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Activation of the serotonin 5-HT_{2C} receptor is involved in the enhanced anxiety in rats after single-prolonged stress

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Abstract

We have recently confirmed that exposure of rats to the single-prolonged stress (SPS) paradigm induces enhanced hypothalamic–pituitary–adrenal (HPA) axis negative feedback and enhanced anxiety, and found that these changes develop time-dependently following stress exposure, suggesting that it could model the neuroendocrinological and behavioral abnormalities of the post-traumatic stress disorder (PTSD) patients. In the present study, microarray analysis was performed using RNA from the hippocampus, amygdala and anterior cingulate cortex of SPS rats and unstressed controls to unveil the molecular changes underlying SPS-induced behavioral changes. Thirty-one genes were found whose time course of expression corresponded to that of behavioral changes. One gene, 5-hydroxytryptamine$_{2C}$ (5-HT_{2C}) receptor, was identified as a putative candidate. The overexpression of the gene in the amygdala of SPS rats was confirmed using real-time PCR 7 days after the SPS exposure. This molecule was then pharmacologically validated using FR260010 (*N*-[3-(4-methyl-1*H*-imidazol-1-yl)phenyl]-5,6-dihydrobenzo[*h*]quinazolin-4-amine dimethanesulfonate), a selective 5-HT_{2C} receptor antagonist. FR260010 (1–10 mg/kg, s.c.) significantly inhibited the enhancement of anxiety in SPS rats. These results demonstrate for the first time that activation of the brain 5-HT_{2C} receptor is involved in the development of behavioral abnormality in this model. This suggests that selective 5-HT_{2C} receptor antagonists might provide novel therapeutic avenues for PTSD treatment.

Keywords: Serotonin 5-HT_{2C} receptor; Post-traumatic stress disorder; Single-prolonged stress; Microarray; Real-time PCR; Fear conditioning

1. Introduction

Post-traumatic stress disorder (PTSD) is an anxiety disorder that develops after exposure to a life-threatening traumatic experience and is characterized by symptoms such as continually re-experiencing the traumatic event, avoidance of stimuli associated with the trauma, numbing of general responsiveness, and increased arousal (American Psychiatric Association. 1994, DSM-IV). Although a number of commercially available drugs (e.g., benzodiazepines, antidepressants, adrenergic antagonists) have been used in clinical settings to explore the possible therapeutic avenues for treating PTSD (Schoenfeld et al., 2004), they still fall short of being ideal due to limited response and remission rates and tolerability issues (Zhang and Davidson, 2007). In addition, there are no drugs that were rationally developed based upon disease-specific changes that were found with gene expression analysis in PTSD patients.

Enhanced hypothalamic–pituitary–adrenal (HPA) axis negative feedback is a putative neuroendocrinological hallmark of PTSD (Stein et al., 1997; Yehuda, 2001, 2005; Yehuda et al., 1993) that can be induced in rats using a trauma-like single-prolonged stress (SPS) paradigm, which consists of 2 h restraint, 20-min forced swim and ether anaesthesia (Liberzon et al., 1997). SPS rats also exhibit behavioral abnormalities (enhanced anxiety) that mimic the symptoms of PTSD (Imanaka et al., 2006; Khan and Liberzon, 2004; Takahashi et al., 2006). We have recently confirmed that SPS rats exhibit both behavioral and neuroendocrinological (enhanced HPA axis negative feedback) abnormalities, and found that these changes develop time-dependently after stress exposure (Kohda et al., 2007). Given that the SPS model reflects at least some of the clinical characteristics of the disease,

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compounds which ameliorate the behavioral changes in SPS model rats may have therapeutic potential for the treatment of PTSD. Thus, gene expression analysis of SPS rats may unveil novel molecular targets for such compounds.

In the present study, comparative microarray analysis was performed using RNA from the brains of both SPS rats and unstressed controls one day or 7 days after the SPS to examine the molecular changes underlying SPS-induced behavioral changes. Since previous findings assert that contextual fear conditioning requires both the amygdala and the hippocampus (Anagnostaras et al., 1999; Kim and Fanselow, 1992; Phillips and LeDoux, 1992, 1994), and that the anterior cingulate cortex is involved in suppression of the amygdala (Morgan and LeDoux, 1995), gene expression changes in these brain regions were examined. Since behavioral changes were observed 7 days after the SPS but not one day after (Kohda et al., 2007), genes with the same time course were considered possible candidates. One such candidate is 5-hydroxytryptamine$_{2C}$ (5-HT$_{2C}$) receptor. This molecular target was subjected to quantitative real-time PCR to confirm the overexpression in the amygdala. Next, the involvement of the receptor was pharmacologically validated using the selective 5-HT$_{2C}$ receptor antagonist FR260010 (*N*-[3-(4-methyl-1*H*-imidazol-1-yl)phenyl]-5,6-dihydrobenzo[*h*]quinazolin-4-amine dimethanesulfonate) which we have recently discovered (Harada et al., 2006).

2. Methods

2.1. Animals

Experiments were carried out using 8-week-old male Sprague–Dawley rats purchased from Japan SLC, Inc. (Hamamatsu, Japan). Animals were housed at 23±2 °C with 55±5% humidity under a 12 h light/dark cycle (lights on at 7:00 a.m.) for at least 1 week before use in experiments, and were allowed free access to food and water. All animal experiments were performed in accordance with the Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharma Inc.) Animal Experiment Committee guidelines.

2.2. Single-prolonged stress (SPS)

Rats were exposed to a SPS event according to a previously reported procedure (Liberzon et al., 1997). In brief, rats were restrained for 2 h in acrylic animal holders (55×45×200 mm, NeuroScience Idea, Osaka, Japan), followed immediately by a 20-min forced swim in 24 °C water. Following a 15-min rest, animals were exposed to ether until loss of consciousness was achieved, and were then left undisturbed in their home cages until used for brain tissue sampling or behavioral study.

2.3. Total RNA isolation

One or 7 days after the SPS, rats were decapitated and the brains were removed immediately for sectioning. Unstressed control rats of the same age were treated likewise. Coronal sections 2 mm thick were prepared using a microslicer and then soaked in RNAlater (Qiagen, GmbH, Hilden, Germany) on ice. The hippocampus was excised from the slices, and tissue samples from the amygdala and the anterior cingulate cortex were excised using a brain punch (diameter: 2 mm), based on coordinates from the brain atlas (Paxinos and Watson, 1986). Tissue samples were immediately frozen in liquid nitrogen and stored at −70 °C before the RNA was isolated. Total RNA from the amygdala and the anterior cingulate cortex was isolated using an RNeasy Mini Kit (Qiagen, GmbH), and total RNA from the hippocampus was isolated using an RNeasy Lipid Tissue Mini Kit (Qiagen, GmbH) according to the manufacturer's protocol. The purity and integrity of the samples was assessed by measuring the OD with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and by viewing the ethidium bromide-stained 28S and 18S ribosomal RNA bands. Each group consisted of 4 or 5 rats. Equal amounts of RNA from the respective brain regions of the rats in each group were mixed and used for microarray analysis. Real-time PCR was performed for the individual RNA samples.

2.4. RNA amplification and microarray analysis

These experiments were conducted by Life Science Group, Hitachi, Ltd. (Kawagoe, Japan). T7 amplification was employed to obtain the amount of RNA required to produce a fluorescent probe (Van Gelder et al., 1990). First-strand cDNA synthesis was performed after attaching a T7 oligo (dT) 24 primer to the poly (A) tail of total RNA (5 μg). Second strand synthesis was employed to produce double strand DNA. cRNA containing aminoallyl-UTP was then synthesized with T7 RNA polymerase. cRNA was then reacted with the *N*-hydroxy succinimide esters of Cy5 or Cy3 (PerkinElmer Life Sciences Inc., Boston, MA) following the manufacturer's instructions. Dye molecules were separated from the labeled products using an RNeasy Mini Kit (Qiagen, GmbH). Cy5-labeled cRNA from SPS rats was mixed with the same amount of Cy3-labeled cRNA from unstressed rats. The mixture was hybridized with Rat Oligo Microarray G4130A (Agilent Technologies, Santa Clara, CA) containing 22,575 probes at 60 °C for 17 h. The hybridization signal was scanned with a confocal laser scanner G2565BA (Agilent Technologies).

Table 1
Summary for number of genes differentially expressed in the hippocampus, amygdala, or anterior cingulate cortex as a result of a single-prolonged stress (SPS) in rats

Group	Expression level		Hippocampus	Amygdala	Anterior cingulate cortex
	Day 1	Day 7	Gene number	Gene number	Gene number
1	⇑	⇑	2	5	0
2	⇑	⇒	0	6	3
3	⇒	⇑	1	13	1
4	⇒	⇓	0	14	3
5	⇓	⇒	0	0	1
6	⇓	⇓	0	0	0

⇑, up-regulated vs. unstressed control; ⇓, down-regulated vs. unstressed control. ⇒, no change.
Criterion for change: 2-fold.

Table 2
List of genes and their fold changes in expression relative to unstressed controls that were differentially expressed only on Day 7 after the SPS

Genbank ID	Day 1	Day 7	Gene
Hippocampus			
CB547655	1.91	2.63	Similar to mouse chloride intracellular channel 6 (Clic6)
Amygdala			
X60661.1	1.60	3.88	Potential ligand-binding protein RYD5
AA900283	1.64	3.61	Similar to mouse 18-day embryo whole body cDNA
NM_012544	1.63	2.73	Angiotensin I-converting enzyme (Ace)
NM_031059	1.84	2.58	Homeo box, msh-like 1 (Msx1)
BM388699	1.42	2.52	Similar to mouse procollagen type VIII α2
NM_031336	1.34	2.46	Klotho (Kl)
AW142560	1.72	2.27	Similar to mouse chloride intracellular channel 6 (Clic6)
BF556962	1.77	2.15	Similar to mouse laminin-2 alpha2 chain
CB544991	0.79	2.06	Similar to human KIAA1424 protein
NM_012765	0.92	2.06	Serotonin 5-HT_{2C} receptor (Htr2c)
AA964289	1.26	2.04	Similar to mouse adult male testis cDNA
CB544321	0.90	2.04	Similar to mouse nuclear X box binding factor 1(NFX.1)
BF393452	1.20	2.02	Similar to mouse orthodenticle homolog 2 (Drosophila) (Otx2)
CB547427	0.89	0.36	Similar to mouse ELK3
CB548357	0.93	0.37	Similar to rat AT motif-binding factor (Atbf)
AW520820	0.96	0.37	Similar to mouse BAC clone RP24-267C20 from chromosome 13
AI704656	0.85	0.38	Laminin, alpha 5 (Lama5)
BF562740	0.86	0.41	Similar to mouse HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1 (Hace1)
NM_019313	1.14	0.43	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1 (Kcnn1)
CB545843	0.91	0.45	Similar to mouse rho/rac guanine nucleotide exchange factor (GEF) 18 (Arhgef18)
BF288712	0.87	0.45	Similar to mouse YTH domain family 2 (Ythdf2)
CB544711	0.92	0.46	Similar to mouse expressed sequence 2 (Es2) protein
AA956956	0.96	0.46	Similar to mouse BAC clone RP24-559P3 from chromosome 5
BF395784	0.90	0.46	Similar to human trophinin associated protein (tastin)
NM_133559	0.91	0.47	Proprotein convertase subtilisin/kexin type 4 (Pcsk4)
CB582080	0.95	0.48	Similar to mouse 18-day embryo whole body cDNA, RIKEN fulllength enriched library, clone:1110060O18
AW914764	1.00	0.49	Similar to mouse exosome component 5 (Exosc5)
Anterior cingulate cortex			
BM388699	1.12	2.05	Similar to mouse procollagen type VIII α2
CB546432	1.66	0.35	Similar to mouse casein kinase 1, gamma 1 (Csnk1g1)
CB545812	0.99	0.44	Similar to mouse plakophilin 2 (Pkp2)
BF403173	1.22	0.45	Similar to mouse adult male testis cDNA

The intensities of the fluorescence on the scanned images were quantified, corrected for background, and normalized using global normalization methods with Feature Extraction software (Agilent Technologies). Genes with a signal intensity of more than 20 were analyzed for differential expression (criteria for differential expression was set at 2-fold). When the names of differentially expressed genes were not annotated, the NCBI database BLAST (Basic Local Alignment Search Tool) was used to search for genes with similar sequences.

2.5. Quantitative real-time PCR

These experiments were conducted by Life Science Group, Hitachi, Ltd. RNA from the amygdala of rats 7 days after the SPS and unstressed control rats was chosen for quantitation using fluorescence-based quantitative real-time PCR with an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Primers and TaqMan probes for the 5-HT_{2C} receptor (Assay ID: Rn00562748_m1) and β-actin (Assay ID: Rn00667869_m1) were purchased from Applied Biosystems. The TaqMan probes, which were designed for use when hybridizing PCR products, were labelled with the fluorescent reporter dye FAM at the 5′-end, and a quenching dye at the 3′-end. Ten nanograms of cDNA was used as a PCR template. Forward and reverse primers were used at 10 μM, and TaqMan probes at 5 μM in Universal PCR Mix (Applied Biosystems) in a 20-μl reaction volume. The thermal cycle conditions used were as follows: 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. All assays were performed in triplicate for verification. PCR assays for unknown samples were performed simultaneously with standard samples [diluted reverse transcript from Rat Whole Brain total RNA (Cat#: 201, Unitech, Kashiwa, Japan)] to construct a standard curve. The relative concentrations of 5-HT_{2C} receptor and β-actin in the unknown samples were calculated from this standard curve using ABI PRISM 7900HT sequence detection system software SDS 2.1

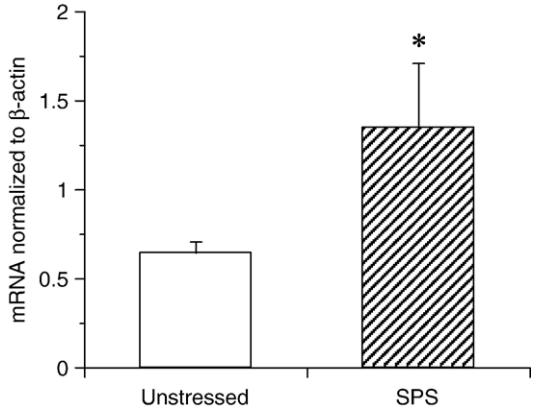

Fig. 1. Expression of 5-HT_{2C} receptor mRNA in the amygdala of unstressed rats and SPS rats 7 days after the SPS. mRNA levels were determined by quantitative real-time PCR. Results were normalized to β-actin mRNA. Values were given as the mean ± S.E.M. ($n=4$). *$P<0.05$ vs. unstressed group (Mann–Whitney's U-test).

(Applied Biosystems). The ratio of the relative concentration of 5-HT_{2C} receptor was calculated relative to the concentration of β-actin.

2.6. Contextual fear conditioning

Rats were subcutaneously administered FR260010 seven days after the SPS. Thirty minutes after this administration, they were subjected to five inescapable scrambled electric footshocks (0.3 mA, duration of 30 s each, variable-interval schedule) in a chamber (24 × 30 × 29 cm) with a grid floor during a 5-min conditioning session. Twenty-four hours after the conditioning session, the animals were again placed in the same chamber, this time without footshock, and observed for 5 min. Behavior was recorded on videotape and scored later. Freezing was defined as the absence of all observable skeletal movements except those related to respiration. The duration of the freezing behavior was recorded using a modified time-sampling procedure. The behavior of each animal was classified as either freezing or active every 10 s. When a rat froze for the entire 10-sec period, behavior was scored as freezing. The percentage of periods frozen during the 5-min observation was then calculated.

2.7. Chemicals

FR260010 was synthesized by Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharma Inc.). It was dissolved and diluted in 0.5% methylcellulose. The injection volume was adjusted to 2 mL/kg.

2.8. Statistical analyses

All values were given as the mean ± S.E.M. In a real-time PCR study, statistical analysis for SPS rats vs. unstressed rats was conducted using Mann–Whitney's U-test. In a fear conditioning study, statistical analysis for SPS control group vs. unstressed group was conducted using t-test, and statistical analysis for drug treatment vs. SPS control was conducted using Dunnett's multiple comparison test. A value of $P<0.05$ was considered significant.

3. Results

Gene expression in the hippocampus, amygdala, and anterior cingulate cortex was compared between SPS rats and unstressed controls either one day or 7 days after the SPS using a microarray technique. Several genes were differentially regulated between the groups at one time point at least (Table 1). The number of differentially expressed genes was greatest in the amygdala. Thirty-one genes were differentially expressed in the brain regions only 7 days after the SPS (one gene was up-regulated in the hippocampus; 13 genes were up-regulated and 14 genes were down-regulated in the amygdala; and one gene was up-regulated and 3 genes were down-regulated in the anterior cingulate cortex), which could be possible candidates (Table 2). Only one gene (Ac.# BM388699, similar to mouse procollagen type VIII α2) was differentially expressed in two regions (amygdala and anterior cingulate cortex).

Next we selected the 5-HT_{2C} receptor and performed quantitative real-time PCR to confirm the overexpression of this gene in the amygdala using the same RNA as microarray analysis. 5-HT_{2C} receptor mRNA significantly increased in the amygdala compared to unstressed controls ($P=0.030$, Mann–Whitney's U-test; Fig. 1).

We then examined the effect of the selective 5-HT_{2C} receptor antagonist FR260010 in a contextual fear conditioning test in SPS rats. In our preliminary study, FR260010 dose-dependently inhibited 1-(*m*-chlorophenyl)-piperazine (*m*-CPP)-induced hypolocomotion in rats, an index of in vivo 5-HT_{2C} receptor antagonism (Kennett and Curzon, 1988; Kennett et al., 1997), with an ID_{50} value of 0.88 mg/kg, s.c. and the minimum effective dose with statistical significance of 3.2 mg/kg, s.c. (unpublished

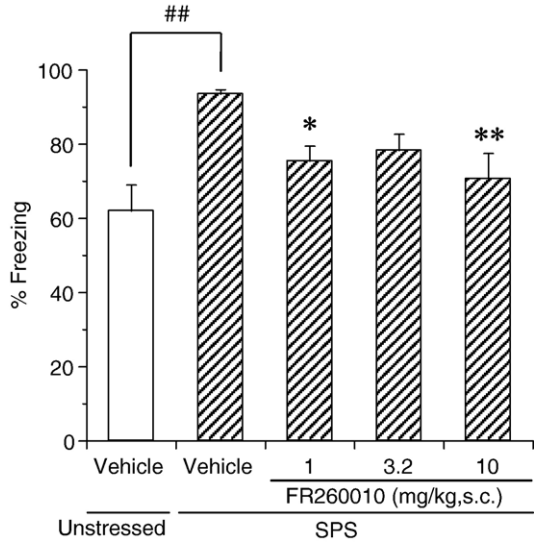

Fig. 2. Effect of 5-HT_{2C} receptor antagonist FR260010 on enhanced contextual fear conditioning in SPS rats. Values were given as the mean ± S.E.M. ($n=18$). $^{\#\#}P<0.01$ vs. unstressed group (t-test). *$P<0.05$, **$P<0.01$ vs. SPS control group (Dunnett's multiple comparison test).

results). Based upon this result, 1, 3.2 and 10 mg/kg, s.c. were selected as doses for fear conditioning test. The SPS produced a significant increase in contextual freezing compared to the unstressed controls 7 days after the SPS ($P=0.0003$, t-test; Fig. 2). FR260010 dosed 30 min before the conditioning session significantly reduced SPS-enhanced freezing at the doses of 1 and 10 mg/kg, s.c. ($P=0.019, 0.060, 0.0022$ for 1, 3.2, 10 mg/kg group vs. vehicle group, respectively, Dunnett's multiple comparison test; Fig. 2). The compound did not affect the sensitivity to footshock in SPS rats at these doses (data not shown).

4. Discussion

Since SPS rats exhibited both behavioral and neuroendocrinological abnormalities that mimicked the symptoms of PTSD (Kohda et al., 2007), they might provide an animal model of PTSD. Comparative microarray analysis using RNA from the brains of both SPS rats and unstressed controls showed that the expression time course of 31 genes (groups 3 and 4 in Table 1) corresponded to the behavioral changes of SPS rats. This indicated that they could be molecular targets. Two of these genes, 5-HT_{2C} receptor (Griebel et al., 1997; Heisler et al., 2007; Kennett et al., 1997) and angiotensin I-converting enzyme (ACE) (Costall et al., 1990) are known to be involved in anxiety. Overexpression of the 5-HT_{2C} receptor gene in the amygdala of SPS rats was confirmed using quantitative real-time PCR. These findings revealed for the first time that 5-HT_{2C} receptor mRNA in the amygdala is up-regulated following exposure to SPS.

As we had discovered the novel 5-HT_{2C} receptor antagonist FR260010 (Harada et al., 2006), which showed high affinity for 5-HT_{2C} receptor (Ki = 1.10 nM) and high selectivity over many other receptors, an attempt was made to pharmacologically validate the functional role of this receptor in the development of enhanced anxiety in this rat model of PTSD. FR260010 significantly inhibited the enhanced freezing in SPS rats at the doses comparable to in vivo 5-HT_{2C} receptor antagonism, without affecting the sensitivity to footshock. As the compound did not affect the contextual freezing in unstressed control rats in our preliminary study (data not shown), the reduction of freezing in SPS rats did not seem to be a result of non-selective motor-enhancing effect. These findings suggest that 5-HT_{2C} receptor is involved in this behavioral change. Since it has been suggested that chronic treatment with paroxetine induces the desensitization of 5-HT_{2C} receptors (Kennett et al., 1994), this finding may correspond to a recent report that chronic, but not acute, administration of paroxetine reduces enhanced anxiety in SPS rats (Takahashi et al., 2006). If this is the case, a selective 5-HT_{2C} receptor antagonist with a faster onset of action might provide an advantage over SSRIs. While there have been no clinical studies on the efficacy of selective 5-HT_{2C} receptor antagonists or even non-selective 5-HT_2 receptor antagonists in PTSD patients, it is known that m-CPP, a putative 5-HT_{2C} receptor agonist, induces panic attacks in PTSD patients (Southwick et al., 1997), which suggests that this receptor is hyperactive. Our studies agree with this result, and so the application of a selective 5-HT_{2C} receptor antagonist seems a reasonable approach for the treatment of PTSD.

5-HT_{2C} receptor mRNA is highly expressed in the amygdala of rats (Pompeiano et al., 1994) and humans (Pasqualetti et al., 1999). Microinjection of m-CPP into the basolateral nucleus of the amygdala (BLA) induces an acute fear-like response in rats, and this effect is blocked by systemic pre-treatment with a selective 5-HT_{2C} receptor antagonist SB-242084 (Campbell and Merchant, 2003). Moreover, systemic administration of 6-chloro-2(1-piperazinyl)-pyrazine (MK-212), a preferential 5-HT_{2C} receptor agonist, induces anxiety in rats, and this effect is blocked by microinjection of ritanserin, a 5-$HT_{2A/2C}$ receptor antagonist, into the BLA (de Mello Cruz et al., 2005). These reports suggest that the 5-HT_{2C} receptor in the amygdala plays a critical role in the anxiety of rats, and the current findings agree with them. 5-HT_{2C} receptor mRNA is known to be edited by adenosine deaminase, which results in changes to the amino acids in the second intracellular loop that couples G proteins (Tohda et al., 2006). Since these changes modify the signal transduction of this receptor (Niswender et al., 1999; Price and Sanders-Bush, 2000), the extent of RNA editing may affect the anxiety level in animals. In fact, the RNA editing profile in the amygdala of two anxious mouse strains (BALB/cJ and DBA/2J) is different from that of C57BL/6J mice (Hackler et al., 2006). In addition to increase in 5-HT_{2C} receptor mRNA levels, the editing state of the 5-HT_{2C} receptor mRNA in the amygdala may affect enhanced anxiety in SPS rats. Further studies are needed to clarify this point.

Rat genes have not been characterized to the extent that mouse genes have. Among 31 differentially expressed genes, only 8 were annotated. From a pharmacological point of view, G protein-coupled receptors can be easily modulated by synthetic compounds, such as FR260010 for 5-HT_{2C} receptor. Therefore, we selected this gene for further analysis. Although this receptor was suggested to be involved in the enhanced anxiety in SPS rats, it does not mean that it is the sole mechanism of behavioral changes. In fact, FR260010 did not completely block the enhanced freezing in SPS rats. The involvement of the other 30 differentially expressed genes remains to be examined and discussed.

In conclusion, using microarray analysis and quantitative real-time PCR of brain tissue from SPS rats, a putative animal model of PTSD, we found a candidate molecular target for the rational treatment of the disease, 5-HT_{2C} receptor. The efficacy of the selective 5-HT_{2C} receptor antagonist was confirmed in the animal model. The present study provides compelling evidence suggesting that selective antagonists of the receptor molecule might provide novel therapeutic avenues for PTSD treatment, as this molecule is also suggested to be involved in PTSD in clinical studies.

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