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Involvement of 5-HT_{2A} receptor in chronic inflammatory pain

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- Background** : *Effect of serotonin (5-HT) on nociception can vary depending on the type of its receptors. Information regarding the roles of 5-HT_{2A} receptor in this process is limited.*
- Objective** : *To investigate the role of 5-HT_{2A} receptors in the development of hyperalgesia secondary to chronic inflammation.*
- Research Design** : *Experimental study in animals.*
- Materials and Methods** : *Complete Freund's adjuvant (CFA) was subcutaneously injected into the hind paws of Wistar rats to elicit inflammation. Three days after injection, ketanserin, a 5-HT_{2A} antagonist, was given. Nociceptive behaviours were scored and thermal hyperalgesia was determined by measuring paw withdrawal latency. The response of neurons in somatosensory cortex to painful stimulus was determined using Fos immunoreactivity as an indicator.*
- Results** : *Inflammation induced nociception and thermal hyperalgesia as well as evoked extensive Fos expression in somatosensory cortices. Pre-treatment with ketanserin did not prolong the paw withdrawal latency in non-inflamed limb, indicating the lack of intrinsic analgesic effect. On the contrary, this drug significantly prolonged the paw withdrawal latency in the inflamed side. Ketanserin also attenuated the nociceptive behaviours induced by CFA. Fos expression in the somatosensory cortex was also suppressed by ketanserin.*

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Conclusion : *This study suggests that 5-HT_{2A} receptor does not play primary role in nociception but may involve in the process of nociceptive facilitation.*

Keywords : *5-HT_{2A} receptor , Inflammation, Nociception, Pain, Hyperalgesia, Fos.*

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- ความเป็นมา** : ผลของซีโรโทนิน (5-HT) ต่อกระบวนการนอซิเซปชันมีความหลากหลาย ทั้งนี้ขึ้นอยู่กับตัวรับที่เกี่ยวข้อง ปัจจุบันยังมีข้อมูลไม่มากนักเกี่ยวกับบทบาทของตัวรับชนิด 5-HT_{2A} ต่อกระบวนการนี้
- วัตถุประสงค์** : เพื่อศึกษาบทบาทของตัวรับ 5-HT_{2A} ต่อการเกิดอาการปวดเกินปกติที่เป็นผลจากการอักเสบของเนื้อเยื่อ
- รูปแบบการวิจัย** : การศึกษาแบบทดลองในสัตว์
- วิธีการทดลอง** : ฉีดสารคอมพลีตฟรอนด์แอดจูแวนท์เข้าในชั้นใต้ผิวหนังบริเวณอุ้งเท้าหลังของหนูแรทสายพันธุ์วิสตา เพื่อกระตุ้นให้เกิดการอักเสบ สามวันหลังจากนั้นให้ยา คีแทนเซอริน ซึ่งเป็นสารยับยั้งตัวรับซีโรโทนินชนิด 5-HT_{2A} ทำการบันทึกพฤติกรรม ความปวด และวัดความไวต่อการเกิดความปวดที่กระตุ้นด้วยความร้อน โดยบันทึกระยะเวลาเท้าของสัตว์ทดลอง ประเมินการตอบสนองของเซลล์ในสมองใหญ่ ส่วนไฮมาโตเซนซอรี โดยวิธีการนับเซลล์ที่มีการแสดงออกของโปรตีนฟอส
- ผลการศึกษา** : การอักเสบสามารถกระตุ้นให้เกิดความไวต่อความร้อน และการแสดงออกของโปรตีนฟอสในเซลล์ประสาทสมองใหญ่ส่วนไฮมาโตเซนซอรี การให้สาร คีแทนเซอรินไม่ทำให้เกิดการเปลี่ยนแปลงของระยะเวลาเท้าในระยางค์ที่ไม่มีการอักเสบ ซึ่งบ่งว่าสารนี้ไม่มีฤทธิ์ลดปวด ในทางตรงข้ามสารนี้สามารถยืดระยะเวลาเท้าในระยางค์ที่เกิดการอักเสบ นอกจากนี้สารคีแทนเซอรินยังสามารถลดพฤติกรรมความปวดที่เกิดจากการฉีดสารคอมพลีตฟรอนด์แอดจูแวนท์ รวมทั้งลดจำนวนเซลล์ประสาทในสมองใหญ่ส่วนไฮมาโตเซนซอรี
- สรุป** : การศึกษานี้แสดงว่าตัวรับซีโรโทนินชนิด 5-HT_{2A} มีได้มีบทบาทโดยตรงในกระบวนการนอซิเซปชัน แต่ น่าจะมีบทบาทในกระบวนการเพิ่มความไวของระบบนี้
- คำสำคัญ** : ตัวรับ 5-HT_{2A}, การอักเสบเรื้อรัง, Nociception, ความปวด

Serotonin (5-hydroxytryptamine, 5-HT) is an important transmitter in the endogenous pain control system. Several lines of evidence show that effect of 5-HT on nociception may vary depending on the receptor types. Regarding the 5-HT_{2A} receptor, this class of receptor is generally believed to play minor roles in nociception processing. This hypothesis is based on the finding that only small numbers of this receptor are expressed in the spinal dorsal horn. The role of 5-HT_{2A} receptor in nociception has been recently reconsidered due to some observations. Anatomical studies demonstrated these receptors are expressed in several brain areas related to nociceptive modulation such as basal forebrain, periaquiductal grey, nucleus raphe magnus nucleus raphe dorsalis, etc.⁽¹⁾ It was also demonstrated that the expression of this receptor can be up-regulated in painful conditions such as tissue inflammation.^(2,3)

Previous studies showed that 5-HT_{2A} receptor became down-regulated in response to analgesic drug administration.⁽⁴⁾ The up-regulation of this pro-nociceptive receptor has been proposed as a molecular event contributing to chronic painful condition.⁽⁵⁾ The role of this receptor in central sensitisation induced by tissue inflammation has not yet been investigated.

The objective of the present study was to determine the role of 5-HT_{2A} receptor in modulation of nociceptive information. In this experiment, chronic chemical inflammation was employed to induce sensitisation of central nociceptive neurones. Nociceptive responses were monitored using behavioural indicators. We also studied the expression of transcription factor, Fos, in the somatosensory cortex in order to determine the effect of chronic

nociceptive stimulation on cortical activity as well as to investigate the role of cerebral cortex on pain modulation related to activity of 5-HT_{2A} receptor.

Materials and Methods

Experimental Animals

Male Wistar rats weighing 200 - 250 g (National Animal Centre, Mahidol University, Thailand) were used. The rats were allowed to acclimate for a week before experiment. All animals were kept in a well ventilated room where the temperature was 25 ± 3 °C. The rats were housed in stainless-steel cages with an automatic lighting schedule which provided darkness from 6.00 pm to 6.00 am and free access to food and water. The conduct of the experiment was conformed to the guideline given by the International Association for the Study of Pain.⁽⁶⁾ The experiment proposal was approved by the Ethics Committee of Chulalongkorn University.

Study Design

In this study, effect of 5-HT_{2A} antagonist, ketanserin, on nociceptive processing was tested in two conditions, namely, physiologic pain perception and nociceptive sensitisation. The rats were divided into four groups (10 rats each) including: (1) control, (2) physiologic saline (NSS) and ketanserin, (3) Complete Freund's adjuvant (CFA) alone, and (4) CFA and ketanserin. The first two groups were used to study the effect of 5-HT_{2A} receptor antagonist, ketanserin, on thermal nociception in the physiologic condition with no prior inflammation. On day 1, both groups received 50 µl of normal physiologic saline injection on the right hind paw. On day 4, ketanserin (0.3 mg/kg BW intraperitoneally) was given to the rats in group

2 (NSS + ketanserin) 1 hour before nociceptive stimulation. Behavioural response to noxious heat was monitored using paw withdrawal test. Nociceptive and non-nociceptive behaviours were also monitored.

The rats in groups 3 and 4 were used to determine the role of ketanserin in modulating pain in condition with nociceptive sensitisation. On day 1, chemical inflammation was introduced in two groups of rat (10 rats each) by subcutaneous injection of 50 μ l of CFA into right hind paw. The rats were kept for three days during which time central sensitisation was fully developed. On day 4, ketanserin (0.3 mg/kg BW, intraperitoneally) was given to the rats in group 4 (CFA + ketanserin). Physiologic saline of the same volume was given to the third group (CFA alone). Nociceptive behaviours (licking, lifting and favouring) were recorded for 30 minutes. Response to noxious stimulation was determined using paw withdrawal latency in response to noxious heat as an indicator.

In both experiments, after completing the measurement of behavioural nociceptive responses, all rats were humanely killed with excessive dose of pentobarbital. Their brains were then removed and were further processed for Fos immunohistochemical study.

Behavioural Assessment

On day 3 post-CFA injection, the animals were placed in the observation chamber for measurement of nociceptive and non-nociceptive behaviours. Animal behaviours were continuously recorded for 30 minutes using video system for playback analysis.

Nociceptive behaviours rating was conducted using the following criteria: favouring – the injured paw rest lightly on the floor with pressure pads not in full

contact; lifting – inflamed paw elevated with the most of nails touching the floor; and, licking – injured paw licked or bitten. ⁽⁷⁾ To study the possible effect of 5-HT_{2A} receptor antagonist on motor activity, non-nociceptive behaviours comprising face or body scratching was also monitored. The time spent for each of these behaviours was recorded as was summed.

Thermal Hyperalgesia Test

In order to assess the effect of inflammation on thermal nociception, paw withdrawal reflection to radiant heat experiment was used. In each trial, the rats were lightly anaesthetised with pentobarbital (25 mg/kg BW, intraperitoneally). The level of anaesthesia was kept comparable in that of testing corneal reflex and paw squeezing. By using Model 33 Tail Flick Analgesia Meter (Harvard Apparatus, Edinbridge, UK), a constant intensity radiant heat originated from 150-Watt infrared bulb was focused on each hind paw. The period from the time when heat was applied to the paw and the time of the paw withdrawal was recorded. The mean value of three measures was used for each experimental animal as the paw withdrawal latency (PWL) and the time interval was 5 minutes. In order to avoid tissue injury, a cut-off time was set at 20 seconds. The test was performed in both hind paws (inflamed and non-inflamed) and data were compared.

Fos Immunohistochemistry

After completing the behavioural testing, the rats were humanely killed by excessive dose of sodium pentobarbital and were perfused transcordially with 250 ml of phosphate buffer and followed by 250 ml of 4 % paraformaldehyde in 0.1 M PBS pH 7.4. Their brains were removed and were immediately immersed

in 4 % paraformaldehyde in 0.1 M phosphate buffer. After overnight fixation, tissue was placed in a cryoprotectant solution (30 % sucrose in 0.1 M phosphate buffer, pH 7.4). Blocks of somatosensory cortex were selected using the coordinates described in the atlas of Paxinos and Watson.⁽⁶⁾ Ten 35 μ m thick coronal sections of parietal cortex were cut with cryomicrotome (Microm HM 505N: Waldrof, Germany) at -20 °C. The sections were collected in cold 0.01 M phosphate buffer saline. To minimise endogenous peroxidase, all sections were incubated with 3 % hydrogen peroxide in 50 % ethanol for 20 minutes. After repeated rinses in PBS, the sections were pre-incubated in PBS containing 3 % normal horse serum, 1 % bovine serum albumin for 90 minutes at room temperature and were incubated overnight with rabbit anti-Fos (Santa Cruz Biotechnology, Santa Cruz, CA) (1:1000 dilution). They were then incubated for 30 minutes with biotinylated goat anti-rabbit (Dako LSAB 2 system, Denmark). Sections were rinsed again in PBS before incubating for 30 minutes in a streptavidin horseradich peroxidase solution (Dako LSAB 2 system, Denmark). Bound peroxidase was revealed by incubating all sections in solution containing 0.05 % 3,3 - diaminobenzidine, 0.005 % hydrogen peroxide for 7 minutes. The reaction was stopped by repeated rinses in PBS. Sections were mounted on gelatinized slides, dehydrated in graded series of ethanol, and coverslipped.

Expression of Fos in the somatosensory cortex was determined by counting Fos-immunoreactive (Fos-IR) neurons from ten sections. A Fos-IR neurons discerned in the grid of 100 μ m x 100 μ m was counted. Total cells observed in ten 100 μ m x

100 μ m squares were counted from each slide. The data were summed and expressed as number of cells per square millimetre. All counting procedures were performed manually by one of the authors (M.W.) who was blinded to the treatment groups.

Data Analysis

All data were presented as mean \pm standard deviation. All data were analysed for possible statistical significance among groups using ANOVA with Bonferroni adjustment. Non-parametric statistical methods were employed where appropriate. Probability values of less than 0.05 were considered statistically significant.

Results

Effect of Ketanserin on Nociceptive Behaviours

The results showed that injection with CFA induced nociceptive behaviours in rats. The behavioural scored in each group was demonstrated in Figure 1. Time dedicated for nociceptive behaviours in CFA-treated rats was 136.3 ± 81.6 seconds. No nociceptive behaviour was observed in the control rats. Administration of ketanserin was able to reduce theses nociceptive behaviours. The total time for nociceptive behaviours in rats receiving CFA with ketanserin was 7.0 ± 14.0 seconds. Statistical testing revealed significant difference when total nociceptive behavioural time between rat receiving CFA with and without ketanserin administration was compared ($p = 0.005$, ANOVA with Bonferroni adjustment). No statistically significant difference was evident when non-nociceptive behavioural time from all groups was compared. (Table 1, Figure 1)

Table 1. Effect of CFA and ketanserin on nociceptive behaviours and thermal hyperalgesia. Subcutaneous CFA injection induced inflammation and nociceptive behaviours in rat. The nociceptive behavioural score was significantly attenuated by administration with ketanserin. Data were expressed as mean \pm standard deviation.

Test	Control	CFA	NSS + Ketanserin	CFA+ Ketanserin	P value
Behavioural score (seconds)					
Non-nociceptive behaviours	1638.2 \pm 252.5	1104.50 \pm 429.5	1108.0 \pm 383.3	944.0 \pm 427.4	0.119
Nociceptive behaviours	0.0 \pm 0.0	136.3 \pm 81.6	0.0 \pm 0.0	7.0 \pm 14.0	0.005
Paw withdrawal test (seconds)					
Inflamed side	8.3 \pm 0.6	5.3 \pm 1.3	9.3 \pm 1.0	14.3 \pm 2.0	< 0.001
Non-inflamed side	9.2 \pm 1.4	9.4 \pm 0.9	11.2 \pm 2.5	9.2 \pm 1.1	0.606

Effect of Ketanserin on Thermal Hyperalgesia

In addition to baseline nociceptive behaviours, chronic inflammation induced by CFA injection also caused hypersensitivity to noxious heat. Table 1 showed that the PWL on inflamed paw was significantly decreased as compared to the non-injured side, $p < 0.001$. Comparing between those receiving CFA and control groups also yielded significant difference, $p < 0.001$. No statistically significant difference was observed when data from non-injured sides from all groups were compared. Among rats without CFA injection, the PWL of those with and without ketanserin was not different. This finding indicated that, in normal physiologic pain perception, 5-HT_{2A} antagonist exerts no intrinsic anti-nociceptive property. On the contrary, ketanserin treatment significantly prolonged PWL in the inflamed limb. The PWL of CFA-treated rats with and without ketanserin administration were 14.3 \pm 2.0 and 5.3 \pm 1.3 seconds, respectively ($p < 0.001$, ANOVA with Bonferroni adjustment). This observation indicated that despite

the lack of anti-nociceptive effect in normal condition, 5-HT_{2A} receptor antagonist may have anti-nociceptive effect in chronic painful condition.

Effect of Ketanserin on Chemical Nociception-evoked Fos Expression

Our immunohistochemical study showed that chronic chemical inflammation evoked extensive Fos expression in all areas of somatosensory cortices. Fos-IR neurones were distributed diffusely and evenly in both hemispheres. No difference in number of Fos-IR neurones was observed comparing between medial (corresponded to hind limb area) and lateral cortical areas. The average number of Fos-IR in ipsilateral and contralateral hemisphere were 252 \pm 83 and 240 \pm 30 cell/mm², respectively ($p = 0.699$, Mann Whitney test). The difference in number of Fos-IR neurones between the two hemispheres was not statistically significant. Only few Fos-IR neurones were observed in the control rats and those receiving normal saline and ketanserin. Significant difference was evident when rats with CFA-

induced chronic inflammation, control rats and those receiving normal saline and ketanserin were compared ($p < 0.001$, Kruskal Wallis test).

Treatment with ketanserin substantially reduced the number of CFA-induced Fos-immunoreactivity in the somatosensory cortex. The numbers of Fos-IR neurones in ipsilateral and contralateral sides

CFA-treated rats with ketanserin administration were 67 ± 42 and 83 ± 5 cell per mm^2 , respectively. Statistical test yielded significant difference when data from CFA treated rats with and without ketanserin was compared. ($p < 0.001$, Mann Whitney test) (Figure 1 and 2)

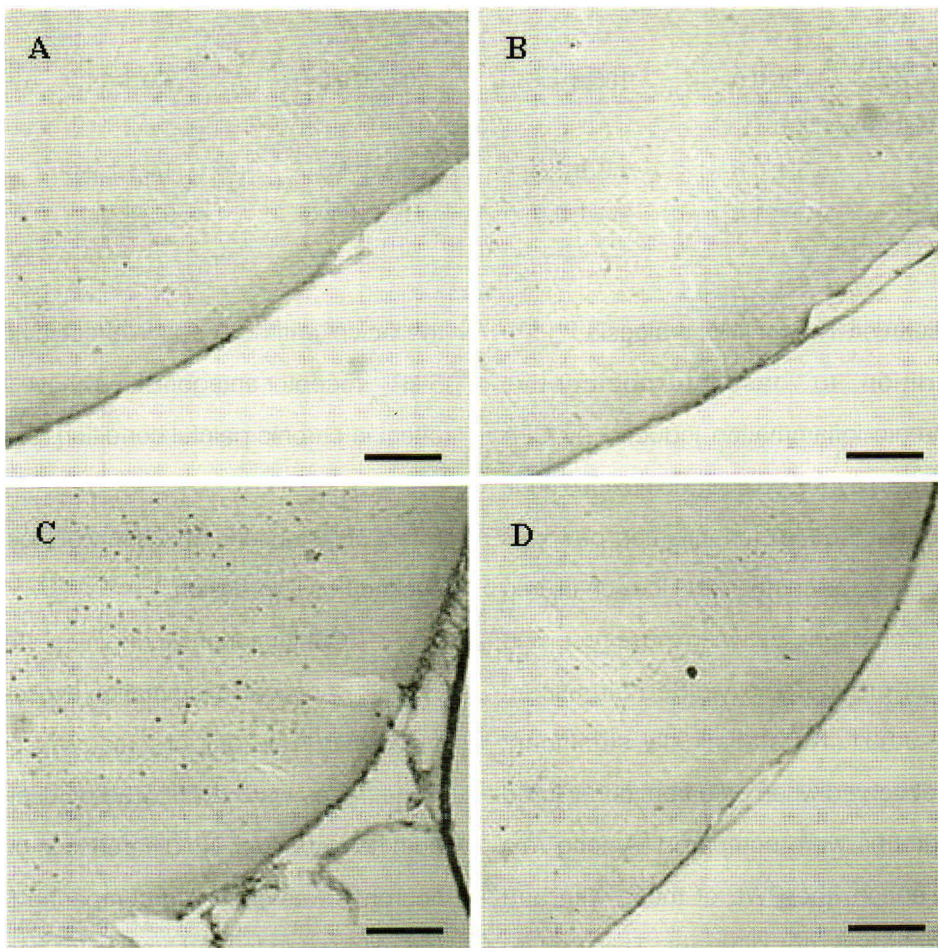


Figure 1. Effect of chronic inflammation with and without ketanserin on Fos expression in cerebral cortex. Only few Fos immunoreactive cells were seen in somatosensory cortex of control group (A) and groups receiving ketanserin without prior inflammation (B). Chronic inflammation induced by CFA injection evoked expression of Fos in all areas of somatosensory cortex (C). The inflammation-evoked Fos expression can be suppressed by a 5-HT_{2A} antagonist, ketanserin (D). (Bar = 500 μm)

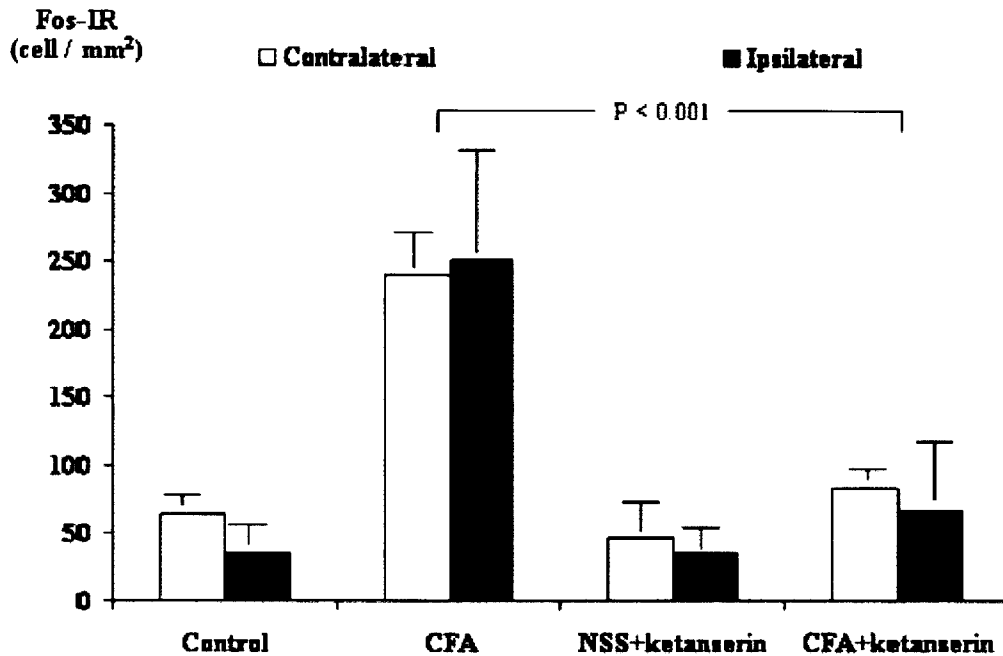


Figure 2. Effect of ketanserin on chronic inflammation-induced Fos expression in cerebral cortex. Chronic inflammation induced expression of Fos in all areas of somatosensory cortex. Ketanserin suppressed this inflammation-evoked Fos expression.

Discussion

The present study was aimed to investigate the role of 5-HT_{2A} receptor in chronic pain model. The result showed that although 5-HT_{2A} antagonist yielded no significant role in reducing nociceptive behaviours in physiologic nociceptive perception, it was effective in reducing hyperalgesia observed in chronic pain state.

The present study revealed that chronic inflammation evoked Fos expression in all areas of the somatosensory cortex. The evoked Fos-IR neurones were not confined in functionally representative the cortical area as expected by its topographic organisation. On the contrary, Fos-IR neurones were distributed evenly throughout both hemispheres. This pattern reflected that, in chronic nociceptive condition, somatosensory cortical neurones are diffusely

sensitised. The wide spread sensitisation of the somatosensory cortex demonstrated in this study may explain the generalised decrease in pain tolerance observed in some chronic painful syndrome such as fibromyalgia. This finding also advocates the role of sensitisation of the somatosensory cortex in the development of chronic pain.

Although our study showed the widespread Fos expression in chronic nociceptive condition, the enhancement in pain perception was not observed in the non-inflamed limb. The PWL on non-inflamed limb of the CFA-treated rats did not differ from those of other groups. The conflicting result may be secondary to the different in level of nervous system involved in initiating behavioural response. The paw withdrawal response is regarded as a spinal reflex which is primarily controlled by circuit in spinal cord. Therefore,

the behavioural effect of changing in the sensitivity of cortical neurones may not be observed by this method.

In this experiment, the extensive Fos expression in the somatosensory cortex induced by CFA injection was attenuated by treatment with 5-HT_{2A} antagonist, ketanserin. This finding corresponded to the decrease in nociceptive behaviours and lengthening of PWL observed in this group. The role of 5-HT_{2A} receptor in nociception is quite controversy. Previous studies were aimed to investigate this issue revealed conflicting results. Some studies show that 5-HT_{2A} receptor may have some role in attenuating nociception.⁽⁹⁾ Recent studies, however, showed that intrathecaly administered 5-HT receptor agonists suppressed inflammatory pain or neuropathic pain, which were reversed by ketanserin.^(10,11) These reports suggested that activation of spinal 5-HT_{2A} receptors mediate analgesia for chronic pain. On the other hand, several studies showed facilitating effect of this receptor in the process of nociception.⁽¹²⁻¹⁴⁾ The present findings of the effect of 5-HT_{2A} antagonist in reducing hyperalgesia secondary to chronic inflammation despite no efficacy in normal physiological nociception indicated that this class of receptor may be of importance in the chronic pain state. Previously, we have shown that activation of 5-HT_{2A} receptor leads to an enhancement of nitric oxide synthase expression.⁽¹⁵⁾ It is known that nitric oxide is strongly implicated in the process of sensitisation of central neurones. Therefore, up-regulation of this receptor may increase the sensitivity of central somatosensory neurones and contributes the development of central sensitisation.

In conclusion, our study suggests that the somatosensory cortex is diffusely sensitised

in the chronic painful condition. Since this central sensitisation can be blocked by 5-HT_{2A} receptor antagonist, it is likely that 5-HT_{2A} receptor may involve in the process of central sensitisation; hence, inducing nociceptive facilitation in chronic painful conditions.

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