Full Paper

Increased DOI-Induced Wet-Dog Shakes in Adrenocorticotropin Hormone–Treated Rats Are Not Affected by Chronic Imipramine Treatment: Possible Involvement of Enhanced 5-HT2A–Receptor Expression in the Frontal Cortex

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Abstract. We examined the influence of imipramine, a traditional tricyclic antidepressant, on the binding to serotonin (5-HT)2 receptors and levels of 5-HT2A–receptor mRNA in the frontal cortex of rats treated with adrenocorticotropin hormone (ACTH). Chronic treatment with ACTH significantly increased the binding of [3H]-ketanserin to 5-HT2 receptors and the expression of 5-HT2A–receptor mRNA in the frontal cortex. However, it did not alter the concentration of 5-HT or 5-hydroxyindole acetic acid. The effect of chronic ACTH treatment on 5-HT2 receptor and 5-HT2A–receptor mRNA levels was not altered by the chronic administration of imipramine. Also, imipramine did not affect the hyperfunction of 5-HT2A receptors caused by chronic ACTH treatment. These findings suggest that chronic treatment with ACTH acts to increase 5-HT2A–receptor synthesis through increased gene transcription, without modulating presynaptic serotonergic neurotransmission.

Keywords: 5-HT2A receptor, adrenocorticotropin hormone, wet-dog shake, receptor binding, receptor mRNA

Introduction

Serotonin (5-HT)2 receptors are widely distributed throughout the brain, in a pattern that implicates their activation in the regulation of mood disorders. Clinical studies have demonstrated that the numbers of 5-HT2 receptors are increased in the brains of depressed subjects postmortem (1–5). On the other hand, psychoneurological studies have previously focused on the regulation of the hypothalamic–pituitary–adrenal (HPA) axis in patients with depression (6). Information on the mechanism whereby steroid hormones regulate 5-HT function will impact our understanding of hypercortisolism, a condition typical of affective disorders and treatment-resistant depression (7). We previously reported that chronic administration of corticosterone promoted the binding of [3H]-ketanserin to 5-HT2 receptors (particularly the 5-HT2A–receptor subtype) in the frontal cortex; this treatment also potentiated the wet-dog shakes induced by (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), a 5-HT2A–receptor agonist (8), suggesting that the 5-HT2A receptor is closely related to the physiological response activating the HPA axis in rats. This animal model of the activation of the HPA axis may shed light on the mechanism governing the 5-HT2 or 5-HT2A–receptor’s up-regula-
tion, a condition associated with the pathophysiology of depression. Furthermore, we reported that chronic treatment with adrenocorticotropic hormone (ACTH) potentiated the wet-dog shakes induced by DOI (9 – 11). We suggested that the chronic administration of ACTH in rats would induce hyperactivity of the 5-HT$_{2A}$ receptor via the activation of the HPA axis.

Imipramine, a noradrenaline/5-HT–reuptake inhibitor, is suggested to achieve its pharmacological effect by modifying the function of the 5-HT$_3$ receptor in rats. The chronic administration of imipramine reduces the density of 5-HT$_3$ receptors (12). Redrobe and Bourin (13) showed that a blockade of the 5-HT$_3$ receptor, particularly 5-HT$_{2A/2C}$–receptor subtypes, led to a potentiation of the antidepressant effects of imipramine in the forced swim test. In addition, a few antidepressant drugs, such as nefazodone, trazodone, and mirtazapine, are 5-HT$_{2A}$–receptor antagonists, a property that may underlie their therapeutic actions (14). These reports may indicate that there is a close relationship between the therapeutic action of antidepressants and the 5-HT$_{2A}$ receptor.

We have already shown that the antidepressant-like effects of tricyclic antidepressants (imipramine and desipramine) in the forced swim test are blocked by chronic treatment with ACTH in rats (15). On the other hand, we reported that the administration of imipramine for 14 days attenuated DOI-induced wet-dog shakes, whereas treatment with ACTH for 14 days exacerbated them. This effect of ACTH, potentiating DOI-induced wet-dog shakes, was not inhibited by a 14-day administration of imipramine (10). However, precisely why the hyperfunction of 5-HT$_{2A}$ receptors is not inhibited by imipramine remains to be elucidated.

In our previous paper, we could not precisely explain how ACTH and imipramine influence the 5-HT$_{2A}$ receptor. Therefore, the present study was designed to investigate the effect on the number of 5-HT$_3$ receptors and especially the level of 5-HT$_{2A}$–receptor mRNA in the frontal cortex of chronic ACTH-treated rats. Furthermore, we examined the effect of imipramine on the 5-HT$_{2A}$–receptor’s hyperfunction in ACTH-treated rats.

Materials and Methods

Animals

Male Wistar rats (Charles River Japan, Yokohama), initial weight 210 – 230 g, were kept on a constant light-dark cycle (lights on: 7:00 – 19:00 h), with standard laboratory food and tap water in an air-conditioned room (23 ± 1°C with approximately 60% humidity). All experiments were conducted according to the guidelines for Animal Experimentation at Okayama University Medical School. Every effort was made to minimize the number and suffering of the animals used.

Drugs

The drugs used were imipramine hydrochloride (Wako, Osaka), DOI (Research Biochemicals, Inc., South Natick, MA, USA), and ACTH-(1-24)-zinc (Cortrosyn-Z; Daiichi Seiyaku, Tokyo). Imipramine and DOI were dissolved in saline. The rats were injected with imipramine (10 mg/kg, i.p.) and DOI (1 mg/kg, s.c.) at 2 ml/kg body weight. ACTH (Cortrosyn-Z) was injected subcutaneously once daily (9:00 to 10:00) at a dose of 100 µg/rat (injection volume was 0.2 ml/rat, s.c.) for 1 – 14 days. Control rats received an equivalent volume of saline, 0.2 ml/rat (s.c.), for the same treatment period. DOI was injected subcutaneously at a dose of 1 mg/kg 1 day after the last drug administration. Four to 6 animals were used for each experimental group.

Measurement of DOI-induced wet-dog shakes

Rats were placed in individual clear polycarbonate home cages (35 × 30 × 17 cm) and treated with DOI (1 mg/kg, s.c.). Immediately after injection, the number of wet-dog shakes was recorded over a 30-min period.

5-HT$_3$–receptor binding assay

At 1 day after the last drug administration, rats were sacrificed by decapitation. The brain was quickly removed and dissected on ice. Samples of the frontal cortex were frozen at −80°C before homogenization. The tissues were thawed and homogenized in 50 mM Tris-HCl buffer (pH 7.4) with a Teflon-glass homogenizer. The homogenate was centrifuged at 1000 × g for 10 min, and the supernatant was collected and recentrifuged at 50,000 × g for 20 min. The resultant pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4 at 25°C) and incubated at 37°C for 15 min. The pellet was resuspended in 50 mM Tris-HCl buffer (about 1 mg/ml protein) and stored at −80°C until assayed. The number of 5-HT$_3$ receptors was measured, using [$^3$H]-ketanserin, according to the method of Leysen et al. (1982). Aliquots (200 µl) of the tissue suspension were incubated in duplicate at 25°C for 30 min with 100 µl of 50 mM Tris-HCl buffer (pH 7.4 at 25°C) containing [$^3$H]-ketanserin (0.25 – 5.0 nM) and 700 µl of buffer. Non-specific binding was defined with the use of 1 µM methysergide. The specific binding was calculated as the difference between total and non-specific binding. The incubation was terminated by rapid filtration through Whatman GF/B filters under reduced pressure. The filters were washed three times with ice-cold Tris-HCl buffer (pH 7.4) and transferred to vials to which a scintillation cocktail was added; radioactivity was measured with a liquid scintillation counter.
Measurement of cortex 5-HT_{2A}–receptor mRNA by real-time quantitative polymerase chain reaction (PCR)

**Total RNA extraction**: At 1 day after the last drug administration, rats were sacrificed by decapitation. The brain was quickly removed and dissected on ice. Samples were frozen at −80°C before homogenization. Total RNA was isolated from the frontal cortex with TRIzol Reagent (GIBCO) according to the manufacturer’s directions. The RNA samples were dissolved in RNase-free water and quantified with UV-spectrophotometer at 260 nm.

**Primers**: Primers for rat 5-HT_{2A} receptors and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed for real-time PCR. The primers and PCR products are listed in Table 1.

**Reverse transcription**: Extracted RNA was reverse transcribed with reverse transcriptase using the procedure of the supplier (Gene AmpRNA PCR kit; Applied Biosystems, Foster City, CA, USA). The reverse-transcription reaction mixture contained 0.02 µg/µl of RNA, 2.5 µM of the oligo-d(T)_{16} primers, 1 mM of each dNTP, 2.5 U/µl of reverse transcriptase, 1 × RT buffer, 1.0 U/µl of RNase inhibitor, and 5 mM MgCl₂. The reverse transcription reaction was performed at 42°C for 60 min, followed by heating at 99°C for 5 min.

**Real-time PCR**: Real-time PCR was performed with the SYBR® Green PCR Core Reagents (Applied Biosystems). For detection and quantification, a GeneAmp 5700 (Applied Biosystems) was used. Reactions were performed in a reaction mixture (25 µl) consisting of 1 × SYBR PCR buffer, 200 µM of each dNTP, 0.025 U/µl of AmpliTaq Gold, 2.5 mM MgCl₂, and 4 pmol of each primer. PCR was performed with a 10 min preincubation at 95°C followed by 40 cycles of 30 s at 95°C, 1 min at 60°C, and 2 min at 72°C. The products were verified using melting curves, agarose gel electrophoresis, and DNA sequencing. The real-time PCR method was validated by using serially diluted cDNA to make a standard curve. To quantify the gene expression profile of each sample, the efficiency of the standard curve was determined from its slope and comparative threshold according to the manufacturer’s instructions. For each sample, the amount of targeted mRNA (arbitrary units) was normalized to that of a housekeeping gene, GAPDH mRNA. Also, PCRs without the RT reaction were performed for each sample in order to exclude genomic DNA contamination. The data analysis was performed with GeneAmp 5700 software.

**Determination of 5-HT and 5-hydroxyindole acetic acid concentrations**

At 1 day after the last drug administration, rats were sacrificed by decapitation. The brain was quickly removed and dissected on ice. Samples of the frontal cortex were homogenized in 0.5 M perchloric acid. Following centrifugation, the supernatant was used for measuring 5-HT and 5-hydroxyindole acetic acid concentrations. The high-performance liquid chromatography system consisted of an EP-300 liquid chromatograph pump (EP-300; Eicom, Kyoto), a DGU-4A degasser (DG-300, Eicom), a reversed phase ODS column, Eicompak SC-5ODS φ2.1 mm × 150 mm (Eicom), and an ECD-300 electrochemical detector (+750 mV against Ag/AgCl reference electrode) (Eicom). The mobile phase was an acetic-citrate buffer (pH 3.5), 190 mg/l sodium 1-octanesulfonate, 5 mg/l EDTA, and 16% methanol; degassed; and pumped at a flow rate of 0.25 ml/min. The peaks were recorded using a Powerchrom integrator (Eicom). A standard solution containing authentic 5-HT and 5-hydroxyindole acetic acid was injected every working day, and the amounts of 5-HT and 5-hydroxyindole acetic acid were determined by comparison with the peak area of the standard.

**Data analyses**

Data are given as the mean ± S.E.M. Data on 5-HT_{2}– receptor binding and 5-HT_{2A}–receptor mRNA expression were analyzed with one-way analysis of variance (ANOVA); the group means were compared using Tukey’s test for multiple comparisons. For the 5-HT and 5-hydroxyindole acetic acid concentrations, statistical comparisons between the two groups were performed using an unpaired t-test (two-tailed). Probability values of less than 0.05 were considered to show a significant difference.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank</th>
<th>Primer</th>
<th>Oligonucleotide sequence 5'-3'</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat 5-HT_{2A} receptor</td>
<td>NM 017254</td>
<td>F</td>
<td>AGC CGC TTC AAC TCC AGA A TTT TGG TCA TCA TTG CTG CTG GA</td>
<td>408</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>ACC ACA GTC CAT GCC ATC AC ACC ACC CTG TTG TA</td>
<td>452</td>
</tr>
</tbody>
</table>

Sequences are shown for forward (F) and reverse (R) primers along with the size of the amplicon (base pairs, bp).
Results

Changes in the DOI-induced wet-dog shakes

At 1 day after the final administration of imipramine (10 mg/kg, i.p., 14 days), the number of wet-dog shakes exhibited during the 30-min period following the injection of DOI (1 mg/kg, s.c.) was significantly decreased compared with the control value. The chronic treatment with ACTH (100 µg/rat, s.c.) for 14 days significantly increased the number of wet-dog shakes induced by DOI. Administration of imipramine (10 mg/kg, i.p.) for 14 days did not decrease the response to DOI when given concurrently with ACTH (100 µg/rat, s.c.) (Fig. 1).

Changes in the 5-HT₂ receptor binding sites

Figure 2 shows the Bₘₐₓ values of the frontal cortical 5-HT₂ receptors labeled by [³H]-ketanserin. Chronic administration of imipramine (10 mg/kg, i.p.) for 14 days significantly decreased the maximal number (Bₘₐₓ) of 5-HT₂ receptors. Chronic treatment with ACTH (100 µg/rat, s.c.) for 14 days significantly increased the Bₘₐₓ of 5-HT₂ receptors. The effect of ACTH was not decreased by the chronic administration of imipramine (10 mg/kg, i.p., 14 days). The treatments did not affect affinity (Kᵦ: nM): control group, 1.541 ± 0.162; imipramine group, 1.787 ± 0.381; ACTH group, 1.321 ± 0.007; ACTH + imipramine group, 1.411 ± 0.102.

Changes in the expression of 5-HT₂A receptor mRNA

Figure 3 shows the expression of 5-HT₂A receptor mRNA. The administration of imipramine (10 mg/kg,
Table 2. Effects of chronic ACTH treatment on 5-HT and 5-hydroxyindole acetic acid concentrations in rat frontal cortex

<table>
<thead>
<tr>
<th></th>
<th>5-HT (ng/mg tissue)</th>
<th>5-Hydroxyindole acetic acid (ng/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>542.4 ± 34.1</td>
<td>296.0 ± 14.0</td>
</tr>
<tr>
<td>ACTH</td>
<td>447.2 ± 44.1</td>
<td>248.3 ± 20.2</td>
</tr>
</tbody>
</table>

Rats were given ACTH (100 µg/rat, s.c.) once daily for 14 days. Control rats were treated with saline (0.2 ml/rat, s.c.) once daily for 14 days. The rats were killed by decapitation and their brains were removed 1 day after the final treatment with saline or ACTH. All values represent the mean ± S.E.M. for six animals per group. Data were analyzed with an unpaired t-test (two-tailed).

i.p.) for 14 days significantly decreased the level of 5-HT<sub>2α</sub>-receptor mRNA. Treatment with ACTH (100 µg/rat, s.c.) for 14 days significantly increased the expression. This effect of ACTH was not decreased by the chronic administration of imipramine (10 mg/kg, i.p., 14 days).

Changes in 5-HT and 5-hydroxyindole acetic acid concentrations

As shown in Table 2, concentrations of 5-HT and 5-hydroxyindole acetic acid in the frontal cortex were not changed by the chronic administration of ACTH (100 µg/rat, s.c.) for 14 days.

Discussion

In a behavioral study, we previously found that chronic treatment with ACTH, which affects the HPA axis, potentiated the wet-dog shakes induced by DOI, a 5-HT<sub>2α</sub>-receptor agonist (9–11). We also reported that the chronic administration of corticosterone increased the binding of [³H]-ketanserin to 5-HT<sub>2</sub> receptors (8). In the present study, we for the first time demonstrated that there is a significant increase in both specific binding to the 5-HT<sub>2</sub> receptor and the expression of 5-HT<sub>2α</sub>-receptor mRNA in the frontal cortex of chronic ACTH-treated rats. We also measured the concentrations of 5-HT and 5-hydroxyindole acetic acid, both of which may reflect presynaptic serotonergic activity to some extent. The chronic ACTH treatment did not alter the concentration of either compound in the frontal cortex. Furthermore, we demonstrated that chronic ACTH treatment did not affect basal extracellular 5-HT concentrations compared with chronic saline treatment in a previous microdialysis-based study of the medial prefrontal cortex (16). Namely, it is possible that chronic ACTH treatment did not affect presynaptic 5-HT activity. Glucocorticoids may be an important regulatory factor for the 5-HT<sub>2α</sub>-receptor gene (17): therefore, the activation of the HPA axis may regulate the function of the 5-HT<sub>2α</sub> receptor via the activation of glucocorticoid receptors in ACTH-treated rats. In the present study, chronic ACTH treatment significantly increased levels of the 5-HT<sub>2α</sub> receptor. Therefore, it is possible that ACTH directly acts to increase 5-HT<sub>2</sub> receptor (probably 5-HT<sub>2α</sub> receptor) numbers without modulating pre-synaptic serotonergic neurotransmission.

Administration of ACTH induced negative feedback to the HPA axis in rats. In regard to the dexamethasone (100 µg/kg, s.c.) suppression test, we observed the plasma corticosterone levels in rats following 14 days of treatment with ACTH (100 µg/rat, s.c.); the corticosterone levels in rats given the various treatments were as follows: saline, 3.3 ± 0.3 µg/dl; dexamethasone, 1.9 ± 0.2 µg/dl; ACTH, 23.9 ± 10.4 µg/dl; and ACTH + dexamethasone, 5.2 ± 4.2 µg/dl. These results suggest that chronic ACTH treatment did not result in disinhibition of the HPA system. Furthermore, we reported the plasma corticosterone levels were significantly higher in chronic ACTH-treated rats than saline-treated rats (15). Namely, chronic ACTH treatment causes a condition termed hypercorticism.

We previously reported that the chronic administration of imipramine decreased the number of DOI-induced wet-dog shakes. However, the effect of ACTH was not inhibited by the chronic administration of imipramine (10). The reason why imipramine did not inhibit the increase in DOI-induced wet-dog shakes in ACTH-treated rats is unknown. It is well recognized that imipramine inhibits the 5-HT uptake carrier. Studies in vivo using microdialysis have found that imipramine increases the output from the 5-HT synapse (15, 18). It is conceivable that the inhibition of imipramine’s effect on the DOI-induced wet-dog shakes in ACTH-treated rats is associated with a decrease in extracellular 5-HT concentrations in the central nervous system. However, we reported that a single administration of imipramine combined with chronic ACTH treatment significantly increased extracellular 5-HT concentrations, compared to imipramine treatment alone, in a microdialysis-based study (16). Namely, the effect of imipramine on the release of 5-HT may not be suppressed by the activation of the HPA axis. However, further study is needed to determine the extracellular 5-HT concentration after the chronic administration of imipramine in ACTH-treated rats using microdialysis. On the other hand, we showed that the chronic administration of imipramine decreased the binding of 5-HT<sub>2</sub> receptors and the expression of 5-HT<sub>2α</sub>-receptor mRNA in naive rats. Also, the enhancing effect on the binding of 5-HT<sub>2</sub> receptors and the expression of 5-HT<sub>2α</sub>-receptor mRNA by the chronic ACTH treatment was not inhibited by the chronic administra-
tion of imipramine. Namely, these findings suggest that
the effect of imipramine on DOI-induced wet-dog
shakes correlates with the binding of the 5-HT$_{2A}$ receptor
and the level of 5-HT$_{2A}$-receptor mRNA in naive and
ACTH-treated rats.

ACTH and α-, β-, and γ-melanocyte–stimulating
hormone are derived from proopiomelanocortin by
enzymatic processing, and they are collectively called
melanocortins. Melanocortins are involved in a wide
range of physiological functions, including responses to
stress (19, 20). Melanocortins act via five receptor
subtypes (MC1–MC5). In the brain, MC3 and MC4
receptors are mainly expressed. The MC4 receptor has
been reported to play important roles in regulation of
the activity of the HPA axis (20, 21). Moreover, a MC4-
receptor antagonist has revealed the involvement of this
receptor in emotional states such as anxiety and depres-
sion (22). On the other hand, an MC4-receptor agonist
has been documented to alter the function of the 5-HT
2A receptor in ACTH-treated rats.

These findings suggest that ACTH acts to promote
the synthesis of 5-HT$_{2A}$ receptors by increasing gene
transcription, without modulating presynaptic serotoner-
gic neurotransmission. This effect of ACTH on the 5-
HT$_{2A}$ receptor was not inhibited by the chronic admin-
istration of imipramine. Further investigation of the
internal signal transduction pathway by which ACTH
alters the function of the 5-HT$_{2A}$ receptor in rats is
needed.

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