Up-regulation of HTR1A reverses stress-induced visceral hypersensitivity through modulating interactions among the anterior cingulate cortex, insular cortex and hippocampus

Abstract: Background: This study aimed to explore the effect of 5-HT1A receptors (HTR1A) on activation of the anterior cingulate cortex and simultaneous regulation of neural activity in the insular cortex and hippocampus.

Methods: The IBS rat model was established via chronic water avoidance stress (WAS). Visceral sensitivity was measured by electromyogram, and anxiety-like behaviours were evaluated by the open field test. HTR1A-specific lentivirus expressing green fluorescent protein was used to overexpress or down-regulate HTR1A expression. Protein expression levels were detected by western blot.

Results: Up-regulation of HTR1A in ACC could inhibit ACC sensitization and reverse the visceral hypersensitivity and anxiety-like behaviours induced by chronic psychological stress. In contrast, down-regulation of HTR1A in ACC might promote these behaviors in IBS rats. Additionally, up-regulation of HTR1A in ACC could inhibit IC and hippocampus sensitization, while down-regulation might have the opposite effect.

Conclusions: In IBS rats, HTR1A could modulate ACC activation and interactions among the ACC, IC and hippocampus. These effects might in turn contribute to the development of visceral hypersensitivity and anxiety-like behaviours induced by chronic psychological stress.

Introduction

Irritable bowel syndrome (IBS), a common functional gastrointestinal disorder marked by abdominal pain and altered bowel habits at least once a week, affects 7% to 21% of the general population, leading to high healthcare costs and lost productivity [1].

Recently, the central nervous system (CNS) has been identified as a key component in the development of IBS [2-4]. Complex interactions between the gastrointestinal system and CNS might underlie the generation of IBS. In addition, the concept of “brain-gut axis disorders,” considered to be one potential mechanism underlying IBS, was mentioned repeatedly in Rome IV, further emphasizing the relationship between the CNS, gastrointestinal system and IBS [5,6].

The limbic system consists of several nuclei, such as the anterior cingulate cortex (ACC), the insular cortex (IC), the hippocampus and the amygdala, and is associated with emotion and emotion-related pain. Among the nuclei in the limbic system, the ACC, IC and hippocampus have been reported to be involved in the formation of visceral
hypersensitivity (VH), one pathophysiological mechanism of IBS. The ACC is considered to be a component of a functional circuit that plays an important role in perception of chronic visceral pain and processing of pain memories [7-9]. Additionally, bidirectional association fibers between the ACC-IC and ACC-hippocampus have been verified in many previous anatomical studies through the use of anterograde or retrograde tracers [10-12]. On the other hand, more and more research suggests that one factor participating in the development of IBS is the disruption of associations between different nuclei through association fibers or projection fibers, which facilitate information flow between brain regions [13,14]. However, there has been a lack of study focusing on the role of associations between different nuclei in the limbic system, especially pain-related brain regions like the ACC, IC and hippocampus, in the development of IBS.

The effect of central 5-hydroxytryptamine (5-HT) and its receptor, 5-HT1A receptor (HTR1A), on perception and processing of pain have become increasingly recognized over time. For example, HTR1A has been associated with chronic stress-induced visceral hypersensitivity and neuropathic pain in pre-clinical rodent studies, and many clinical trials have suggested that activation of HTR1A (either directly or via 5-HT reuptake inhibition) in the CNS might ameliorate psychiatric and gastrointestinal symptoms of IBS [15,16]. Recently, down-regulation of HTR1A in some nuclei of the limbic system (including the ACC and IC) was observed in rats with visceral hypersensitivity induced by chronic water avoidance stress (WAS), and local up-regulation of HTR1A using lentiviral constructs or direct activation with a selective agonist could reverse the visceral hypersensitivity and anxiety-like behaviours [17,18]. All research mentioned above demonstrated the crucial role that 5-HT and HTR1A in the CNS, especially in some nuclei of the limbic system, play in the formation and maintenance of visceral hypersensitivity or IBS. However, few studies have focused on the role of 5-HT and HTR1A in disrupting associations between different nuclei in the limbic system. Whether up-regulation or selective activation of HTR1A in the ACC could simultaneously change the activation of other nuclei in the limbic system remained uncertain.

In our current study, in order to investigate the effect of HTR1A in the ACC on development of visceral hypersensitivity and disruption of associations between the ACC and other nuclei in the limbic system, we aim to: (i) identify the effect of HTR1A in the ACC on the formation and maintenance of visceral hypersensitivity using lentiviral constructs; (ii) explore the role of HTR1A in the ACC on disrupting associations among the ACC, IC and hippocampus through observing simultaneous changes in expression of c-fos, a neuronal activity marker.

Materials and methods

Animals

Male Wistar rats were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. All animals were housed at the vivarium of our institute and were recorded as having a range of weights between 200 and 250g at the beginning of the experiment. Rats were maintained on a 12-h light/dark cycle and housed in cages of four during the experiment.

Ethical approval: The research related to animals’ use has been complied with all the relevant national regulations and the ethical standards of the Committee on Use and Care of Animals at Tongji Hospital (ethics approval number: 2015-DW-005)

Experimental design

A total of forty rats were divided into four groups: ten rats in the Water Avoidance Stress (WAS) group, ten rats in the WAS+Lentiviral Vector-Green Fluorescent Protein(LV-GFP) group, ten rats in the WAS+LV-HTR1A group and ten rats in the WAS+LV-shRNA-HTR1A group. On Days 1 to 10, all rats were exposed to water avoidance stress (WAS) for one hour per day. On Day 11, all rats were subjected to the open field test (OFT). Electromyogram (EMG) electrode implantation was performed on Day 12, and visceromotor response (VMR) to colorectal distension (CRD) was evaluated via EMG recording. After the conclusion of the experiment, all rats were euthanized via intraperitoneal (i.p.) injection of 0.05mg/g sodium pentobarbital and sacrificed.

Visceral hypersensitivity rat model by chronic stressor

WAS was used as the chronic stressor in the following experiments. In the WAS group, rats were placed on a vertical platform in a water tank for one hour per day for ten consecutive days. The water surface was 1 cm below the platform surface. In the sham-WAS group, rats were placed on the same platform, but there was no water in the water tank. Normal Control (NC) rats were placed without any intervention. The wire implantation surgery was
preformed on the eleventh day, while the electromyogram (EMG) recording was performed three days after the surgery.

**Visceral Motor Response (VMR) and EMG recording in rats.**

In order to measure visceral sensitivity, the VMR to colon rectum distension (CRD) was monitored via EMG recording, as described in our previous studies. Briefly, a pair of Teflon-coated stainless wires were surgically implanted into the left external abdominal oblique muscles three days before EMG recording. On the test day, rats were subjected to CRD. A flexible latex balloon was tied around a urethral catheter and inserted into the descending colon with the distal tip 1 cm from the anal verge. In all groups, 60 mmHg CRD was achieved by injecting gas into the colonic balloon within one second and maintaining the distention for twenty seconds. Three cycles of CRD (20 sec duration, 4 min inter-stimulus interval) were applied to each rat. The EMG recording signal was amplified (Gain=5000) and filtered (Lowpass=5000 Hz; Highpass=50Hz) using PowerLab System (Chart 7.0, AD Instruments, Bella Vista, NSW, Australia). The results of electromyography were quantified by calculating the area under the curve per (AUC/s). AUC was calculated as the sum of all recorded data points multiplied by the sample interval (in seconds) after baseline subtraction and was regarded as the rats’ visceral sensitivity.

**Open Field Test**

All rats were subjected to OFT before wire implantation surgery. Rats were placed in an open box with black bottom and white walls (40 cm length × 40 cm width × 40 cm height) for five minutes after thirty minutes of adaptation in the testing room to allow the animals to adapt to the testing environment. The test process was recorded using a video camera above the open box. The ANY-maze system (Stoelting Co, Wood Dale, IL, USA) was used to measure frequency of crossing and entering into the central zone, as well as total distance travelled.

**Western blot**

Protein was extracted from anterior cingulate cortex tissue, and protein concentration was determined using a BCA Protein Assay Kit (Beyotime Biotechnology Co, Ltd, Shanghai, China). Then, electrophoresis and membrane transfer were performed. Immunoblots were probed using the following primary antibodies: HTR1A (1 : 1000, Abcam Co), c-fos (1 : 1000, Abcam Co) and GAPDH (1 : 10000, Sigma Co). A goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Abcam co) was used. Enhanced chemiluminescence reagents were used for detection. The autoradiographed films were scanned and processed using Alphaview SA software. The densitometric values of each protein band were normalized to expression of GAPDH.

**Statistical analyses**

All analyses were performed using R language v3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). All data were expressed as mean(SEM). One-way analysis of variance (one-way ANOVA) was used to compare AUCs of EMG recordings, expression of proteins and IOD values among different groups. TukeyHSD post-hoc multiple comparisons test was also used. All reported P values were two-sided, with P<0.05 defined as statistical significance.
Result

Comparing visceral sensitivity among different HTR1A intervention groups.

There were significant differences in VMR amplitude among WAS, WAS+LV-GFP, WAS+LV-HTR1A and WAS+LV-shRNA-HTR1A groups (F=16.61, P=0.004, Fig.1A). Post-hoc analysis showed that the VMR amplitude of the WAS+LV-HTR1A group was significantly lower than those of the WAS and WAS+LV-GFP groups (WAS+LV-HTR1A vs WAS, 0.465±0.115 vs 1±0.322, p<0.01; WAS+LV-HTR1A vs WAS+LV-GFP, 0.465±0.115 vs 1.2±0.41, p<0.001), while the VMR amplitude of the WAS+LV-shRNA-HTR1A group was significantly higher than those of the WAS and WAS+LV-HTR1A groups (WAS+LV-shRNA-HTR1A vs WAS, 1.532±0.443 vs 1±0.322, p<0.01; WAS+LV-shRNA-HTR1A vs WAS+LV-HTR1A, 1.532±0.443 vs 0.465±0.115, p<0.001).

Electromyographic recordings for 60mmHg CRD in WAS, WAS+LV-GFP, WAS+LV-HTR1A and WAS+LV-shRNA-HTR1A groups are presented in Fig.1B.

Comparing changes in anxiety-like behaviours among different HTR1A intervention groups.

Anxiety-like behaviours in rats were tested via OFT. Rats that avoided the central area of the open field were considered to be more anxious. Number of instances of line crossing, center travelled distance, average speed, immobile time and mobile time were recorded in all rats during the OFT. The number of instances of line crossing (P=0.002), average speed (P<0.001) and mobile time(P<0.001) were significantly different in the WAS, WAS+LV-GFP, WAS+LV-HTR1A and WAS+LV-shRNA-HTR1A groups. In the WAS+LV-HTR1A group, we observed fewer line crossings (WAS+LV-HTR1A vs WAS, p<0.05), a lower center travelled distance (WAS+LV-HTR1A vs WAS, p<0.05) and reduced mobile time (WAS+LV-HTR1A vs WAS, p<0.01) compared with the WAS and WAS+LV-GFP group s. Additionally, a higher frequency of line crossing (WAS+LV-shRNA-HTR1A vs WAS+LV-GFP, p<0.01; WAS+LV-shRNA-HTR1A vs WAS+LV-HTR1A, p<0.01), higher average speed (WAS+LV-shRNA-HTR1A vs WAS+LV-GFP, p<0.001; WAS+LV-shRNA-HTR1A vs WAS+LV-HTR1A, p<0.001) and increased mobile time (WAS+LV-shRNA-HTR1A vs WAS, p<0.01; WAS+LV-shRNA-HTR1A vs WAS+LV-GFP, p<0.001; WAS+LV-shRNA-HTR1A vs WAS+LV-HTR1A, p<0.001) were found in the WAS-LV-shRNA-HTR1A group compared
Up-regulation of HTR1A reverses stress-induced visceral hypersensitivity through modulating interactions with the WAS, WAS+LV-GFP or WAS+LV-HTR1A groups. Detailed results are presented in Table I and Fig. 2 A-D.

Comparing HTR1A expression levels in the ACC among different HTR1A intervention groups.

Protein expression levels of HTR1A in the ACC among different intervention groups were compared using western blot. The results showed that expression of HTR1A was significantly higher in the WAS+LV-HTR1A group compared with the WAS group (WAS+LV-HTR1A vs WAS, left ACC, 2.196±0.454 vs 1±0, p<0.001; right ACC, 1.748±0.327 vs 1±0, p<0.001) and the WAS+LV-GFP group (WAS+LV-HTR1A vs WAS+LV-GFP, left ACC, 0.725±0.117 vs 1.119±0.264, p<0.05, right ACC, 0.735±0.211 vs 1.204±0.275, p<0.001) and the WAS+LV-HTR1A group (WAS+LV-shRNA-HTR1A vs WAS+LV-HTR1A, left ACC, 0.725±0.117 vs 2.196±0.454, p<0.001, right ACC, 0.735±0.211 vs 1.748±0.327, p<0.001). Detailed results are presented in Fig. 3 A, B, D, E.

Comparing expression levels of c-fos in the ACC, IC and hippocampus among different HTR1A intervention groups.

Protein expression of c-fos in the ACC, IC and hippocampus among different intervention groups was compared using western blot. The results showed that expression of c-fos was significantly lower in the WAS+LV-HTR1A group in the ACC, IC and hippocampus compared with the WAS+LV-GFP group (WAS+LV-shRNA-HTR1A vs WAS+LV-GFP, left ACC, 0.725±0.117 vs 1.119±0.264, p<0.05, right ACC, 0.735±0.211 vs 1.204±0.275, p<0.001) and the WAS+LV-HTR1A group (WAS+LV-shRNA-HTR1A vs WAS+LV-HTR1A, left ACC, 0.725±0.117 vs 2.196±0.454, p<0.001, right ACC, 0.735±0.211 vs 1.748±0.327, p<0.001). Detailed results are presented in Fig. 3 A, B, D, E.
Figure 3: Effects of lentivirus intervention on expression of HTR1A and c-fos in the ACC. (A) Representative immunoblots from the left ACC. (B)(C) Protein expression levels of HTR1A and c-fos in the left ACC were compared among WAS, WAS+LV-GFP, WAS+LV-HTR1A and WAS+LV-shRNA-HTR1A groups. (D) Representative immunoblots from the right ACC. (E)(F) Protein expression levels of HTR1A and c-fos in the right ACC were compared among WAS, WAS+LV-GFP, WAS+LV-HTR1A and WAS+LV-shRNA-HTR1A groups. * P<0.05, ** P<0.01, *** P<0.001.

Table 1: Comparison of anxiety-like behavior among different HT1AR intervention groups.

|                          | WAS     | WAS+LV-GFP | WAS+LV-HT1AR | WAS+LV-shRNA-HT1AR | P value |
|--------------------------|---------|------------|--------------|--------------------|---------|
| Line crossing (times)    | 70.75(17.63) | 49.2(16.3) | 48(15)         | 81.2(21.6)         | 0.002   |
| center travelled distance | 0.234(0.158) | 0.254(0.033) | 0.264(0.025) | 0.230(0.052)       | 0.784   |
| Average speed (m/s)      | 0.026(0.011) | 0.015(0.009) | 0.015(0.007) | 0.033(0.008)        | <0.001  |
| Mobile time (s)          | 79.85(28.93) | 70.52(16.78) | 48.3(20.6)     | 110.83(3.5)        | <0.001  |

a, aa, aaa represent P value<0.05, P<0.01 and P value<0.001 respectively compared with WAS group;
b, bb, bbb represent P value<0.05, P<0.01 and P value<0.001 respectively compared with WAS+LV-GFP group;
c, cc, ccc represent P value<0.05, P<0.01 and P value<0.001 respectively compared with WAS+LV-HT1AR group;
Up-regulation of HTR1A reverses stress-induced visceral hypersensitivity through modulating interactions ... vs 1±0, p<0.01; right ACC, 0.63±0.01 vs 1±0, p<0.05; right hippocampus, 0.675±0.054 vs 1±0, p<0.01) and the WAS+LV-GFP group (WAS+LV-HTR1A vs WAS+LV-GFP, left ACC, 0.2±0.2 vs 1.074±0.235, p<0.001; right ACC, 0.63±0.01 vs 1.211±0.206, p<0.001; right IC, 0.675±0.054 vs 1.032±0.257, p<0.01; left hippocampus, 0.560±0.115 vs 1.429±0.537, p<0.01). Additionally, expression levels of c-fos were significantly higher in the WAS+LV-shRNA-HTR1A group in the ACC, IC and hippocampus compared with the WAS group (WAS+LV-shRNA-HTR1A vs WAS, left ACC, 1.250±0.258 vs 1±0, p<0.05; right ACC, 1.564±0.511 vs 1±0, p<0.001; left IC, 1.523±0.478 vs 1±0, p<0.01; left hippocampus, 1.719±0.924 vs 1±0, p<0.05), the WAS+LV-GFP group (WAS+LV-shRNA-HTR1A vs WAS+LV-GFP, right ACC, 1.564±0.511 vs 1.211±0.206, p<0.05; left IC, 1.523±0.478 vs 1.014±0.337, p<0.01; right hippocampus, 1.235±0.234 vs 0.747±0.302, p<0.001) and the WAS+LV-HTR1A group (WAS+LV-shRNA-HTR1A vs WAS+LV-HTR1A, left ACC, 1.250±0.258 vs 0.2±0.2, p<0.001; right ACC, 1.564±0.511 vs 0.63±0.01, p<0.001; left IC, 1.523±0.478 vs 0.669±0.325, p<0.001; right IC, 1.116±0.308 vs 0.675±0.054, p<0.001; left hippocampus, 1.719±0.924 vs 0.560±0.115, p<0.01; right hippocampus, 1.235±0.234 vs 0.593±0.166, p<0.001). Detailed results are presented in Fig.3 A,C,D,E and Fig.4 A-F.

Discussion

Chronic psychological stress plays a crucial role in the generation and maintenance of visceral hypersensitivity and anxiety-like behaviours, the most common symptoms of IBS. The factors have been reported to be associated with the central nervous system and functional connectivity between different brain regions, especially nuclei in the limbic system. The nuclei of the limbic system, such as
the ACC, IC and hippocampus, have been reported to be major components of emotional responses and pain perception, disorders of which might result in both visceral hypersensitivity and anxiety-like behaviours. In our previous research, HTR1A in the ACC was demonstrated to be a key receptor in psychological stress-induced IBS, mediating the generation of visceral hypersensitivity and anxiety-like behaviours [18]. However, it remained unclear whether HTR1A mediated these biological processes via change in connectivity between the ACC and other nuclei.

In our present study, we used lentiviral vectors to regulate expression of HTR1A in the ACC, as in our previous study. It was found that up-regulation of HTR1A in the ACC could reverse the visceral hypersensitivity and change in anxiety-like behaviour, indicating that up-regulation of HTR1A in the ACC could partially inhibit the generation of IBS. On the other hand, down-regulation of HTR1A in the ACC could intensify the visceral pain induced by noxious stimuli and promote feelings of anxiety, suggesting that down-regulation of HTR1A in the ACC might promote the development of IBS and aggravate IBS-related symptoms.

In accordance with previous research, our present study proves that up-regulation of HTR1A could reverse visceral hypersensitivity and anxiety-like behaviours in chronic psychological stress-induced IBS rats. In addition, expression levels of c-fos protein in the ACC, IC and hippocampus among these four lentivirus-treated groups were compared. The results showed that up-regulation of HTR1A in the ACC could significantly decrease expression levels of c-fos in all three of the studied brain regions. These results suggest that up-regulation of HTR1A in the ACC could modulate ACC, IC and hippocampus activation simultaneously and provide evidence that both of these functional circuits (ACC-IC and ACC-hippocampus) might participate in the development of stress-related visceral hypersensitivity.

However, there are some limitations to this study. Due to a lack of financial support and experimental appliances, we had to use c-fos expression as a biomarker of neural activity, rather than utilizing electrophysiologic technologies to obtain direct evidence of increased spike frequency or synchronization across different nuclei. More direct evidence about the effect of HTR1A on functional connections among the ACC, IC and hippocampus based on electrophysiological data is still needed.

Notwithstanding these limitations, our study illustrates that up-regulation of HTR1A in the ACC could reverse visceral hypersensitivity and anxiety-like behaviours induced by chronic psychological stress, further supporting the idea proposed in our previous research: that HTR1A in the ACC mediates the development of stress-induced visceral hypersensitivity. Moreover, simultaneous changes in c-fos expression levels in the ACC, IC and hippocampus after regulation of HTR1A in the ACC was shown in our study, indicating an effect of HTR1A on interactions between the ACC, IC and hippocampus. However, direct evidence based on electrophysiology needs further research.

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