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Expression of 5-HT_{2A} receptor mRNA in rat nucleus raphe magnus neurons after peripheral inflammation[1]

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KEY WORDS serotonin receptors; messanger RNA; carrageenan; fluorescence *in situ* hybridization; immunohistochemistry; raphe nuclei

ABSTRACT

AIM: Examine the expression of 5-HT_{2A} receptor mRNA and co-localization of 5-HT_{2A} receptor mRNA with 5-HT immunoreactivity in nucleus raphe magnus (NRM) neurons following carrageenan inflammation. **METHODS**: *In situ* hybridization (ISH) and combining fluorescent ISH with immunofluorescent histochemical double staining technique were used in the present study. **RESULTS**: 5-HT_{2A} receptor mRNA was expressed in moderate level in NRM neurons. Following carrageenan inflammation, the expression of 5-HT_{2A} receptor mRNA in NRM was greatly increased. The peak occurred at 3 - 8 h and then there was a clear decrease at 24 h after injection of carrageenan. Only a few 5-HT_{2A} receptor mRNA and 5-HT immunoreactive double labeled cells were observed in NRM, and the number of the double labeled cells had no obvious change following carrageenan inflammation. **CONCLUSION**: The synthesis of 5-HT_{2A} receptor was increased in non-serotonin rather than serotonin neurons of NRM during peripheral inflammation.

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) receptors are currently classified into seven families comprising 14 different receptor subtypes. The 5-HT_2 family includes three subtypes: 5-HT_{2A} (previously called 5-HT_2), 5-HT_{2B} (previously termed 5-HT_{2F}) and 5-HT_{2C} (previously included in the 5-HT_1 family and termed 5-HT_{1C}). They are G-protein-coupled receptors, and share a high degree of structural similarity[1,2]. Within this family, the 5-HT_{2A} receptor was the first to be described and has been extensively studied. It is well known that 5-HT_{2A} receptor is widely distributed in brain and spinal cord[3-6]. However, to our knowledge, the data concerning the level of expression of 5-HT_{2A} receptor in brain in painful states is so far limited. Our Study recently confirmed that the expression of 5-HT_{2A} receptor mRNA in ipsilateral dorsal horn, bilateral ventrolateral periaqueductal gray (vPAG), and dorsal raphe nucleus (DRN) was markedly increased following peripheral inflammation, suggesting that expression of 5-HT_{2A} receptor mRNA in brain and spinal cord be involved in inflammatory process.

Nucleus raphe magnus (NRM) is a neurochemically heterogenous nucleus with most 5-HT neurons[7,8]. It is involved in many physiologic functions, but the most significant is its antinociceptive function. Recent studies from our laboratory have shown that 5-HT_{1A} receptor mRNA was expressed with moderate level in NRM and some of them expressed in 5-HT neurons. Following carrageenan inflammation, the expression of 5-HT_{1A} receptor mRNA in NRM, especially in 5-HT neurons was increased[9]. It is not known at present whether 5-HT_{2A} receptor in NRM will change following peripheral inflammation. The present study was designed to examine the expression of 5-HT_{2A} receptor mRNA in NRM in the carrageenan model of inflammatory pain in rat.

MATERIALS AND METHODS

Animal preparation Male Sprague-Dawley rats (220 - 250 g, Grade Ⅱ, Certificate No 02-22-2, Experimental Animal Center, Fudan University) were

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used in this study. The peripheral inflammation was induced by intraplantar injection of carrageenan [λ-carrageenan, Sigma, 3 mg in 150 μL of normal saline (0.9 % NaCl, NS)] in the right hindpaw of non-anesthetized rats. Rats were perfused at 1, 3, 8, or 24 h after intraplantar injection of carrageenan. Vehicle-injected control rats received an intraplantar injection of 150 μL NS and were perfused at 3 h after NS injection. Non-stimulated control rats kept free movement in their home cages before perfusing. In order to assess the development of inflammation, rats were tested for thermal hyperalgesia by a method described in detail by Hargreaves *et al*[10] immediately before perfusion. All animals were given an over dose of urethane (1.5 g/kg, ip) and perfused transcardially with 200 mL NS followed by 4 % paraformaldehyde in phosphate-buffer 0.1 mol/L (PB, pH 7.4). The brains were removed and post-fixed in the same fixative solution for 4 h at 4 ℃, and immersed in 30 % sucrose in PB for 24 – 48 h at 4 ℃ for cryoprotection. Coronal sections (30 μm thick) were cut and collected in cryoprotectant solution (PB 0.05 mol/L, 30 % sucrose, 30 % ethylene glycol)[11] and then stored at – 20 ℃ until use.

***In situ* hybridization** A 48 mer oligonucleotide probe for rat 5-HT_{2A} receptor mRNA containing the sequence 5′-AGTGTTAAGCATCTCTGGAGTTGAAG-TCATTATGGTAGAGCCTCCTCGGGC-3′ (nucleotides 3 – 51)[12] was synthesized and purified by Beijing Bioneer Corporation. The probe was labeled at 3′-end with DIG-11-UTP using a DIG oligonucleotide tailing labeling kit (Boehringer Mannheim Biochemica, Germany).

Free-floating tissue sections were processed for 5-HT_{2A} receptor mRNA *in situ* hybridization (ISH). Sections were removed from storage at – 20 ℃ and placed directly into 4 % paraformaldehyde at room temperature for 10 min. Following 4 times for 15 min rinses in phosphate-buffered saline 0.1 mol/L (PBS, pH 7.4), sections were treated with proteinase K (1 mg/L in Tris-HCl 0.1 mol/L with edetic acid 1 mmol/L, pH 8.0) for 20 min at 37 ℃. Subsequently, sections were rinsed twice for 15 min in PBS, triethanolamine 0.1 mol/L (pH 8.0, plus 0.25 % acetic anhydride) for 10 min and 50 % formamide in 4 × SSC (saline sodium citrate, pH 7.2) for 20 min. Sections were then incubated in hybridization solution (50 % formamide, 5 × SSC, 0.02 % SDS, 2 % blocking reagent, and 1 mg/L of the DIG-labeled oligonucleotide probe complimentary to nt 3 – 51 of 5-HT_{2A} receptor cDNA) for 20 h at 37 ℃. After hybridization, sections were subsequently rinsed twice for 15 min in 2 × SSC at 37 ℃, twice for 15 min in 0.1 × SSC at 42 ℃, and 30 min in buffer Ⅱ (Tris-HCl 0.1 mol/L with NaCl 0.15 mol/L, 0.3 % Triton X-100, and 2 % normal goat serum) at room temperature. Sections were treated with alkaline phosphates-labeled anti-DIG antibody (diluted 1 : 3000) for 2 h. After 2 times for 15 min rinses in buffer Ⅰ (Tris-HCl 0.1 mol/L with NaCl 0.15 mol/L) and 3 min in buffer Ⅲ (Tris-HCl 0.1 mol/L with NaCl 0.15 mol/L and $MgCl_2$ 0.05 mol/L), sections were visualized with NBT/BCIP in buffer Ⅲ for over 6 h in dark. Finally sections were mounted on gelatin-coated slides, air-dried, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped. An adjacent series of sections to hybridization were stained with Cresyl Violet to aid in anatomic localization.

Fluorescent *in situ* hybridization and immunofluorescent histochemical double stain Referring to previous description[13], FISH and IFH double staining was performed. For FISH, the procedures of pre-hybridization, hybridization, and post-hybridization wash were essentially the same as ISH. After post-hybridization wash, sections were incubated in fluorescein isothiocyanate (FITC)- conjugated anti-DIG antibody (1 : 10 diluted in PBS containing 0.3 % Triton X-100, 1 % normal goat serum, Boehringer Mannheim) for 1 h at 37 ℃. Sections were then rinsed in PBS 0.01 mol/L, incubated with rabbit antiserum against 5-HT (1 : 2000, Sigma) overnight at 4 ℃. After washing in PBS, sections were incubated with rhodamine-labeled goat anti-rabbit IgG (1 : 200, Sigma) for 1 h at 37 ℃, then rinsed in PBS. All sections were cover-slipped with a mixture of 50 % glycerin in PBS, and then observed with confocal laser scanning microscope (CLSM) (Leica TCS-NT, Germany) by using laser beams of 488 nm and 543 nm with an appropriate emission filters for FITC (510 – 525 nm) and rhodamine (590 – 610 nm). The CLMS system was operated by Leica TCS-NT 4.0 software. The zoom was 1.0 and the thickness of each optical scan slice was 1.25 μm. The scan images were saved with 512 × 512 pixel type. After the confocal observation, the same series of sections were exposed and counterstained with Cresyl Violet to aid in anatomic localization.

The specificity of DIG-labeled antisense oligonucleotide probe was examined by hybridizing with labeled sense probe, overdose unlabeled probe, or

omitting probe in hybridization solution as well as RNase pretreatment. The specificity of the antibody was verified by both omitting the primary antibody during the overnight incubation and by the preabsorption experiment.

Data analysis 5-HT_{2A} receptor mRNA positive cells, 5-HT positive somata, and double labeled cells of 5-HT_{2A} receptor mRNA with 5-HT were counted using a computerized image analysis system (Leica Q 500 IW, Germany). The number of positive cells present in NRM was evaluated in 6 sections through NRM (10.3 – 11.3 mm caudal to bregma). Results were expressed as $x \pm s$ of positive cells per section. Statistical analysis was carried out on these samples by ANOVA followed by the Newman-Keuls post-hoc test. A P-value of 0.05 or less was considered statistically significant.

RESULTS

Specificity of the methods Sections incubated without the probe or in sense probe showed no or very faint signals. When the unlabelled probe was added in excess (30-fold) to hybridization solution or the sections were preincubated in RNase (200 mg/L), the signal was abolished. Sections processed by omitting primary antiserm showed no obvious immunostaining. Sections incubated in antiserum (at working dilution) preabsorbed overnight with 5-HT (0.1 mmol/L) showed very faint staining confirmed the specificity of the antiserum. No cross-reaction was observed between the secondary antibodies and the primary probe or antibody (figure not shown).

The expression of 5-HT_{2A} receptor mRNA in NRM In non-stimulated and vehicle-injected control rats, the 5-HT_{2A} receptor mRNA was expressed in moderate level in NRM neurons (Fig 1A). It showed that 5-HT_{2A} receptor mRNA positive cells were blue purple, and had the hybridized reactive products in the cell cytoplasm. No hybridized stain was observed in nuclei and axons. The number of 5-HT_{2A} receptor mRNA positive cells were (49 ± 4) and (50 ± 8) per section in contralateral NRM of non-stimulated and vehicle-injected rats respectively ($n = 8$). No significant difference was found between either the two control groups (Fig 2) or the both sides of NRM. One hour after carrageenan injection, the number of 5-HT_{2A} receptor mRNA positive cells were significantly increased in bilateral NRM [(81 ± 19) *vs* (49 ± 4) per section

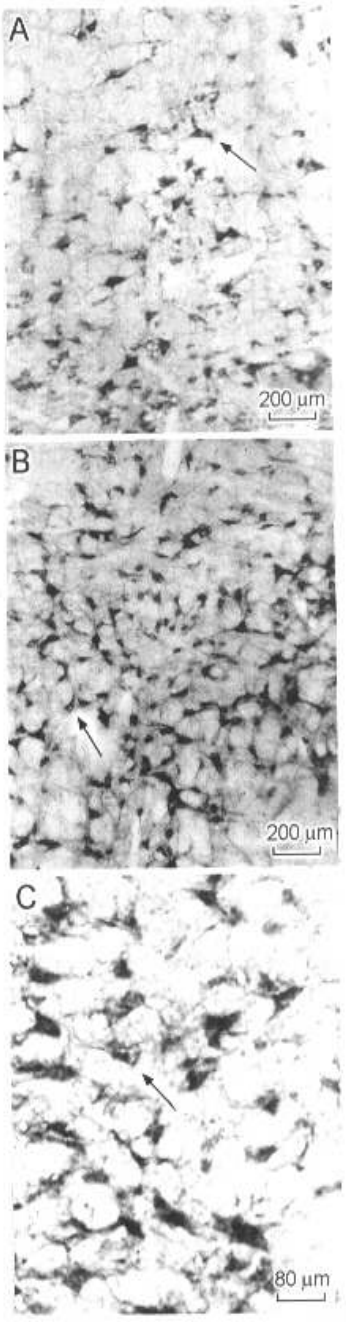

Fig 1. Photomicsographs showing the expression of 5-HT_{2A} receptor mRNA in the rat NRM at 3 h after intraplantar injection of vehicle (A) and carrageenan (B and C).

(non-stimulated rats) or (50 ± 8) per section (vehicle-injected rats) for contralateral NRM; KW-ANOVA, $H = 9.922$, $P = 0.007$]. At 3 h after carrageenan injection,

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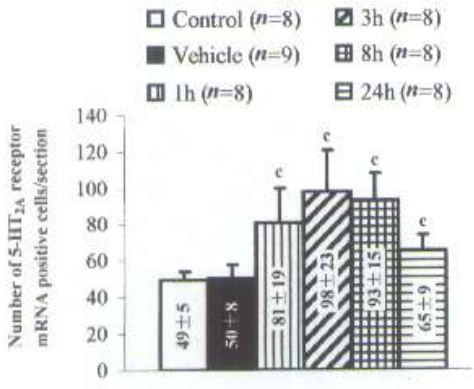

Fig 2. Time-course of 5-HT_{2A} receptor mRNA expression in NRM after an intraplantar injection of carrageenan. $\bar{x} \pm s$. $^cP < 0.01$ *vs* vehicle-injected control.

the expression of 5-HT_{2A} receptor mRNA reached the highest level (Fig 1B) (98 ± 23 per section for contralateral NRM) and thereafter, a very little decrease at 8 h and a clear decrease at 24 h were observed (Fig 2). No significant difference was found between the contralateral and ipsilateral NRM (data not shown).

The co-localization of 5-HT_{2A} receptor mRNA with 5-HT immunoreactivity (5-HT-IR) in NRM

Using the appropriate filters with confocal laser scanning microscopes, 5-HT_{2A} receptor mRNA appeared as green hybridized reactive products in cell cytoplasm. A nuclear profile with no hybridized stain could be clearly seen. 5-HT-IR products were seen as red in cell somata and some axon terminals or dendritic endings. 5-HT_{2A} receptor mRNA and 5-HT-IR double labeled cells were yellow (Fig 3). In non-stimulated and vehicle-injected control group, moderate 5-HT_{2A} receptor mRNA positive cells and 5-HT-IR cells as well as few 5-HT_{2A} receptor mRNA and 5-HT-IR double labeled cells were observed in NRM (Fig 3A). Three hours after carrageenan injection, 5-HT_{2A} receptor mRNA single labeled cells in NRM were markedly greater than that obtained either from non-stimulated or vehicle-injected rats (Fig 3B, 4). No significant difference was found between the both sides of NRM. Whereas 5-HT_{2A} receptor mRNA and 5-HT-IR double labeled cells had no obvious change. Although 5-HT-IR single labeled cells had an increase, the change was not statistically significant.

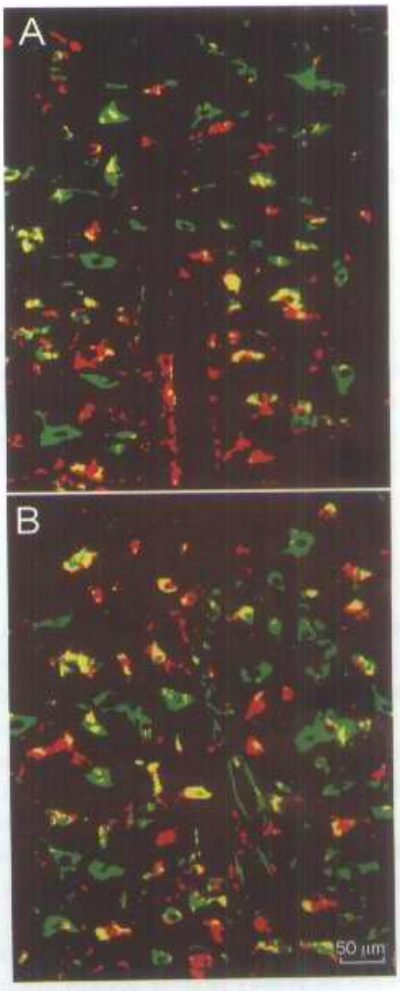

Fig 3. Confocal photomicrographs showing the colocalization of 5-HT_{2A} receptor mRNA with 5-HT-IR in NRM. 5-HT_{2A} receptor mRNA positive cells are exhibited by green color; 5-HT-IR cells by red color and double labeled cells by yellow. A: vehicle-injected control; B: at 3 h after injection of carrageenan.

DISCUSSION

The present study revealed that 5-HT_{2A} receptor mRNA was expressed in moderate level in NRM neurons. This result was not consistent with the autoradiographic mapping of 5-HT_2 receptor in rats, in which 5-HT_2 receptors appeared to be scarce or absent in NRM[14]. Such a discrepancy may be explained in technical terms as that ISH using DIG-labeled affords increased sensitivity as compared to radioligands. Besides, it is possible that this disproportionality may reflect a specific neurobiological process, perhaps related to transport of the receptor protein to site distant from the cell body or possible

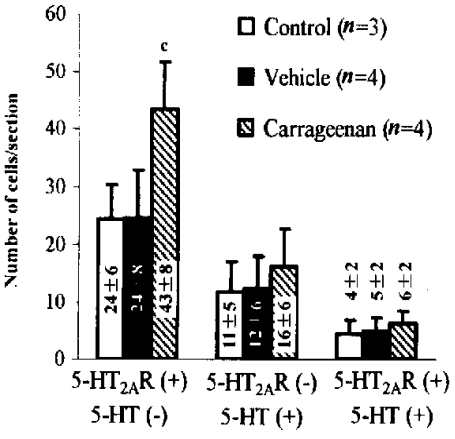

Fig 4. Correlation of 5-HT_{2A} receptor (5-HT_{2A} R) mRNA positive cells with 5-HT-IR cells in NRM. $\bar{x} \pm s$. $^cP < 0.01$ *vs* vehicle-injected control.

related to 5-HT_{2A} receptor turnover mechanisms in the nucleus.

It is the first report demonstrating that the expression of 5-HT_{2A} receptor mRNA in NRM neurons was markedly increased following carrageenan injection, suggesting that the synthesis of 5-HT_{2A} receptor be increased. The peak occurred at 3 h, and then there was a clear decrease at 24 h. This time scale of development corresponds to the time course of hyperalgesia as shown by behavioral and electrophysiologic studies[15,16], suggesting that 5-HT_{2A} receptor may be involved in inflammatory pain. There has been abundant evidence for the involvement of bulbospinal 5-HT neurons and 5-HT receptors including 5-HT_2/5-HT_{2A} receptor in nociceptive mechanism. Takeshita and Yamaguchi demonstrated that systemic administration of m-CCP (a 5-HT receptor agonist) dose-dependently attenuated both early and late phases of the formalin-induced nociceptive responses. The antinociception of m-CCP was reversed by ketanserine (a 5-HT_2/5-HT_{2A} receptor antagonist), suggesting that 5-HT_2/5-HT_{2A} receptor play an attenuating role in acute as well as chronic pain transmission[17]. This effect was regarded as related to peripheral, spinal and supraspinal 5-HT_2/5-HT_{2A} receptor. Moreover, it has been reported that microinjection of 5-HT_2 antagonist, ritanserin into NRM reduced morphine analgesia elicited from PAG, indicating that 5-HT_2 receptor in NRM modulate the transmission of opioid pain-inhibitory signals from PAG[18-20].

Recent studies in our laboratory[9] revealed that 5-HT_{1A} receptor mRNA was also expressed in moderate level in NRM, and some of them were expressed in 5-HT neurons, suggesting that 5-HT_{1A} receptors in NRM may, at least in part, be autoreceptors. This supported the studies of Sotelo *et al*[21], in which the existence of 5-HT_{1A} autoreceptor on the perikarya and dendrites of 5-HT neurons in NRM was demonstrated using double immunohistochemical staining with the anti-5-HT_{1A} receptor antibodies and an anti-5-HT antiserum. However, it has been not known whether some of 5-HT_{2A} receptors in NRM are autoreceptors. The present study demonstrated that only a few 5-HT_{2A} receptor mRNA positive cells were 5-HT neurons, whereas most of 5-HT_{2A} receptor mRNA positive cells were non-5-HT neurons. Following carrageenan inflammation, the colocalization of 5-HT_{2A} receptor mRNA with 5-HT-IR had no obvious change, suggesting the 5-HT_{2A} autoreceptor in NRM may not be related to peripheral inflammatory process.

Together with the findings that activity of 5-HT_2/5-HT_{2A} receptor in spinal and supraspinal structures can produce the antinociceptive effect[17,18,22,23], it is reasonable to assume that the enhanced expression of 5-HT_{2A} receptor mRNA following carrageenan inflammation may contribute to suppress the inflammatory pain. Anyway, further investigation will be required for elucidating the significance and mechanism of the expression increase of 5-HT_{2A} receptor mRNA in NRM with peripheral inflammation.

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外周致炎后 5-羟色胺 2A 受体 mRNA 在大鼠中缝大核神经元中的表达[1]

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关键词 血清素受体; 信使 RNA; 角叉菜胶; 荧光原位杂交; 免疫组织化学; 中缝核

目的: 观察角叉菜炎症时, 5-羟色胺 2A(5-HT_{2A})受体 mRNA 在中缝大核(NRM)表达的水平及其与 5-羟色胺(5-HT)免疫阳性神经元的共存. **方法**: 实验采用原位杂交和荧光原位杂交结合荧光免疫组织化学双标技术. **结果**: 正常大鼠 NRM 内有中等水平的 5-HT_{2A}受体 mRNA 表达; 角叉菜致炎后, 5-HT_{2A}受体 mRNA 在双侧 NRM 内的表达明显增加, 3 – 8 h 达峰值, 24 h 后显著降低. 在正常大鼠的 NRM 内, 仅有少量 5-HT_{2A}受体 mRNA 和 5-HT 免疫阳性双标神经元, 双标神经元的数量在角叉菜致炎后无明显变化. **结论**: NRM 内 5-HT_{2A}受体主要分布在非 5-HT 神经元, 且随外周炎症的发展, 5-HT_{2A}受体的合成增加.

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