Hallucinogenic 5-Hydroxytryptamine 2A Receptor Agonist Effects in Senescence-Accelerated Mice

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Abstract: Many neuropharmacological agents modulate the activity and conformation of heptahelical G protein-coupled receptors and activate ligand-specific signaling pathways. The hallucinogenic chemical 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI), a serotonin receptor 2A (5-HT2AR) agonist, evokes extracellular signal-regulated kinase 1/2 (ERK1/2) signaling and head-twitch behavior. We previously reported that the senescence accelerated-prone mouse 6 (SAMP6) exhibited altered emotional behavior and increased levels of a serotonin-biosynthesizing enzyme compared to the senescence accelerated-resistant mouse 1 (SAMR1); however, the mechanism underlying the relationship between specific receptor signaling and behavioral phenotypes was unclear. In this study, we performed head-twitch tests and examined the total and phosphorylated levels of ERK1/2 and cAMP-responsive element-binding protein (CREB) in the bilateral somatosensory cortex to assess the differences between SAMP6 and SAMR1 using DOI. Although DOI dose-dependently increased the head-twitch response in both strains, the responses of SAMP6 given 0.3 and 1.0 mg/kg DOI were significantly greater than those of SAMR1 given DOI at the same doses. Although no dose-dependent increase in total ERK1/2 and total CREB expression was detected in response to DOI, the levels of phospho-ERK1/2 and -CREB increased in both strains. These results indicate that SAMP6 increases DOI-dependent ERK1/2-CREB signaling leading to more head-twitch responses than SAMR1, and that SAMP6 could provide a useful model for examining the relationship between 5-HT2AR regulatory signaling and behavioral phenotypes.

Key words: DOI, SAM, Signaling pathway

Introduction

Many therapeutic drugs and chemical compounds interact with heptahelical G protein-coupled receptors (GPCRs), and the effects of these ligands originate from changes in receptor conformation that modulate cellular signaling [4, 14]. Many receptor subtypes are coupled to multiple signaling pathways [11], and specific agonists acting at the same receptor have been discovered that vary their relative efficacy in activating downstream signals [2, 12]. The heptahelical G protein-coupled serotonin receptor 2A (5-HT2AR) is present at high con-
centrations in the frontal cortex, including the bilateral somatosensory cortex and basal ganglia, and at lower levels in some components of the limbic system, including the hippocampus, septum, and amygdala [17]; moreover, it has been implicated in a variety of behaviors [6]. Hallucinogens such as 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) are potent 5-HT2AR and serotonin receptor 2C (5-HT2CR) agonists, and their effects, which include the stimulation of a head-twitch behavioral response [23], appear to reflect their actions at 5-HT2AR [15, 19]. The two agonists have been reported to direct differential signaling via 5-HT2AR [18]. Serotonin (5-HT) induces extracellular signal-regulated kinase 1/2 (ERK1/2) signaling and head-twitch behavior by a β-arrestin-2-dependent mechanism. In contrast, DOI invokes ERK1/2 signaling and head-twitch behavior in a β-arrestin-2-independent manner.

The senescence accelerated mouse (SAM) was developed through selective breeding of the AKR/J strain based on a graded score for senescence and is known as a murine model of aging [20]. SAM strains consist of the senescence-accelerated prone mouse (SAMP) and senescence-accelerated resistant mouse (SAMR). We previously reported that SAMP6 exhibited reduced anxiety and increased expression of the serotonin-biosynthesizing enzyme tryptophan hydroxylase (TPH), and that TPH is more phosphorylated at serine-58 (pTPH) compared to SAMR1 [16]. However, the cellular mechanisms mediating receptor signaling of the specific behavioral phenotype are not well understood.

As a first step toward understanding whether or not SAMP6 has an alteration in the 5-HT2AR ligand-directed specific signaling pathway, we performed a head-twitch response test and examined both the 5-HT2AR levels and ERK1/2 signaling to assess the differences between SAMP6 and SAMR1 using western blot analysis. We also used the synthetic 5-HT2AR hallucinogenic agonist, DOI, to conduct a neuropharmacological analysis.

Materials and Methods

Animals
Male mice of SAMR1 and SAMP6 were purchased at 3 weeks of age from Japan SLC (Shizuoka, Japan). The mice were housed individually in micro-isolation cages (19.1 x 29.2 x 12.7 cm; Allentown Caging Equipment, Allentown, NJ, USA) with bedding (Tek-Fresh; Harlan Teklad, Madison, WI, USA). The mice were given access to water and food pellets (CRF-1; Oriental Yeast, Tokyo, Japan) ad libitum and were maintained at room temperature (23 ± 1°C) and 55 ± 5% humidity under a 12:12-h light-dark cycle (lights on from 08:00 to 20:00). Eight-week-old mice were used in our experiments. The mice were injected intraperitoneally with saline 1 week before the administration of DOI to reduce the effect of stress caused by a novel stimulus on their behavioral responses and protein expression. Behavioral analyses were conducted between 09:00 and 14:00 by a well trained experimenter who was blinded to the mouse strains. All experimental procedures were approved by the RIKEN Institutional Animal Care and Use Committee.

Drug
DOI (Sigma-Aldrich, St. Louis, MO, USA) was suspended in saline and injected intraperitoneally 15 min before the behavioral tests began. The injected doses were 0, 0.3, and 1.0 mg/kg. For administration of DOI, 0.3 ml of 30 or 100 µg/ml DOI was intraperitoneally injected to a mouse weighing 30 g, resulting in a dose of 0.3 or 1.0 mg/kg, respectively. For the dose of 0 mg/kg, a similar volume of saline was used instead of DOI suspension.

Head-twitch response test
Three dose groups were created for each strain: 0 (SAMR1, SAMP6: n=8, 8), 0.3 (SAMR1, SAMP6: n=8, 8), and 1.0 mg/kg DOI (SAMR1, SAMP6: n=8, 7). Mice were placed in the center of a plexiglass cage (17.0 x 27.0 x 13.0 cm, Clean-Cage; CLEA Japan Inc., Tokyo, Japan) for 20 min, during which the behavioral responses of the mice were recorded using a video camera. The videotapes were scored for head-twitch responses by an experienced observer blinded to the strains. The experiment was conducted under a light intensity of 80 lx.

Western blot analysis
Thirty-five to sixty minutes after DOI administration,
the mice were sacrificed by cervical dislocation. Brains were removed and washed in ice-cold saline for 1–2 min. The proteins from the bilateral somatosensory cortex were extracted using PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology Inc., Gyeonggi, Korea) according to the manufacturer’s instructions. Total proteins (20 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoresis was performed onto a polyvinylidene fluoride membrane. The membranes were then incubated with rabbit anti-5HT2AR polyclonal antibodies (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-total-ERK1/2 polyclonal antibody (1:500, Cell Signaling Technology), mouse anti-phospho-ERK1/2 (Thr202/Tyr204) monoclonal antibody (1:1000, Cell Signaling Technology), mouse anti-total-CREB monoclonal antibody (1:500, Cell Signaling Technology), or rabbit anti-phospho-CREB (Ser133) polyclonal antibody (1:500, Cell Signaling Technology), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG, 1:25,000; anti-rabbit IgG, 1:1000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The protein concentrations were standardized using rabbit polyclonal anti-α-tubulin antibody (1:250; Abcam, Cambridge, MA, USA). The blots were developed using an ECL Plus Western Blotting Detection System (GE Healthcare, Princeton, NJ, USA). Protein signals were quantified using ImageJ software (NIH, Bethesda, MD, USA). The phosphorylation level of each protein was normalized to the total protein level.

Statistical analysis

All statistical analyses were conducted using Excel Statistics 2006 (SSRI, Tokyo, Japan). The data were analyzed using a factorial two-way analysis of variance (ANOVA) with the Bonferroni correction and Tukey’s multiple-comparison test. The alpha level was 0.05.

Results

Head-twitch response test

First, we performed the head-twitch response test (Fig. 1). DOI significantly increased the head-twitch responses in both SAMP6 and SAMR1. A significant interaction between strain and dose (F_{2,41}=63.61, P<0.001), a strain effect (F_{1,41}=193.29, P<0.001), and a significant dose effect (F_{2,41}=346.31, P<0.001) were detected (Fig. 1). Both doses of 0.3 and 1.0 mg/kg significantly increased the responses of both strains compared to 0 mg/kg (0.3 vs. 0 mg/kg: SAMP6; P<0.001, SAMR1; P<0.01, 1.0 vs. 0 mg/kg: SAMP6; P<0.001, SAMR1; P<0.001, Tukey test). The response counts of SAMP6 given 0.3 and 1.0 mg/kg DOI were significantly higher than those of SAMR1 given DOI at the same doses (0.3 mg/kg, P<0.001; 1.0 mg/kg, P<0.001, Bonferroni correction).

Expression analysis

The expression levels of 5-HT2AR in the bilateral somatosensory cortex were not significantly different between SAMP6 and SAMR1 (data not shown). To determine whether systemic administration of DOI results in the activation of ERK1/2 and cAMP responsive element-binding protein (CREB), we measured the expression levels of ERK1/2, phospho-ERK1/2, CREB, and phospho-CREB in the bilateral somatosensory cortex by western blot analysis (Table 1). DOI dose-dependently increased phospho-ERK1/2 (F_{2,41}=49.91, P<0.001) and phospho-CREB (F_{2,41}=118.08, P<0.01) in SAMP6 and SAMR1, whereas no dose-dependent increase in total ERK1/2 (F_{2,41}=0.16, P=0.856) or total CREB (F_{2,41}=0.75, P=0.477) was detected in either strain. DOI also dose-dependently increased the phosphorylation level of ERK1/2 (phospho-ERK1/2 per total ERK1/2) in SAMP6 and SAMR1 (Fig. 2A). A significant interaction between strain and dose (F_{2,41}=3.26, P<0.05),
a significant strain effect \( F_{1,41}=48.1, P<0.001 \), and a significant dose effect \( F_{2,41}=142.02, P<0.001 \) were detected. Both doses of 0.3 and 1.0 mg/kg significantly increased the phosphorylation levels of ERK1/2 compared to 0 mg/kg (0.3 vs. 0 mg/kg: SAMP6; \( P<0.001 \), SAMR1; \( P<0.001 \), 1.0 vs. 0 mg/kg: SAMP6; \( P<0.001 \), SAMR1; \( P<0.001 \), Tukey test). The phosphorylation levels of ERK1/2 in SAMP6 given 0.3 and 1.0 mg/kg DOI were significantly higher than those in SAMR1 (0.3 mg/kg, \( P<0.001 \); 1.0 mg/kg, \( P<0.001 \), Bonferroni correction), whereas the phosphorylation levels of ERK1/2 were similar between SAMP6 and SAMR1 at 0 mg/kg (\( P>0.05 \), Bonferroni correction). DOI also dose-dependently increased the phosphorylation level of CREB (phospho-CREB per total CREB) in SAMP6 and SAMR1 (Fig. 2B). A significant interaction between strain and dose \( F_{2,41}=32.06, P<0.001 \), a significant strain effect \( F_{1,41}=189.48, P<0.001 \), and a significant dose effect \( F_{2,41}=261.09, P<0.001 \) were detected. Both doses of 0.3 and 1.0 mg/kg significantly increased the phosphorylation levels of CREB compared to 0 mg/kg (0.3 vs. 0 mg/kg: SAMP6; \( P<0.001 \), SAMR1; \( P<0.001 \), 1.0 vs. 0 mg/kg: SAMP6; \( P<0.001 \), SAMR1; \( P<0.001 \), Tukey test). The phosphorylation levels of CREB in SAMP6 given 0.3 and 1.0 mg/kg DOI were significantly higher than those in SAMR1 (0.3 mg/kg, \( P<0.001 \); 1.0 mg/kg, \( P<0.001 \), Bonferroni correction), whereas the phosphorylation levels of CREB were similar between SAMP6

**Table 1.** Expression levels of phospho-ERK1/2, total ERK1/2, phospho-CREB, and total CREB

<table>
<thead>
<tr>
<th>DOI dose (mg/kg)</th>
<th>SAMR1</th>
<th>SAMP6</th>
<th>SAMR1</th>
<th>SAMP6</th>
<th>SAMR1</th>
<th>SAMP6</th>
<th>SAMR1</th>
<th>SAMP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.51 ± 0.03</td>
<td>0.62 ± 0.05</td>
<td>1.27 ± 0.06</td>
<td>1.31 ± 0.04</td>
<td>0.06 ± 0.01</td>
<td>0.15 ± 0.04</td>
<td>1.93 ± 0.06</td>
<td>1.99 ± 0.08</td>
</tr>
<tr>
<td>0.3</td>
<td>0.64 ± 0.03*</td>
<td>0.84 ± 0.02**</td>
<td>1.20 ± 0.05</td>
<td>1.32 ± 0.04</td>
<td>0.24 ± 0.04**</td>
<td>0.52 ± 0.03***</td>
<td>1.96 ± 0.16</td>
<td>1.87 ± 0.07</td>
</tr>
<tr>
<td>1.0</td>
<td>0.86 ± 0.06***</td>
<td>1.13 ± 0.06***</td>
<td>1.23 ± 0.07</td>
<td>1.32 ± 0.06</td>
<td>0.49 ± 0.04***</td>
<td>0.97 ± 0.07***</td>
<td>2.09 ± 0.09</td>
<td>2.00 ± 0.12</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *, **, and *** mean \( P<0.05 \), \( P<0.01 \), and \( P<0.001 \), respectively, compared with value for 0 mg/kg in each strain. §§ and §§§ mean \( P<0.01 \) and \( P<0.001 \), respectively, compared with value for 0.3 mg/kg in each strain.
Discussion

In this study, we found that injecting DOI, a hallucinogenic chemical, into SAMP6 increased ERK1/2 signaling and head-twitch responses more than in SAMR1.

5-HT2AR is thought to be essential for mediating a large number of physiological processes in the central nervous system and to have different signaling profiles depending on the nature of the bound ligand [1, 9, 10]. To examine the correlation between ligand-directed 5-HT2AR signaling and behavioral phenotypes, extensive pharmacological and gene-targeting tools have been used [7, 8]. The results of those studies showed that 5-HT2AR-knockout mice do not exhibit head-twitch responses following the administration of DOI and other hallucinogenic drugs, supporting the idea that the 5-HT2AR binding agents that induce hallucinations in humans produce a head-twitch response in mice.

Our results also show that SAMP6 and SAMR1 exhibited a dose-dependent increase in head-twitch responses. Receptor regulation and the signaling pathway activated are determined by the proteins that interact with the receptors [21], and β-arrestin-2 has been reported to promote GPCR signaling [22]. Previous studies showed that 5-HT2AR is co-localized with β-arrestin-2 in cortical neurons [9] and that 5-HT2AR internalization is mediated by β-arrestin-2 [5]. These findings suggest that β-arrestin-2 plays a significant role in 5-HT2AR ligand-directed functional signaling and behavioral responsiveness. The head-twitch response induced by 5-HT was previously shown to be greatly attenuated in β-arrestin-2-knockout mice [18]. Moreover, treatment with DOI produced head-twitch responses of equal magnitude in β-arrestin-2-knockout and wild-type mice [18]. These data suggest that β-arrestin-2 mediates endogenous agonist-induced head-twitch responses, whereas the synthetic agonist DOI produces this behavior in a β-arrestin-2-independent manner. In this study, the head-twitch responses of SAMP6 given 0.3 and 1.0 mg/kg DOI were significantly greater than those of SAMR1 given DOI at the same doses. These results suggest that SAMP6 increases β-arrestin-2-independent signaling.

A recent study reported that 5-HT2AR signaling can be transduced by the ERK1/2 pathway [18]. The results of the present study indicate that DOI dose-dependently increased the phosphorylation levels of ERK1/2 in SAMP6 and SAMR1, and that SAMP6 given 0.3 and 1.0 mg/kg DOI showed higher phosphorylation levels of ERK1/2 than SAMR1 given DOI at the same doses. These data indicate that SAMP6 has alterations in the ERK1/2 pathway. CREB is thought to be a transcription factor [13], and CREB phosphorylation is a downstream event in the ERK1/2 pathway [24]. In addition, DOI has been reported to increase the level of phospho-CREB in primary isolated cerebellar granule cells [3]. Our expression analysis of CREB in the bilateral somatosensory cortex also showed that DOI dose-dependently increased the phosphorylation levels of CREB in SAMP6 and SAMR1, and that SAMP6 given 0.3 and 1.0 mg/kg DOI showed higher phosphorylation levels of CREB than SAMR1 given DOI at the same doses. These results indicate that injection of DOI increases ERK1/2-CREB signaling activity leading to greater head-twitch responses in SAMP6 mice.

In this study, we used 4-month-old SAMP6 and SAMR1. We previously used the elevated plus maze test and the light-dark exploration test to compare SAMP6 and SAMR1. In those tests, 1-, 4-, and 8-month-old SAMP6 exhibited reduced anxiety as measured by the time spent on the open arms in the elevated plus maze test and the time spent in the light box in the light-dark exploration test [16]. These results indicate that although SAMP6 express senile osteoporosis after 4 months of age [20], central nervous alterations such as emotional disorders might be a non-senescence-accelerated phenotype, and that the change in brain function appears to be innate. Indeed, western blot analysis showed increased TPH and pTPH levels in 1-month-old SAMP6 [16], suggesting that the altered emotional behavior of SAMP6, at least after 1 month of age, is due to increased serotonin levels. After binding with 5-HT2AR and 5-HT, its natural ligand, the receptor is known to β-arrestin-2-dependently traffic from the cell surface to the intracellular vesicles [18]. As SAMP6 seems to have an increased level of endogenous 5-HT, it is presumed that
less 5-HT2AR exists on the cell surface because of a β-arrestin-mediated internalization mechanism leading to decreased sensitivity to its ligands. Contrary to expectation, in the present study, an increased response to DOI, a synthetic 5-HT2AR ligand, by SAMP6 was found. This suggests the possibility that SAMP6 has an abnormality in the β-arrestin-2 function. This might leave sufficient 5-HT2AR for binding with DOI on the cell surface. Alternatively, it is also a possibility that 5-HT2AR is insufficiently phosphorylated in SAMP6. This might lead to the preferential activation of the β-arrestin-2-independent signaling pathway in SAMP6. To test these hypotheses, further investigations such as measuring the β-arrestin-2 expression level, examining 5-HT2AR localization (cell surface or intracellular site), or comparing the phosphorylation levels of 5-HT2AR between sham- and 5-HTP (precursor of 5-HT) or DOI administered SAMP6 mice are needed.

In summary, we showed that after the injection of DOI, SAMP6 had increased β-arrestin-2-independent ERK-CREB activity leading to increased head-twitch responses. SAMP6 may be a useful model for studies of the mechanisms involved in the relationship between the 5-HT2AR signaling pathway and behavioral phenotypes.

References


