevented by prior treatment with 30  $\mu$ g yohimbine showing that this dose blocks antihyperalgesia produced by activation of  $\alpha_2$ -adrenoceptors. Since intrathecal yohimbine did not affect the antihyperalgesic effects of low or high frequency TENS in the current study, we conclude that the analgesic mechanism of TENS does not involve spinal  $\alpha_2$ -adrenoceptor activation.

#### 4.3. Spinal serotonin receptor subtypes

In the present study, methysergide attenuated the effects of low frequency TENS but did not affect high frequency TENS antihyperalgesia. Methysergide is a non-selective antagonist at 5-HT receptors acting on 5-HT<sub>1</sub> and 5-HT<sub>2</sub> families. Since low frequency TENS antihyperalgesia was attenuated by methysergide, we conclude that serotonin released in response to low frequency TENS in the spinal cord acts on one or more 5-HT receptor subtypes to reduce hyperalgesia. Serotonin has seven types of receptors identified so far (5-HT<sub>1-7</sub>) and a total of 14 subtypes. All types, except 5-HT<sub>3</sub>, are G-protein coupled and 5-HT<sub>3</sub> is ligand-gated ion channel receptor (Hoyer et al., 1994). 5-HT<sub>1</sub>, 5-HT<sub>2</sub> and 5-HT<sub>3</sub> serotonin receptor types are located in the spinal dorsal horn (Hamon et al., 1989; Marlier et al., 1991). The role of spinal serotonin receptor subtypes, especially those in 5-HT<sub>1</sub> family, in pain processing is controversial (Obata et al., 2001). 5-HT<sub>1</sub> receptors have been shown to be antinociceptive (Eide et al., 1990; Oyama et al., 1996), nociceptive (Solomon and Gebhart, 1988; Alhaider and Wilcox, 1993) or have no role (Bardin et al., 2000; Sasaki et al., 2001) in spinal pain modulation. In our study, although the 5-HT<sub>1A</sub> antagonist NAN-190 had no effect on low frequency TENS antihyperalgesia, the 5-HT<sub>1A</sub> agonist 8-OH-DPAT (50  $\mu$ g) significantly reduced the hyperalgesia induced by injection of kaolin—carrageenan into the knee joint of rats. These data suggest that spinal 5-HT<sub>1A</sub> receptors are not activated by the physiological levels of serotonin produced by low frequency TENS stimulation, but can be activated by an exogenous agonist at a pharmacological dose.

Our results clearly show the involvement of 5-HT<sub>2</sub> and 5-HT<sub>3</sub> receptors in low frequency TENS-induced anti-hyperalgesia in rats and are in agreement with other investigators that show spinal antinociception produced by 5-HT agonists is mediated by 5-HT<sub>2</sub> (Solomon and Gebhart, 1988; Danzebrink and Gebhart, 1991a; Sasaki et al., 2001; Obata et al., 2001) and 5-HT<sub>3</sub> (Danzebrink and Gebhart, 1991a; Bardin et al., 2000; Paul et al., 2001; Peng et al., 1996; Sasaki et al., 2001) receptors. In contrast, a recent study by Zeitz et al. (2002) shows that 5-HT<sub>3</sub> receptor knockout and spinal blockade of spinal 5-HT<sub>3</sub> receptors reduces nocifensive behaviors associated with the second phase of the formalin test, but not hyperalgesia associated with CFA or partial sciatic nerve lesion. The difference in observation could be due to the different antagonists, different testing methods, different animal models and species used.

#### 4.4. TENS, opioids and serotonin

Endogenous opioids are involved in both low and high frequency TENS analgesia. Low frequency TENS activates  $\mu$ -opioid receptors and high frequency TENS activates  $\delta$ -opioid receptors in the spinal cord and rostral ventral medulla (Sluka et al., 1999; Kalra et al., 2001). Similarly, human studies show increased endogenous opioid concentrations in cerebrospinal fluid (CSF) following PES (Hughes et al., 1984; Almay et al., 1985). Further, in rats, repeated application of low or high frequency TENS can lead to development of tolerance of spinal  $\mu$ - and  $\delta$ -opioid receptors, respectively (Chandran and Sluka, 2003). Also, in rats which were made tolerant to morphine, low frequency TENS was ineffective (Sluka et al., 2000). Thus, the involvement of endogenous opioids in TENS-induced analgesia is clear. Activation of  $\mu$ -opioid receptors in the supraspinal structures such as PAG and RVM exerts antinociceptive effects through either serotonin or norepinephrine receptors spinally (Aimone et al., 1987; Fields and Basbaum, 1999). Further, spinal antinociceptive effects of 5-HT are blocked by naloxone, an opioid receptor antagonist (Yang et al., 1994; Goodchild et al., 1997). Conversely, spinal morphine analgesia is blocked by serotonin receptor subtype antagonists (Crisp et al., 1991). In either case, an interaction between opioid receptors and serotonin receptors in the spinal cord plays a significant role in antinociception. Lastly, supraspinal administration of morphine produces increased release of GABA spinally that is blocked by 5-HT<sub>3</sub> receptor antagonists (Kawamata et al., 2002) suggesting that activation of supraspinal opioid receptors produces inhibition through spinal serotonin release. Since low frequency TENS-induced analgesia is mediated by both opioid and serotonin receptors the interaction spinally and/or supraspinally between these receptors could be important in TENS analgesia.

#### 4.5. Clinical relevance

This study provides direct evidence for spinal serotonin receptor mediation in low frequency TENS analgesia in rats. As such, there exists a possibility that serotonin agonists, serotonin selective reuptake inhibitors (SSRIs) or monoamine oxidase (MAO) inhibitors could enhance the analgesic effects of low frequency TENS clinically. Also, analgesic effects of low frequency TENS could be attenuated in patients who are under treatment with serotonin antagonists like methysergide, cyproheptadine or granisetron for unrelated conditions.

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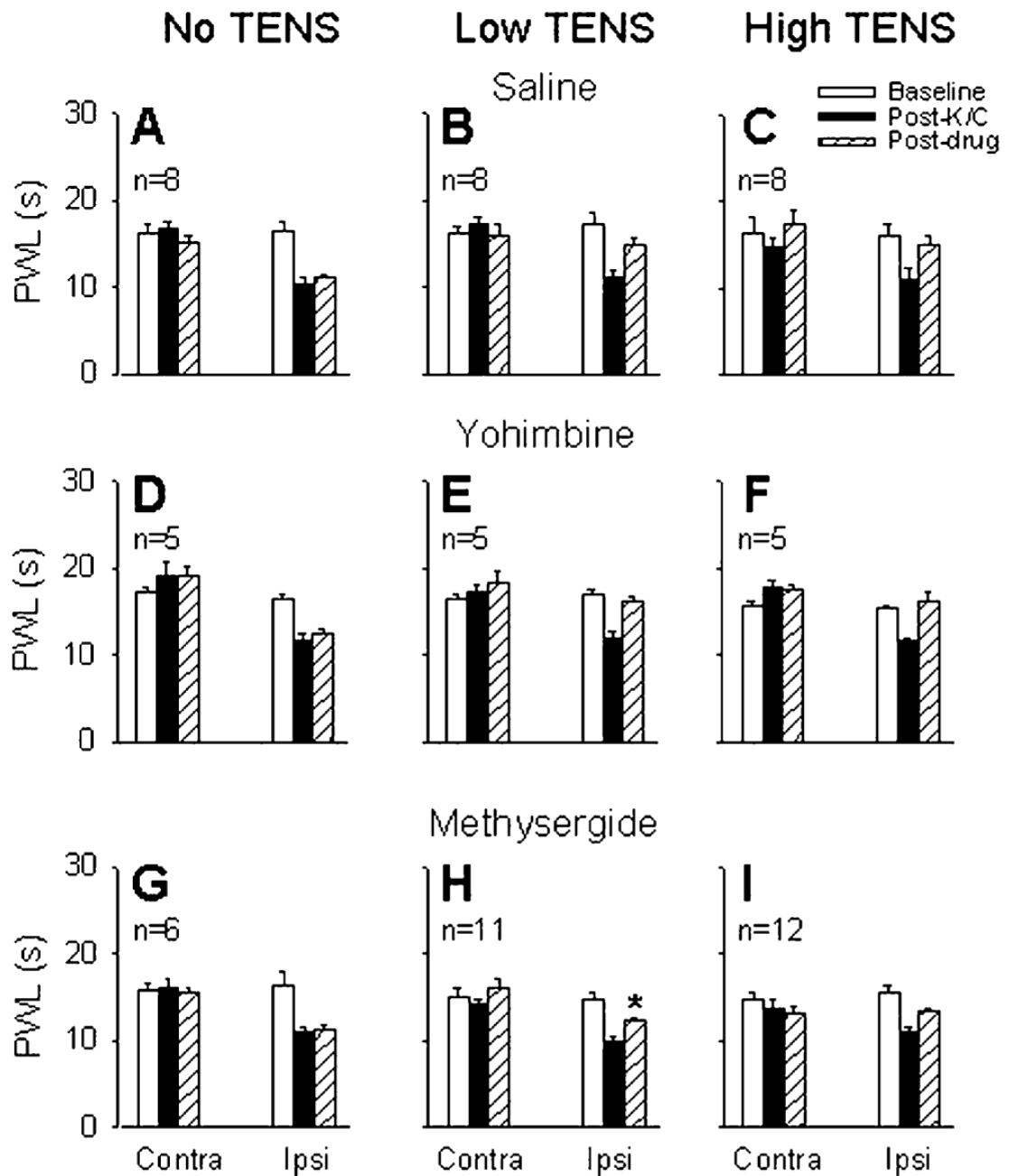


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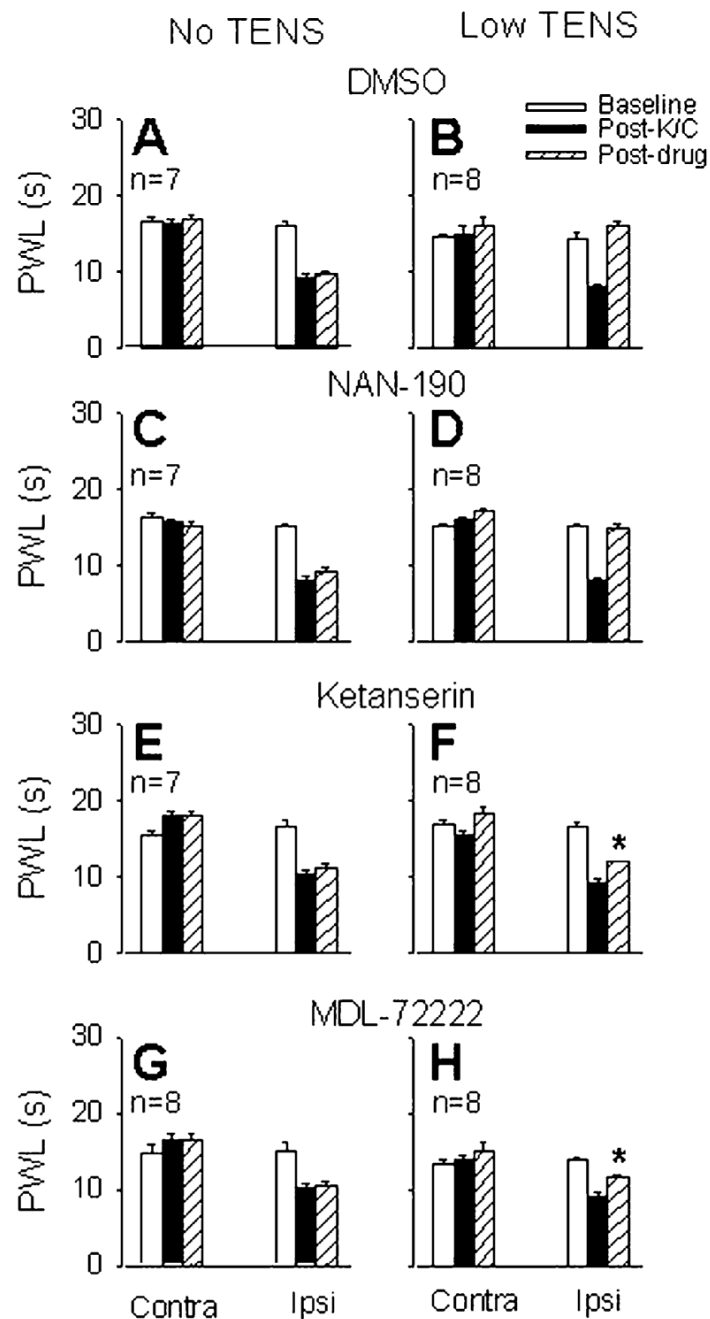
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**Fig. 1.**

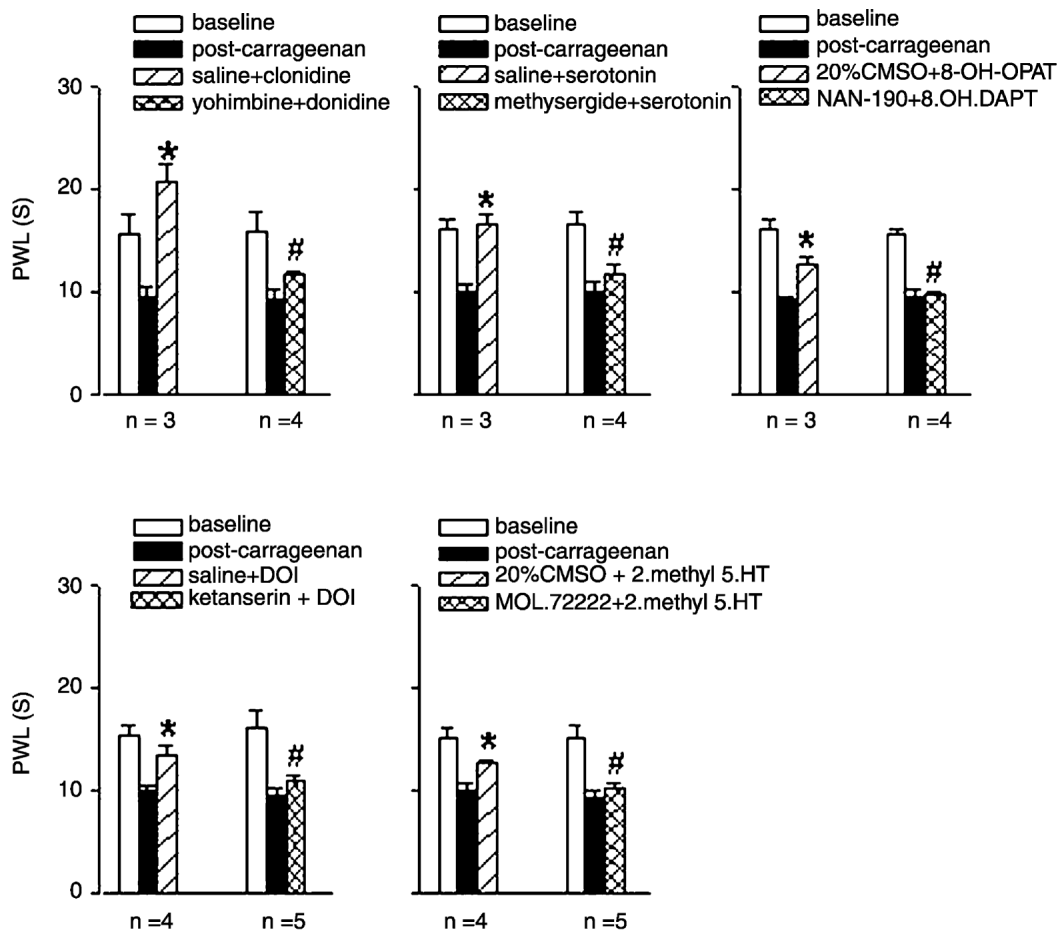
Bar graphs show the PWLs before (open bars), after joint inflammation (solid bars) and after treatment with drug and TENS/no TENS (hatched bars) for the ipsilateral and contralateral paws. Effects of intrathecal yohimbine and methysergide on carrageenan-induced hyperalgesia compared to saline control are shown in the graphs on the left panel (no TENS; graphs A, D, G). Effects of intrathecal saline, yohimbine or methysergide on low frequency TENS antihyperalgesia are shown in the middle panel (low TENS; graphs B, E, H), and effects of saline, yohimbine (30  $\mu$ g) or methysergide (30  $\mu$ g) on high frequency TENS are shown in the right panel (high TENS; graphs C, F, I). \*, Significantly different from vehicle control group



and also from its own baseline. K/C, kaolin and carrageenan; Contra, contralateral; Ipsi, ipsilateral.

**Fig. 2.**

Bar graphs show the PWLs before (open bars), after joint inflammation (solid bars) and after treatment with drug and TENS/no TENS (hatched bars) for the ipsilateral and contralateral paws. Effects of intrathecal 20% DMSO, NAN-190 (15  $\mu$ g, 5-HT<sub>1A</sub> antagonist), ketanserin (30  $\mu$ g, 5-HT<sub>2</sub> antagonist) and MDL-72222 (12  $\mu$ g, 5-HT<sub>3</sub> antagonist) on the carrageenan-induced decrease in PWL are shown in the left panels (no TENS; graphs A, C, E, G). Effects of intrathecal 20% DMSO, NAN-190, ketanserin and MDL-72222, on low frequency TENS antihyperalgesia are shown in the right panels (low TENS; graphs B, D, F, H). \*, Significantly different from vehicle control group and also from their own baseline. K/C, kaolin and carrageenan; Contra, contralateral; Ipsi, ipsilateral.



**Fig. 3.** The left cluster of bars in each graph shows ipsilateral PWLs before (open bar), 4 h after injection of kaolin—carrageenan injection into the knee joint (solid bar), and after intrathecal vehicle + agonists (hatched bar). Right cluster shows ipsilateral PWLs before (open), 4 h after kaolin—carrageenan injection (solid), and after intrathecal antagonist + agonist (hatched). \*, PWLs were significantly increased compared to PWLs after kaolin—carrageenan. #, PWLs for antagonist + agonist were significantly less than PWLs from vehicle + agonist.