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Changes in 5-HT1A receptor in the dorsal raphe nucleus in a rat model of post-traumatic stress disorder

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Abstract. Post-traumatic stress disorder (PTSD) is characterized mainly by symptoms of re-experiencing, avoidance and hyperarousal as a consequence of catastrophic and traumatic events that are distinguished from ordinary stressful life events. Single-prolonged stress (SPS) is an established animal model for post-traumatic stress disorder (PTSD). The dorsal raphe nucleus (DR)-serotonin (5-HT) system is markedly affected by swim stress and has been implicated in affective disorders. The 5-HT1A receptor (5-HT1AR) is critically involved in regulating mood and anxiety levels. In this study, we investigated changes in the expression of 5-HT1AR in the DR of rats after SPS that may reveal part of the pathogenesis of PTSD. 5-HT1AR expression in the DR was examined using immunohistochemistry, Western blotting and reverse transcription polymerase chain reaction. The expression of 5-HT1AR in the DR after SPS exposure was increased when compared to that in the control group ($P<0.05$). These findings indicate an increase in 5-HT1AR in the DR of SPS rats, which may play important roles in the pathogenesis of PTSD rats.

Introduction

The definition of post-traumatic stress disorder (PTSD) in DSM-IV (1) links a specific syndrome characterized mainly by symptoms of re-experiencing, avoidance and hyperarousal with catastrophic and traumatic events that are distinguished from ordinary stressful life events. Although extensive research has already been carried out, the etiology of PTSD remains unclear. Several clinical neuroendocrinological studies have significantly improved our understanding of PTSD. One of the core neuroendocrine abnormalities associated with PTSD is the dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, characterized by low levels of adrenocorticotropic hormone (ACTH), plasma cortisol and urinary cortisol and enhanced suppression of cortisol in response to low-dose dexamethasone administration (2-4). These neuroendocrine findings specific to PTSD have served as the basis for animal models that are useful for elucidating the pathophysiology of PTSD. Single prolonged stress (SPS) is a reliable animal model of PTSD based on the time-dependent dysregulation of the HPA axis and has been developed and employed for PTSD research patients.

Furthermore, the serotonin (5-HT) system and the HPA axis have complex interrelationships (5). In particular, the 5-HT1AR is markedly susceptible to modulation by stress and HPA-axis activation and is known to play a significant role in the pathophysiology of mood disorders (6-8).

It has been established that the serotonergic system plays an important role in the pathophysiology of anxiety and depression (9,10). In the dorsal raphe nucleus (DR) of rats whose brains were approximately half-composed of serotonergic neurons, numerous forebrain regions involved in the regulation of anxiety-related behaviour were innervated, including the hippocampus, amygdala, hypothalamus and prefrontal cortex (11,12). Among the various types of receptors for serotonin (5-HT) present in the brain, the 5-HT1AR is known to be involved in affective disorders and the mechanism of action of antidepressants (13). The 5-HT1AR is present in high density in the mesencephalic raphe nuclei as well as in cortical and limbic areas (e.g., frontal cortex, entorhinal cortex, hippocampus, amygdala and septum) (14,15). In the raphe nuclei, the 5-HT1AR is located on serotonergic cell bodies and dendrites (16), whereas in the projection areas, it is located postsynaptically. This receptor, which is present on the soma and dendrites of DR 5-HT neurons, inhibits the activity of these neurons as well as 5-HT synthesis within the neurons and 5-HT release in projection regions (17). 5-HT1AR is present in cortical and limbic areas, in which it plays a crucial role in the regulation of neuroendocrine function and responses to stress (18).

DR 5-HT neurons are particularly responsive to intense stressors such as catastrophic and traumatic events, probably since this region receives a unique set of inputs (19). These neurons are critical in the mediation of the behavioral sequelae of intense stressors, which is probably due to the unique projections of these neurons (20). This study therefore focused on the observation of changes in 5-HT1AR in the DR to ascertain the involvement of 5-HT1AR in the DR in PTSD.

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Materials and methods

Experimental animals. A total of 150 male Wistar rats weighing 150-180 g and aged 8 weeks at the start of the study, were supplied by the Animal Experimental Center, China Medical University, China. All animal procedures were carried out in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, the Ministry of Science and Technology of the People's Republic of China.

Model establishment and grouping. Animals were divided randomly into four groups: i) the control group; ii) SPS 1d (1-day) group; iii) SPS 4d (4-day) group, and iv) SPS 7d (7-day) group. Control animals remained in their home cages with no handling for 7 days and were sacrificed at the same time as the SPS groups. SPS rats underwent the SPS procedure on the first day. The SPS protocol was based on a combined plural stress paradigm (21): immobilization (compression with plastic bags) for 2 h, forced swimming for 20 min (24±1˚C), rest for 15 min, followed by drying and ether anesthesia (until consciousness was lost).

Perfusion-based sections. Rats of both normal control and SPS groups were prepared by perfusion of the left ventricle and fixation. The animals were post-fixed in the same fixative at 4˚C for 6-10 h and were then immersed in a 20% sucrose solution in 0.01 M phosphate buffer (PB, pH 7.4) at 4˚C. Samples were snap-frozen in liquid nitrogen and sectioned. Coronal sections of 12 μm were prepared for morphological studies.

Immunohistochemical analysis of 5-HT1AR. The sections were treated with 5% bovine serum albumin (BSA) and 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 30 min at room temperature (RT) to block non-specific staining. The sections were then incubated with goat monoclonal antibody against 5-HT1AR (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200) in 2% BSA-PBS overnight at 4˚C. After being washed with PBS, the sections were incubated with rabbit anti-goat IgG (Boster, China; 1:100) for 2 h and then with the streptomycin-avidin-biotin-peroxidase complex for 1 h. The sections were washed three times with PBS after each incubation and subsequently incubated with 3,3'-diaminobenzidine and H_2O_2. To assess non-specific staining, several sections in each experiment were incubated in buffer without primary antibody.

A total of 5 slides were randomly selected from each group, and 5 visual fields of the DR were randomly selected in each slide (x400). The optical density (OD) of positive cells in each field was recorded to evaluate the average OD. The OD of immunoreactivity of 5-HT1AR-immunopositive cells was analyzed using the MetaMorph/DPIO/BX41 morphology image analysis system.

Western blotting used to detect 5-HT1AR. Materials were obtained as above. Samples of normal control rats and SPS rats were respectively homogenized with a sample buffer containing 200 mM tris-buffered saline, pH 7.5, 4% sodium dodecyl sulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol, and denatured by boiling for 3 min. The protein fraction (30 μg/lane) prepared from each sample was separated by 12% (w/v) gradient SDS-polyacrylamide gel electrophoresis and electroblotted to a PVDF membrane (Millipore, Bedford, MA, USA) from the gel by a semi-dry blotting apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table I. The primer sequences of 5-HT1AR and β-actin.

Name	Upstream primer	Downstream primer	Product size (bp)
5-HT1AR	5'-tggctttctcatctccatcc-3'	5'-ctcactgcccca ttagtgc-3'	357
β-actin	5'-atcacccacact gtgcccatc-3'	5'-acagagtacttg cgctcagga-3'	542

The membrane was blocked with 5% dried skim milk, 0.05% Tween-20 in tris-buffered saline and Tween-20 (TBST) at RT for 2 h and incubated with goat monoclonal antibody 5-HT1AR (1:1,000) overnight at 4˚C.

Blots were washed three times with TBST, and then incubated with anti-goat IgG-HRP (Santa Cruz; 1:5000) for 2 h at RT and washed with TBST. After the incubation, the PVDF membrane was washed three times with TBST prior to visualization using enhanced chemiluminescence (ECL), (Amersham Pharmacia Biotech, Buckinghamshire, UK). To confirm equal protein loading, the same blots were reincubated with antibodies specific for β-actin (Abcam, UK; 1:1,000). Immunoreaction for β-actin was detected with ECL. The OD was analyzed on a Gel Image Analysis System. The levels of 5-HT1AR were determined by calculating the OD ratio of 5-HT1AR /β-actin.

Reverse transcription-polymerase chain reaction (RT-PCR) used to detect 5-HT1AR. Total mRNA was extracted from the DR using the TRIzol kit according to the manufacturer's instructions. The primers were designed by Shenggong Biotech Company (Shanghai, China) according to the serial number from Genbank (Table I). The reaction was started at 94˚C for 4 min and amplified for 5-HT1AR for 36 cycles of 45 sec at 94˚C, 45 sec at 60˚C, 40 sec at 72˚C and ended with a 7-min extension at 72˚C. β-actin mRNA, used as an internal control, was co-amplified with 5-HT1AR. The products were observed after electrophoresis on a 1.2% agarose gel, and the density of each band was analyzed on the Gel Image Analysis System. The levels of 5-HT1AR mRNA were determined by calculating the density ratio of 5-HT1AR mRNA/β-actin mRNA.

Statistical analysis. Data were expressed as the mean ± SD. Data among groups were analyzed by one-way analysis of variance using SPSS 13.0 software. $P<0.05$ was considered to be statistically significant.

Results

Immunohistochemical analysis of 5-HT1AR. 5-HT1AR was widely distributed throughout the DR, mainly at the plasma membrane, and appeared as buff-colored particles (Fig. 1A-D). Evaluation of the 5-HT1AR content by immunohistochemistry indicated a significant increase in the SPS model groups compared with the normal control group (Fig. 1E).

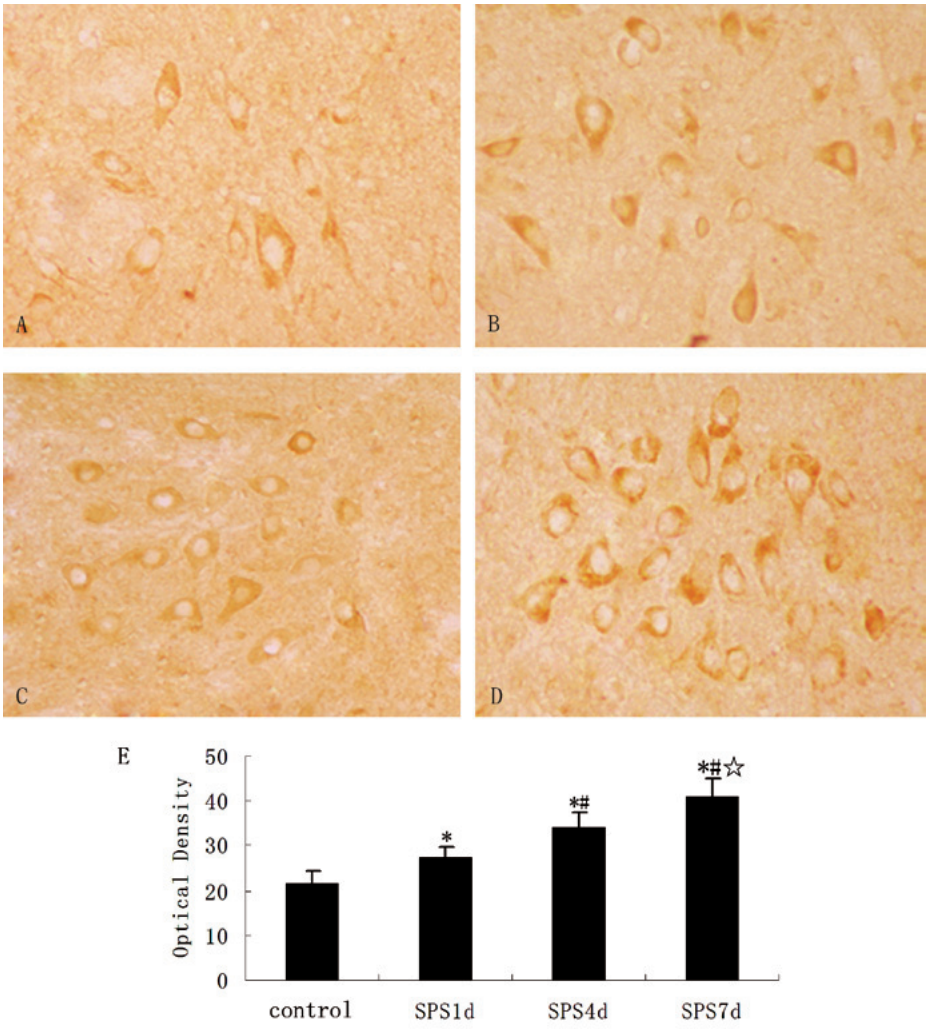

Figure 1. Presentation of 5-HT1AR expression in the DR in each group (A-D, magnification, x400). (A and B) The quantity of 5-HT1AR-immunoreactive cells in SPS 1d was greater than that of the control rats, then (C) gradually increased in SPS 4d and (D) in SPS 7d rats. (E) Quantitative analysis of the optical density. *P<0.05 vs. control group, #P<0.05 vs. SPS 1d group, ☆P<0.05 vs. SPS 4d group.

Western blotting of 5-HT1AR. Immunoreactive signals for 5-HT1AR and β-actin appeared at 56 and 42 kDa, respectively (data not shown), and the mean value of band densities of the control group was set as 100%. Data were expressed as normalized OD. Changes in 5-HT1AR expression in the DR between the control and SPS groups are shown in Fig. 2. The 5-HT1AR protein expression of the DR revealed a significant up-regulation after SPS stimulation in comparison with the control rats.

5-HT1AR mRNA expression. The levels of 5-HT1AR mRNA were normalized with the β-actin mRNA level. The levels of 5-HT1AR mRNA increased more significantly in the SPS group than in the control group (Fig. 3).

Discussion

Generalization of anxiety response to an ambiguous environment containing both threatening and non-threatening contextual cues is a characteristic of PTSD.

Anxiety is a mental state elicited in anticipation of threat. Feelings of anxiety are accompanied by behavioral and physiological responses that facilitate coping with danger, including avoidance and arousal. Perturbations of two genes of the serotonergic system have been associated with increased anxiety. Polymorphisms in the promoter of the human serotonin transporter and 5-HT1AR that alter the transcriptional activity of these genes are associated with increased trait anxiety (24-27).

Within the brain 5-HT1AR, two principal types of 5-HT1AR can be distinguished: the 5-HT1A autoreceptor and the postsynaptic 5-HT1AR. 5-HT1AR was found to be the inhibitory autoreceptor at the soma and dendrites of the 5-HT neurons in the raphe nuclei (22). In the raphe nuclei, 5-HT1AR is localized somatodendritically at 5-HT neurons (20,27). Their localization is mostly extrasynaptic at the plasma membrane, supporting the hypothesis of a volume transmission activation of these receptors (22,28) reported in the DR a ratio of 40:1 of the membrane associated with cytoplasmatic 5-HT1AR. The source of 5-HT1A autoreceptor activation is 5-HT, which is released from 5-HT neurons within one raphe nucleus, or from 5-HT neurons projecting from other raphe nuclei. Several studies observed that stimulation of the tonically activated 5-HT1A-autoreceptors (29) consistently inhibited 5-HT cell firing in the raphe nuclei and reduced 5-HT synthesis and 5-HT release in the raphe nuclei

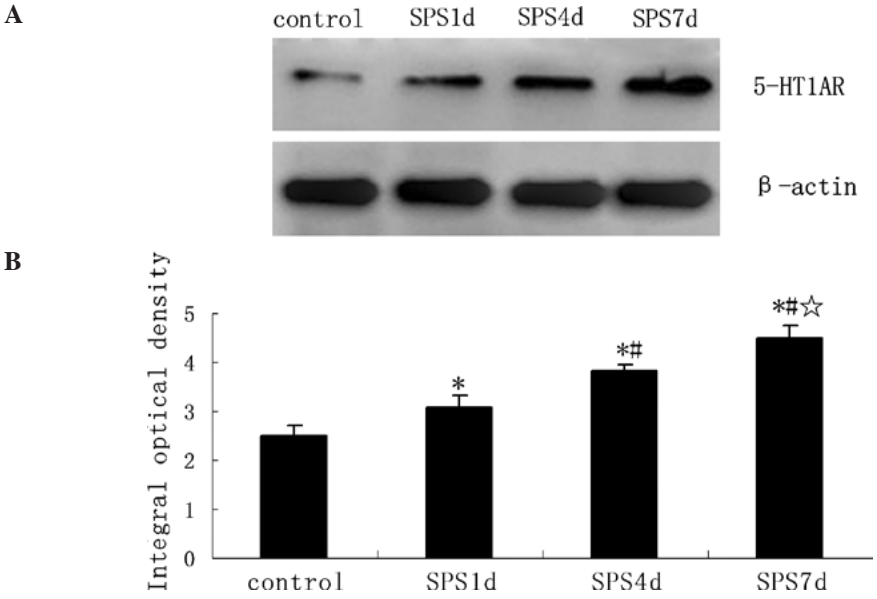

Figure 2. 5-HT1AR expression in the DR by Western blotting. (A) Bands show 5-HT1AR protein levels. (B) Relative quantitative levels of 5-HT1AR. *P<0.05 vs. control group, #P<0.05 vs. SPS 1d group, ☆P<0.05 vs. SPS 4d group.

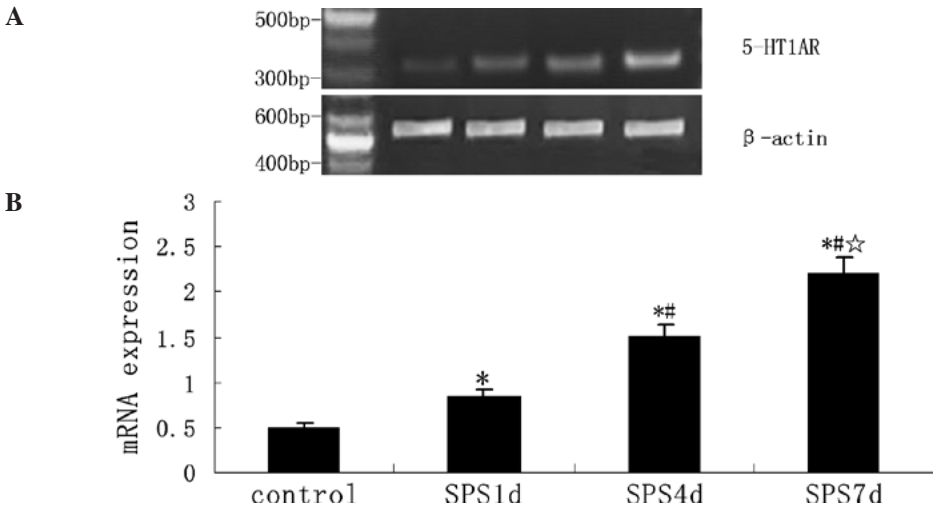

Figure 3. (A) Representative gel pattern of 5-HT1AR, β-actin cDNA bands (lane 0, marker; lane 1, control; lane 2, SPS 1d; lane 3, SPS 4d; lane 4, SPS 7d). (B) Relative 5-HT1AR mRNA expression. *P<0.05 vs. control group, #P<0.05 vs. SPS 1d group, ☆P<0.05 vs. SPS 4d group.

and in terminal areas of the DR projections (30). Overall, 5-HT1A-autoreceptors in the raphe nuclei appear to be in a crucial position to regulate the 5-HT activity in the terminal regions of the 5-HT projections by modulating the activity of 5-HT neurons (31). In a number of forebrain terminal regions of the ascending 5-HT projections, which arise from the DR, 5-HT1AR was observed to be a postsynaptic receptor (27,32). 5-HT1AR is located either at the dendrites or soma of post-synaptic neurons, or at non-serotonergic synapses, where it serves as a heteroreceptor. The stimulation of postsynaptic 5-HT1AR, in particular in the hippocampus, yielded anxiolytic, as well as anxiogenic effects in rats (33,34).

It was reported that SPS, a putative PTSD animal model, presents behavioral alterations resembling and shows the most consistent neuroendocrinologic characteristics with PTSD patients (35,36). Rats exposed to SPS exhibited enhanced inhibition of the HPA system and alteration in the glucocorticoid/mineralocorticoid receptor. Dysfunction of the HPA axis is one of the core neuroendocrine abnormalities of PTSD (37).

The present study examined the 5-HT1AR function in an animal model of SPS in Wistar rats. It was revealed that the expression of 5-HT1AR in the DR gradually increased after SPS stimulation. Several studies observed a high level of 5-HT1AR in the DR reserved for the inhibition of DR projection cell firing and 5-HT synthesis activity (38). The increase of 5-HT1AR in the DR, most likely by reducing the serotonergic tone in terminal areas, leads to an increase in anxious behavior and mood disorders. Since 5-HT1AR is susceptible to modulation by stress and is known to play an important role in the pathophysiology of anxious behavior and mood disorders, the dysfunction of 5-HT1AR in the DR may contribute

to the important pathobiological basis for abnormality of affective behavior induced by PTSD.

Our results indicate that the symptoms of PTSD require proper signalling by serotonin via DR 5-HT1AR. Presumably, dysfunction of the receptor is essential in order to set in motion a cascade of events that lead to long-lasting changes in brain chemistry or structure that are essential for the symptoms of PTSD throughout life.

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