Gene transfer of GLT-1, a glutamate transporter, into the nucleus accumbens shell attenuates methamphetamine- and morphine-induced conditioned place preference in rats

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Abstract

Several lines of evidence have suggested that the glutamatergic system in the nucleus accumbens (NAc) plays an important role in the conditioned rewarding effect of drugs of abuse. In addition, it is recognized that extracellular glutamate is rapidly removed from the synaptic cleft by Na⁺-dependent glutamate transporters in neurons and glial cells, thereby maintaining physiological levels of glutamate. We previously reported that activation of glutamate uptake by a glutamate transporter activator attenuated the acquisition of conditioned place preference induced by methamphetamine and morphine in mice. In the present study, we examined the effects of gene transfer of a glial glutamate transporter, GLT-1, into the NAc shell by recombinant adenoviruses on methamphetamine- and morphine-induced conditioned place preference in rats. Bilateral infusion of the recombinant adenoviruses into the NAc shell efficiently increased GLT-1 expression surrounding the infusion site, at least during the period 2–8 days after the infusion. In the conditioned place preference paradigm, animals were conditioned with repeated subcutaneous injections of methamphetamine (2 mg/kg) or morphine (3 mg/kg). Intra-NAc shell overexpression of GLT-1 before the conditioning significantly attenuated the conditioned place preference induced by methamphetamine or morphine, when compared with control. However, it had no effect on the somatic signs of naloxone-precipitated morphine withdrawal. These results suggest that GLT-1 within the NAc shell plays an inhibitory role in the conditioned rewarding effects of methamphetamine and morphine but not the physical dependence on morphine.

Introduction

Several lines of evidence suggest that the nucleus accumbens (NAc), which receives a prominent dopaminergic input from the ventral tegmental area, plays an essential role in the rewarding effects and reinforcing properties of most drugs of abuse, including methamphetamine and morphine (Bardo, 1998; Koob et al., 1998). The NAc can be divided into two anatomically and functionally distinct parts, the shell and the core, and it has been suggested that the shell subdivision plays a role in emotional and motivational functions, while the core subdivision is involved in somatic motor functions (Heimer et al., 1991; Zahm, 2000). Several studies have suggested that the NAc shell, rather than the core, is a preferential target for the rewarding effects of drugs of abuse (Pontieri et al., 1995; Heidbreder et al., 1999; Sellings & Clarke, 2003; Ikemoto et al., 2005).

In addition to the dopaminergic inputs, the medium spiny neurons in the NAc are innervated by glutamatergic inputs from limbic and cortical areas, such as the prefrontal cortex, hippocampus and amygdala (Sesack & Pickel, 1990; Smith & Bolam, 1990; Sesack et al., 2003), and are thought to have integrative functions. The glutamatergic system in the NAc has been demonstrated to take part in addiction-related behaviors (Kelley & Throne, 1992; Pulvirenti et al., 1992; Cornish & Kalivas, 2000). It has been reported that glutamate release was increased in the NAc of amphetamine- and morphine-conditioned animals (Reid & Berger, 1996; Hotsenpiller et al., 2001; Sepulveda et al., 2004). In the conditioned place preference (CPP) paradigm, intra-NAc injection of several types of glutamate receptor antagonists inhibited acquisition and/or expression of CPP induced by amphetamine and morphine (Layer et al., 1993; Popik & Kolasiewicz, 1999). These findings suggest that the conditioned rewarding effects of drugs of abuse involve activation of glutamate receptors and subsequent glutamate-dependent neuronal adaptation in the NAc (White & Kalivas, 1998; Everitt & Wolf, 2001).

It is recognized that extracellular glutamate is rapidly removed from the synaptic cleft by a family of Na⁺-dependent high-affinity glutamate transporters in neurons (EAAC1, EAAT4 and EAAT5) and glial cells (GLT-1 and GLAST), thereby maintaining physiological levels of glutamate (Tanaka et al., 1997; Robinson, 1998; Conti & Weinberg, 1999). We previously showed that the mRNA expression of the glial glutamate transporter GLT-1 decreased in the striatum of morphine-dependent rats (Ozawa et al., 2001). Furthermore, we reported that a glutamate transporter inhibitor facilitated and its activator inhibited the acquisition of methamphetamine- and morphine-induced CPP (Sekiya et al., 2004; Nakagawa et al., 2005). These findings suggest that glutamate transporters, such as GLT-1, play inhibitory roles in the methamphetamine- and morphine-induced CPP. However, the lack of specific inhibitors and/or activators for
the internal ribosome entry site of the encephalomyocarditis virus, which allows translation of two open reading frames from a single mRNA by a promoter, all cells expressing EGFP protein also express GLT-1 protein concomitantly (Rees et al., 1996).

**Materials and methods**

**Subjects**

A total of 142 male Sprague-Dawley rats initially weighing 180–220 g were used. They were kept at a constant ambient temperature of 24 ± 1 °C under a 12-h light/dark cycle with free access to food and water. After their arrival, the rats were individually housed in plastic cages with woodchip bedding for at least 1 day before surgery. The experiments were conducted in accordance with the ethical guidelines of the Kyoto University animal experimentation committee and the guidelines of the Japanese Pharmacological Society. All possible efforts were made to minimize the number of animals used.

**Materials**

D,L-Methamphetamine hydrochloride and morphine hydrochloride were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan) and Takeda Chemical Industries (Osaka, Japan), respectively. Naloxone hydrochloride was purchased from Sigma (St Louis, MO, USA). These drugs were freshly dissolved in saline each day and injected subcutaneously in a volume of 1 mL/kg. Morphine pellets each containing 75 mg of morphine base were prepared as previously described (Watanabe et al., 2002).

**Construction of recombinant adenoviruses**

Construction of the recombinant adenoviruses was described previously (Ozawa et al., 2004). Briefly, fragments of rat GLT-1 and enhanced green fluorescence protein (EGFP) cDNAs were subcloned into multiple cloning sites 1 and 2 of the vector pIRES (Clontech), respectively. A 3.5-kb fragment containing the GLT-1-internal ribosome entry site (IRES)-EGFP sequence was then subcloned into the cosmid vector pAXCAwt (Takara, Kyoto, Japan), which contains the CAG promoter (cytomegalo virus enhancer and β-actin promoter) and the rabbit β-globin polyadenylation signal (Miyake et al., 1996). For the control adenovirus, the only EGFP fragment was subcloned into pAXCAwt. The recombinant adenoviruses were generated using an Adenovirus Expression Vector kit (Takara) according to the manufacturer’s instructions and named Ad-GLT-1 + EGFP and Ad-EGFP, respectively. They were propagated in HEK293 cells and purified by caesium chloride gradient centrifugation. The titres of Ad-GLT-1 + EGFP and Ad-EGFP were 5.6 × 10^8 and 8.7 × 10^8 pfu/mL, respectively. As the expression unit of Ad-GLT-1 + EGFP contains the rabbit β-globin polyadenylation signal, all cells expressing EGFP protein also express GLT-1 protein concomitantly (Rees et al., 1996).

**Infusions of recombinant adenoviruses into the nucleus accumbens shell**

Infusions of the recombinant adenoviruses were performed as previously described (Ozawa et al., 2004) with slight modifications. Briefly, under thiamylal sodium (50 mg/kg, i.p.) anesthesia, the recombinant adenoviruses or phosphate-buffered saline were bilaterally infused at 1.7 mm anterior to the bregma, 1.0 mm lateral to the midline and 7.0 mm below the surface of the skull according to the atlas of Paxinos & Watson (1998) in a volume of 2 μL/side over 5 min via a stainless steel injection cannula (33 gauge, o.d. 0.2 mm) by a microinfusion pump. The injection cannulas were left in place for an additional 2 min to prevent backflow. The rats were then individually returned to their respective cages.

**Western blotting**

Ten animals from a separate group used in behavioral experiments were killed by decapitation at 2 days after the infusion of phosphate-buffered saline, 5 days after the infusion of Ad-EGFP or 2, 5 and 8 days after the infusion of Ad-EGFP + GLT-1. The brain was rapidly removed and the NAc shell samples dissected from coronal brain slices (500 μm thick) were immediately frozen and stored at −80 °C until use. The tissues from two pools of animals were homogenized by sonication in 20 mM Tris buffer (pH 7.5) containing 2 mM EDTA, 0.5 mM EGTA and protease inhibitors (Calbiochem, San Diego, CA, USA), and protein concentrations were determined. Aliquots of protein (5 μg) were diluted with an equal volume of sample buffer (124 mM Tris-HCl, pH 7.5, 4% sodium dodecyl sulfate, 10% glycerol, 4% 2-mercaptoethanol and 0.02% bromophenol blue), subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% acrylamide gels) and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Blots were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 for 1 h and then incubated with goat anti-GLT-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-actin (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, respectively. The blots were then incubated with horseradish peroxidase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:10 000 for 1 h at room temperature. The immunoreactive proteins were finally detected by enhanced chemiluminescence (Amersham Biosciences) and visualized by exposure to X-ray film.

**Histology**

After all behavioral experiments, histological analyses were performed. Animals were anesthetized with diethyl ether and perfused transcardially through the ascending aorta with 0.1 M phosphate buffer (pH 7.4) followed by 4% paraformaldehyde in phosphate buffer. The brains were removed, postfixed in the same fixative overnight at 4 °C, cryoprotected with 20% sucrose in 0.05 M phosphate buffer overnight at 4 °C and then frozen in powdered dry ice. Coronal sections (50 μm) including the NAc shell were prepared on a cryostat and thaw-mounted onto gelatin-coated slides. The slides were lightly stained with cresyl violet and each section was examined by fluorescent microscopy for EGFP.
Conditioned place preference paradigm

Eighty-four animals were divided into four groups, i.e. 16 Ad-EGFP-infused and methamphetamine-conditioned, 24 Ad-GLT-1 + EGFP-infused and methamphetamine-conditioned, 24 Ad-EGFP-infused and morphine-conditioned and 20 Ad-GLT-1 + EGFP-infused and morphine-conditioned animals.

Apparatus

Conditioned place preference was conducted as previously described (Sekiya et al., 2004) with slight modifications. The place-conditioning apparatus (Natsume Seisakusho, Tokyo, Japan) consisted of a shuttle box (30 x 60 x 30 cm, width x length x height) divided into two equal-sized compartments with distinctive visual color and floor texture. The inner surface of one compartment was black with a smooth floor and the other was white with a textured floor. The infrared beam sensors were positioned on each cover from the seam to 5 cm and, when the animal interrupted the beam, it was considered to enter the compartment. The time spent in each compartment during a period of 900 s was measured automatically in a blind fashion using a computer system. The apparatus was enclosed by a sound- and light-attenuated box under conditions of dim illumination and white noise masking.

Preconditioning session

The experimental process consisted of three distinct sessions. On the first day (day 1), the partition separating the two compartments was raised 12 cm above the floor and a neutral platform (5 x 2 x 12 cm) was inserted along the seam separating the compartments. The rats were individually placed on the platform and allowed to climb down and then freely explore the two compartments. After the rats got their whole body off the platform, they habituated themselves to the apparatus for 900 s. The platform was large enough so that the animals had a choice of remaining on the platform or exploring either side of the CPP apparatus. On day 2, rats were infused with adenoviruses into the NAc shell bilaterally as described above. One Ad-EGFP-infused and methamphetamine-conditioned, one Ad-EGFP-infused and morphine-conditioned and two Ad-GLT-1 + EGFP-infused and morphine-conditioned rats died during the surgery. On day 3, the same trial as on day 1 was performed and the time spent in each compartment for 900 s was measured automatically in a blind fashion using a computer system. The rats that spent more than 720 s in one side were excluded from further analysis.

Conditioning session

Although the animals showed a little preference bias in the apparatus used in this study (see Results), we selected an unbiased design in order to circumvent the interpretational problems because the biased design is considered to be susceptible to yielding false-positive results (see review, Tzschentke, 1998). Thus, assignment of the drug-paired compartment was performed randomly and counterbalanced across the subjects. The conditioning session was conducted once daily for six consecutive days from day 4 (2 days after the adenoviral infusion) to day 9 (7 days after the adenoviral infusion). The neutral platform was removed and the compartments were closed by the partition. The rats were injected subcutaneously with either drug (2 mg/kg methamphetamine or 3 mg/kg morphine) or saline and confined to one compartment for 1 h. The next day, the rats that had been given drug were injected subcutaneously with saline, while those that had received saline were injected with drug and confined to the other compartment for 1 h. The conditioning was repeated three times from day 4 to 9.

Test session

On day 10 (8 days after the adenoviral infusion), the rats were individually placed on the neutral platform and allowed to freely explore each compartment in the drug-free state. The time spent in each compartment for 900 s was then measured. Data are expressed as means ± SEM of the time spent in the drug-paired compartment in the preconditioning and test session.

Measurement of naloxone-precipitated morphine withdrawal-induced somatic signs

Forty-eight animals were divided into two groups, i.e. 20 Ad-EGFP-infused and 28 Ad-GLT-1 + EGFP-infused animals. Measurement of somatic signs of naloxone-precipitated morphine withdrawal was performed as previously described (Watanabe et al., 2002). On day 1, rats were infused with adenoviruses into the NAc shell bilaterally as described above. One Ad-EGFP-infused rat died during the surgery. Two days after the infusion (day 3), under light ether anesthesia, the rats had a morphine pellet implanted subcutaneously in the back of their neck. On day 6 (5 days after the adenoviral infusion), each animal was placed in a Plexiglass cylinder to acclimatize it to the experimental environment. After a 60-min habituation period, naloxone (1 mg/kg) was injected intraperitoneally and then the specific behaviors of the animals for naloxone-precipitated morphine withdrawal (rearing, stretching, wet-dog shaking, teeth chattering, jumping, paw shaking, head shaking, ejaculation, penile grooming, backwards walking, grooming, diarrhea, salivation, lacrimation, rhinorrhea and ptosis) were observed for 1 h. Body weight was measured just before and 1 h after naloxone injection and is presented as the means ± SEM of percentage body weight loss. The number of occurrences of rearing, stretching, wet-dog shaking, teeth chattering, jumping, paw shaking, head shaking, ejaculation, penile grooming, backwards walking and grooming were counted and data are presented as means ± SEM of total numbers. The occurrence of diarrhea, salivation, lacrimation, rhinorrhea and ptosis was monitored and is presented as the number of rats showing positive signs divided by the total number of rats tested.

Statistical analysis

In the case of CPP experiments, the data on the initial preference for the compartment (black/white and drug/saline-paired compartments) were analysed statistically using Student’s t-test. Statistical significance from the data on methamphetamine- and morphine-induced CPP was then calculated using two-way ANOVA, with group [Ad-EGFP, Ad-GLT-1 + EGFP and Ad-GLT-1 + EGFP (extra-NAc shell)] x conditioning (preconditioning and test session), followed by the Bonferroni posthoc test. In the case of morphine withdrawal-induced somatic signs, the data on weight loss and counted signs were analysed using one-way ANOVA followed by the Student-Newman-Keuls posthoc test and the data on the checked signs were compared using the chi-square test. Differences with *P < 0.05* were considered significant.

Results

Recombinant adenovirus-induced GLT-1 expression in the nucleus accumbens shell

Eight days after the intra-NAc shell infusion of Ad-GLT-1 + EGFP, fluorescence from EGFP expression was observed within the medial NAc shell. The EGFP expression was restricted to a small elliptic area.
around the needle track with an observed spread approximately 0.1–0.4 mm² from the injection tip (Fig. 1A). The infusion of adenoviruses was accompanied by minimal tissue damage and gliosis in the NAc shell, similar to that produced by infusion of phosphate-buffered saline, as revealed by cresyl violet staining (Fig. 1B). In western blot analysis, although immunoreactivity for endogenous GLT-1 was observed in the NAc shell of the rats infused with phosphate-buffered saline and Ad-EGFP, the expression of GLT-1 within the NAc shell was increased by infusion of Ad-GLT-1 + EGFP at 2 and 5 days and remained stable up to 8 days after the infusion (Fig. 1C). Infusion of adenoviruses into the bilateral NAc shell produced no apparent behavioral abnormalities.

Preconditioning session in conditioned place preference test
In the preconditioning session on day 3, four Ad-EGFP-infused and morphine-conditioned and one Ad-GLT-1 + EGFP-infused and morphine-conditioned rats spent more than 720 s in one side and they were excluded from further analysis. The other rats showed a little preference for the black compartment with a smooth floor (481.4 ± 14.4 s, n = 75) rather than the white compartment with a textured floor (413.9 ± 15.0 s, n = 75) and there was a significant difference between them (t = 3.24, P < 0.01). However, assignment of the drug-paired compartment was performed randomly and counterbalanced across the subjects in an unbiased design and therefore there was no significant difference between the time spent in the drug-paired compartment (439.1 ± 14.9 s, n = 75) and that in the saline-paired compartment (456.3 ± 15.5 s, n = 75) in the preconditioning session on day 3 (t = 0.80, P = 0.425).

Effect of recombinant adenovirus-induced GLT-1 overexpression within the nucleus accumbens shell on methamphetamine-induced conditioned place preference
Illustrations of adenoviral infusion sites in all animals ultimately used in the methamphetamine-induced CPP test are shown in Fig. 2A. Fluorescence microscopy for EGFP accurately showed the EGFP expressions in the bilateral NAc shell in eight of 15 rats infused with Ad-EGFP and 12 of 24 rats infused with Ad-GLT-1 + EGFP, from which experimental data were analysed. The expressions of five additional rats infused with Ad-GLT-1 + EGFP were improperly located outside the NAc shell bilaterally, i.e. in the NAc core and lateral or medial septal nucleus, and experimental data from these rats were grouped as Ad-GLT-1 + EGFP (extra-NAc shell) and analysed.
The other data (i.e. from the other seven rats infused with Ad-EGFP and the other seven rats infused with Ad-GLT-1 + EGFP) were excluded from the final analysis because of improper or incomplete expression (data not shown).

The effect of adenoviral infusions on the methamphetamine-induced CPP is shown in Fig. 3. In the preconditioning session, the times that the rats spent in the methamphetamine-paired compartment were almost the same between groups. The conditioning with methamphetamine increased the times. Two-way ANOVA demonstrated a significant difference in the times between the preconditioning and test session ($F_{1,44} = 21.3, P < 0.0001$), with significant increases in Ad-EGFP within the bilateral NAc shell ($t = 3.23, P < 0.01$) and Ad-GLT-1 + EGFP (extra-NAc shell) ($t = 3.32, P < 0.01$) but not Ad-GLT-1 + EGFP within the bilateral NAc shell ($t = 1.12, P > 0.05$) by the Bonferroni posthoc test. Although two-way ANOVA showed no significant difference between groups ($F_{2,44} = 2.52, P = 0.092$), the Bonferroni posthoc test revealed that there were significant differences in the times spent in the test session between Ad-EGFP and Ad-GLT-1 + EGFP within the bilateral NAc shell ($t = 2.56, P < 0.05$) and between Ad-GLT-1 + EGFP within the bilateral NAc shell and Ad-GLT-1 + EGFP (extra-NAc shell) ($t = 2.85, P < 0.05$) but not between Ad-EGFP within the bilateral NAc shell and Ad-GLT-1 + EGFP (extra-NAc shell) ($t = 0.61, P > 0.05$).

As shown in Table 1, the conditioning with methamphetamine significantly decreased the times that the rats spent in the saline-paired compartment ($F_{1,44} = 21.2, P < 0.0001$), with significant increases in Ad-EGFP ($t = 3.20, P < 0.01$) and Ad-GLT-1 + EGFP (extra-NAc shell) ($t = 3.32, P < 0.01$) but not Ad-GLT-1 + EGFP ($t = 1.13, P > 0.05$). Although there was no significant difference between groups ($F_{2,44} = 2.46, P = 0.097$), the Bonferroni posthoc test showed significant differences in the times spent in the test session between Ad-EGFP and Ad-GLT-1 + EGFP ($t = 2.53, P < 0.05$) and between
TABLE 1. Effects of infusion of recombinant adenoviruses into the bilateral nucleus accumbens (NAc) shell and extra-NAc shell on the time that methamphetamine-conditioned rats spent in the saline-paired compartment.

<table>
<thead>
<tr>
<th>Time spent in saline-paired compartment (s)</th>
<th>Ad-EGFP (NAc shell)</th>
<th>Ad-GLT-1 + EGFP (NAc shell)</th>
<th>Ad-GLT-1 + EGFP (extra-NAc shell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preconditioning session (day 3)</td>
<td>465.8 ± 37.1</td>
<td>467.5 ± 35.4</td>
<td>481.2 ± 52.1</td>
</tr>
<tr>
<td>Test session (day 10)</td>
<td>300.5 ± 20.1*</td>
<td>419.8 ± 30.8††</td>
<td>264.4 ± 35.6*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of the time that the same animals as Fig. 3 spent in the saline-paired compartment. *P < 0.01 compared with respective time that the rats spent in the preconditioning session, ††P < 0.05 compared with Ad-enhanced green fluorescence protein (EGFP) and Ad-GLT-1 + EGFP (extra-NAc shell) in the test session, respectively (Bonferroni posthoc test).

Ad-GLT-1 + EGFP and Ad-GLT-1 + EGFP (extra-NAc shell) (t = 2.83, P < 0.05) but not between Ad-EGFP and Ad-GLT-1 + EGFP (extra-NAc shell) (t = 0.61, P > 0.05).

Effect of recombinant adenovirus-induced GLT-1 overexpression within the nucleus accumbens shell on morphine-induced conditioned place preference

Illustrations of adenoviral infusion sites in all animals ultimately used in the morphine-induced CPP test are shown in Fig. 2B. The expressions were determined in the bilateral NAc shell in six of 19 rats infused with Ad-EGFP and eight of 17 rats infused with Ad-GLT-1 + EGFP, from which experimental data were analysed. The expressions of nine other rats infused with Ad-GLT-1 + EGFP were located outside the NAc shell bilaterally and were grouped as Ad-GLT-1 + EGFP (extra-NAc shell), i.e. from the NAc core, dorsal peduncular cortex and lateral septal nucleus, and analysed. The other data (i.e. the other 13 rats infused with Ad-EGFP) were excluded from the final analysis because of improper or incomplete expression (data not shown).

The effect of adenoviral infusions on the morphine-induced CPP is shown in Fig. 4. In the preconditioning session, the times that the rats spent in the saline-paired compartment were almost the same between groups. There was a significant difference in the times between the precon- ditioning and test session (F1,40 = 32.9, P < 0.0001), with significant increases in Ad-EGFP within the bilateral NAc shell (t = 3.60, P < 0.01) and Ad-GLT-1 + EGFP (extra-NAc shell) (t = 4.73, P < 0.001) but not Ad-GLT-1 + EGFP within the bilateral NAc shell (t = 1.68, P > 0.05). Furthermore, two-way ANOVA showed a significant difference between groups (F2,40 = 3.37, P < 0.05), with significant differences in the times spent in the test session between Ad-EGFP and Ad-GLT-1 + EGFP within the bilateral NAc shell (t = 2.56, P < 0.05) and between Ad-GLT-1 + EGFP within the bilateral NAc shell and Ad-GLT-1 + EGFP (extra-NAc shell) (t = 3.13, P < 0.01) but not between Ad-EGFP within the bilateral NAc shell and Ad-GLT-1 + EGFP (extra-NAc shell) (t = 0.27, P > 0.05).

As shown in Table 2, the conditioning with morphine significantly decreased the times that the rats spent in the saline-paired compartment (F1,40 = 32.9, P < 0.0001), with significant increases in Ad-EGFP (t = 3.61, P < 0.01) and Ad-GLT-1 + EGFP (extra-NAc shell) (t = 4.69, P < 0.01) but not Ad-GLT-1 + EGFP (t = 1.69, P > 0.05).

Effect of recombinant adenovirus-induced GLT-1 overexpression within the nucleus accumbens shell on physical dependence on morphine

Illustrations of adenoviral infusion sites in all animals ultimately used in the experiments for physical dependence on morphine are shown in

FIG. 4. Effects of infusion of recombinant adenoviruses into the bilateral nucleus accumbens (NAc) shell and extra-NAc shell on morphine-induced conditioned place preference (CPP) in rats. Animals were infused with Ad-enhanced green fluorescence protein (EGFP) (n = 6) or Ad-EGFP + GLT-1 (n = 8) into the bilateral NAc shell. Infusions of Ad-EGFP + GLT-1 that missed the bilateral NAc shell are indicated as extra-NAc shell (n = 9). The animals were conditioned with morphine (3 mg/kg) in the CPP paradigm as described in Materials and methods. Each column represents the time spent in the morphine-paired compartment in the preconditioning session on day 3 (□) and test session on day 10 (■). Data are expressed as mean ± SEM. n.s., not significant; **P < 0.01, ***P < 0.001, #P < 0.05, ###P < 0.01 (Bonferroni posthoc test).

TABLE 2. Effects of infusion of recombinant adenoviruses into the bilateral nucleus accumbens (NAc) shell and extra-NAc shell on the time that morphine-conditioned rats spent in the saline-paired compartment.

<table>
<thead>
<tr>
<th>Time spent in saline-paired compartment (s)</th>
<th>Ad-EGFP (NAc shell)</th>
<th>Ad-GLT-1 + EGFP (NAc shell)</th>
<th>Ad-GLT-1 + EGFP (extra-NAc shell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preconditioning session (day 3)</td>
<td>464.2 ± 54.4</td>
<td>477.3 ± 31.5</td>
<td>461.8 ± 35.8</td>
</tr>
<tr>
<td>Test session (day 10)</td>
<td>275.3 ± 30.1*</td>
<td>400.6 ± 24.1‡‡</td>
<td>261.6 ± 20.9†</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of the time that the same animals as Fig. 4 spent in the saline-paired compartment. *P < 0.01, †P < 0.001 compared with respective time that the rats spent in the preconditioning session, ††P < 0.05, ‡‡P < 0.01 compared with Ad-enhanced green fluorescence protein (EGFP) and Ad-GLT-1 + EGFP (extra-NAc shell) in the test session, respectively (Bonferroni posthoc test).

Furthermore, there was a significant difference between groups (F2,40 = 3.52, P < 0.05), with significant differences in the times spent in the test session between Ad-EGFP and Ad-GLT-1 + EGFP (t = 2.56, P < 0.05) and between Ad-GLT-1 + EGFP and Ad-GLT-1 + EGFP (extra-NAc shell) (t = 3.16, P < 0.01) but not between Ad-EGFP and Ad-GLT-1 + EGFP (extra-NAc shell) (t = 0.29, P > 0.05).

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TABLE 3. Effects of infusion of the adenoviruses into the bilateral nucleus accumbens (NAc) shell and extra-NAc shell on the naloxone-precipitated morphine withdrawal-induced somatic signs

<table>
<thead>
<tr>
<th>Withdrawal signs</th>
<th>Ad-EGFP (NAc shell)</th>
<th>Ad-GLT-1 + EGFP (NAc shell)</th>
<th>Ad-GLT-1 + EGFP (extra-NAc shell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight loss (%)</td>
<td>2.3 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Rearing</td>
<td>10.3 ± 2.8</td>
<td>13.7 ± 3.9</td>
<td>12.9 ± 2.6</td>
</tr>
<tr>
<td>Stretching</td>
<td>1.7 ± 0.5</td>
<td>0.5 ± 0.3</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Wet-dog shaking</td>
<td>7.2 ± 1.8</td>
<td>7.8 ± 2.2</td>
<td>10.8 ± 2.3</td>
</tr>
<tr>
<td>Teeth chattering</td>
<td>34.6 ± 5.4</td>
<td>49.9 ± 12.0</td>
<td>49.6 ± 10.4</td>
</tr>
<tr>
<td>Jumping</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Paw shaking</td>
<td>2.7 ± 1.0</td>
<td>8.2 ± 2.9</td>
<td>7.5 ± 1.7</td>
</tr>
<tr>
<td>Head shaking</td>
<td>3.4 ± 1.0</td>
<td>4.5 ± 1.3</td>
<td>7.5 ± 3.3</td>
</tr>
<tr>
<td>Ejaculation</td>
<td>0.8 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Penile grooming</td>
<td>1.0 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Backwards walking</td>
<td>1.0 ± 0.4</td>
<td>0.1 ± 0.1</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>Grooming</td>
<td>2.3 ± 0.9</td>
<td>3.3 ± 1.0</td>
<td>5.4 ± 1.4</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>9/13</td>
<td>8/10</td>
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<tr>
<td>Salivation</td>
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<td>Lacrimation</td>
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Animals were infused with Ad-enhanced green fluorescence protein (EGFP) (n = 13) or Ad-EGFP + GLT-1 (n = 10) into the bilateral NAc shell. Infusions of Ad-EGFP + GLT-1 that missed the bilateral NAc shell are indicated as extra-NAc shell (n = 11). After 2 days, the rats were implanted subcutaneously with a morphine pellet for 3 days. After 3 days, they were injected intraperitoneally with naloxone (1 mg/kg) and somatic signs of naloxone-precipitated morphine withdrawal were observed for 1 h. Weight loss is presented as the mean ± SEM of percentage body weight loss during 1 h. Signs are shown as the mean ± SEM of total numbers for 1 h. The occurrence of diarrhea, salivation, lacrimation, rhinorrhea and prois is expressed as the number of rats showing positive signs divided by the total number of rats tested.

Discussion

We previously constructed recombinant adenoviruses, designated Ad-GLT-1 + EGFP, which could deliver the GLT-1 gene both in vitro and in vivo (Ozawa et al., 2004). In this study, we confirmed that infusion of Ad-GLT-1 + EGFP into the NAc shell efficiently increased the expression of EGFP and GLT-1 surrounding the infusion site, at least during the period 2–8 days after the infusion. Furthermore, Nissl-stained sections showed no evidence of toxicity or gliosis from the adenoviral infection. This is supported by our previous finding that infusion of Ad-GLT-1 + EGFP had no effect on the immunoreactivity of glial fibrillary acidic protein (Ozawa et al., 2004). These results suggest that the recombinant adenovirus Ad-GLT-1 + EGFP could successfully transfer genes for GLT-1 and EGFP into the NAc shell without detectable toxicity. It was reported that an adenovirus transferred the LacZ gene efficiently into both neurons and glial cells (Le Gal La Sale et al., 1993). As GLT-1 is known to be expressed mainly in astrocytes in the rat brain (Robinson, 1998; Conti & Weinberg, 1999), the adenovirus-mediated expression system is likely to be suitable for the present study. In the present study, we found that significant methamphetamine- and morphine-induced CPPs were observed in the rats that infused with Ad-EGFP within the NAc shell and Ad-GLT-1 + EGFP outside the NAc shell, while they disappeared in the rats that infused with Ad-GLT-1 + EGFP within the NAc shell. In addition, the increased times spent in the drug-paired compartment in the test session were significantly attenuated by intra-NAc shell infusion of Ad-GLT-1 + EGFP compared with intra-NAc shell infusion of Ad-EGFP and improper infusion of Ad-GLT-1 + EGFP outside the NAc shell. Although the numbers of animals used in the final analyses were unequal and relatively small for the usual intrinsic variable CPP paradigm, these two statistical analyses certainly suggest that gene transfer of GLT-1 within the NAc shell could inhibit methamphetamine- and morphine-induced CPP. On the other hand, we showed that gene transfer of GLT-1 within the NAc shell had no effect on naloxone-precipitated morphine withdrawal-induced somatic signs. Taken together, these results suggest that local overexpression of GLT-1 within the NAc shell could inhibit the conditioned rewarding effects of methamphetamine and morphine but not physical dependence on morphine.

Many studies have investigated the roles of two major distinct subregions of the NAc, the shell and core, in the effects of drugs of abuse. Intravenous administration of drugs of abuse including amphetamine and morphine produced a preferential increase in extracellular dopamine levels in the NAc shell as compared with the NAc core (Pontieri et al., 1995). It has been shown that rats learn to self-administer certain dopaminergic drugs, cocaine and amphetamine into the NAc shell but not the core (Carlezon et al., 1995; Ikemoto et al., 1997, 2005; Ikemoto, 2003). Furthermore, amphetamine-induced CPP was inhibited by selective dopaminergic denervation of the NAc shell but not the core (Sellings & Clarke, 2003). These behavioral and in vivo microdialysis studies have suggested that the NAc shell, rather than the core, plays a critical role in the rewarding effects of drugs of abuse, although recent evidence shows the anatomical and functional heterogeneity even in the NAc shell subterritories, the rostral and caudal shell (Heidbreder et al., 1999). The present study further suggests the role of the NAc shell in the conditioned rewarding effects of methamphetamine and morphine. However, there is a body of evidence for the involvement of the NAc core. For example, intra-NAc core, rather than shell, injection of an N-methyl-D-aspartate receptor antagonist impaired response reinforcement learning in the acquisition of a simple lever-press task to obtain food (Kelley et al., 1997). A challenge with cocaine elevated extracellular glutamate level in the NAc core, but not the shell, only in rats sensitized to cocaine and the expression of cocaine sensitization was prevented by an intra-NAc core injection of a non-N-methyl-D-aspartate receptor antagonist (Pierce et al., 1996). In addition, the dopamine release in the NAc core, but not the shell, was facilitated in rats sensitized to morphine, amphetamine and cocaine (Cadoni & Di Chiara, 1999; Cadoni et al., 2000). Recently, Li et al. (2004) showed that the induction of cocaine sensitization was accompanied by...
structural plasticity of the medium spiny neurons in the NAc core but not the shell. Our present results could not exclude the possibility of the involvement of GLT-1 in the NAc core, as unfortunately there was no animal gene transferred with GLT-1 in the bilateral NAc core even in the extra-NAc shell group used in the CPP tests. Clearly, further investigation will be needed to evaluate the role of GLT-1 in the NAc core in the methamphetamine- and morphine-induced CPP.

Adaptive changes in the central glutamatergic system represent a potential mediator of long-term drug effects, given the proposed role of this system in neural plasticity in general. A role for the glutamatergic system in the addiction-related behaviors is supported by numerous pharmacological studies. Regarding the CPP paradigm, many studies have reported that systemic administration of various types of glutamate receptor antagonists prevented the acquisition and/or expression of amphetamine- and morphine-induced CPP (Del Pozo et al., 1996; Kim & Jang, 1997; Tschantke & Schmidt, 1997; Papp et al., 2002), although there are also conflicting results (Hoffman, 1994). In addition, systemic administration of riluzole, a functional inhibitor of glutamatergic transmission, attenuated the acquisition of both amphetamine- and morphine-induced CPP (Tschantke & Schmidt, 1998). Recent studies using genetically modified animals have indicated the involvement of various glutamate receptors in addiction-related behaviors (Chiamulera et al., 2001; Vekovisicheva et al., 2001; Miyamoto et al., 2004). Furthermore, intra-NAc injection of glutamate receptor antagonists inhibited the acquisition and/or expression of amphetamine-, morphine- and cocaine-induced CPP (Layer et al., 1993; Popik & Kolasiewicz, 1999), as well as the amphetamine-potentiated responding for conditioned reward (Kelley & Thorne, 1992), cocaine self-administration (Pulvirenti et al., 1992) and cocaine-induced relapse to drug-seeking behavior (Cornish & Kalivas, 2000). These findings suggest that the glutamate receptors in the NAc play important roles in the addiction-related behaviors (White & Kalivas, 1998; Everitt & Wolf, 2002). The present results further support the notion of the roles of the glutamatergic system in the NAc shell in the conditioned rewarding effects of methamphetamine and morphine. However, as glutamate is known to involve various forms of learning and conditioning (Collingridge & Singer, 1990), it is difficult to determine whether the inhibitory effect of gene transfer of GLT-1 is due to a reduction of the primary rewarding effects or due to impairment of learned associations during conditioning. Indeed, glutamate transporters could regulate long-term potentiation and contextual fear conditioning (Katagiri et al., 2001; Levenson et al., 2002). On the other hand, it has been shown that some glutamate receptor antagonists inhibited the amphetamine- and morphine-induced CPP without affecting the spatial memory and the CPP to food, sucrose, etc. (Popik & Danyzs, 1997; Papp et al., 2002). Additional experiments, such as assessment of the effect on various learning tasks and the CPP to the natural rewards, are needed to elucidate whether the gene transfer of GLT-1 affected the learned associations with drug-taking context.

It should be noted that findings about the subtypes of glutamate receptors involved in the acquisition and/or expression of amphetamine- and morphine-induced CPP are not consistent. For example, the acquisition of morphine-induced CPP was prevented by non-competitive N-methyl-D-aspartate receptor antagonists such as MK-801 (Del Pozo et al., 1996; Tschantke & Schmidt, 1997), while MK-801 failed to affect the acquisition of amphetamine-induced CPP (Hoffman, 1994; but see Kim & Jang, 1997). Intra-NAc injection of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate/kainate glutamate receptor antagonists inhibited the expression of both amphetamine- and morphine-induced CPP and acquisition of amphetamine-induced but not morphine-induced CPP (Layer et al., 1993). On the other hand, in the present study, the overexpression of GLT-1 was observed at least 2 days and up to 8 days after the adeno viral infusion, indicating that it remained stable during the conditioning and test session. As the overexpression of GLT-1 might lower the extracellular glutamate level by increasing glutamate uptake, it is suggested that the functional suppression of all glutamate receptor subtypes could attenuate both the acquisition and expression of methamphetamine- and morphine-induced CPP. Further investigations will be needed to assess whether the overexpression of GLT-1 reduced the acquisition and/or expression of CPP.

It is known that Na⁺-dependent glutamate transporters play crucial roles in physiological functions such as neuronal plasticity in addition to the onset of neurological disorders by regulating extracellular glutamate levels (Robinson, 1998; Hansson et al., 2000). Studies have shown that the altered expression and glutamate uptake activity of glutamate transporters could modulate glutamatergic signal transmission (Rothstein et al., 1996; Tanaka et al., 1997) and neuronal plasticity-based events (Katagiri et al., 2001; Levenson et al., 2002). We have reported that GLT-1 mRNA expression was decreased in the striatum/NAc of morphine-dependent rats (Ozawa et al., 2001), suggesting the enhancement of glutamatergic transmission following a decrease in glutamate uptake in these regions. Furthermore, other groups have also suggested the involvement of changes in the expression of glutamate transporters in drug addiction to morphine as well as other drugs of abuse (Shirai et al., 1996; Elkins et al., 2003; Melendez et al., 2005), although there are also conflicting results (Armstrong et al., 2004). Recent studies have demonstrated the roles of functional changes of glutamate transporters by protein kinases and their translocation from the cell surface (Levenson et al., 2002; Xu et al., 2003; Guillet et al., 2005). On the other hand, GLT-1 has been shown to localize almost exclusively in astrocytic processes in the vicinity of the synaptic cleft (Minelli et al., 2001) and represents the predominant route for the clearance of extracellular glutamate in the regulation of glutamatergic synaptic transmission (Robinson, 1998; Conti & Weinberg, 1999). Zhou & Sutherland (2004) showed that GLT-1 trafficking and cluster formation in astrocytic processes play an important role in the regulation of glutamate uptake at the synaptic cleft. It has been shown that repeated treatments with methamphetamine and morphine caused morphological changes in astrocytes to the reactive form in vivo (Song & Zhao, 2001; Armstrong et al., 2004) and in vitro (Stiene-Martin et al., 1991; Stadlin et al., 1998). It is possible that the morphological changes in astrocytes induce retraction of astrocytic processes spatially away from synaptic cleft, which may contribute to the inhibition of glutamate uptake activity and thus to enhancing glutamatergic transmission (Iino et al., 2001). These findings support the notion that changes in the expression, function and distance from the synaptic cleft of GLT-1 on astrocytic processes in the NAc shell resulting from repeated treatments with methamphetamine and morphine could regulate glutamatergic transmission and subsequent glutamate-dependent neuronal adaptation, related to their conditioned rewarding effects. Furthermore, it was shown that a glutamate transporter inhibitor, t-trans-pyrrolidine-2,4-dicarboxylic acid, increased extracellular glutamate as well as dopamine levels in the striatum (Del Arco et al., 1999), indicating that glutamate transporters could regulate dopamine release from the dopaminergic nerve terminals. We have shown that dl-threo-β-benzylxoyaspartate, a glutamate transporter inhibitor, facilitated the acquisition of morphine-induced CPP (Sekiya et al., 2004) and MS-153, a glutamate transporter activator, attenuated the acquisition of CPP induced by methamphetamine and morphine (Nakagawa et al., 2005). Taken together, these facts make it conceivable that the increase of glutamate uptake resulting from the overexpression of GLT-1 within the NAc shell could attenuate the


Kelley, A.E., Smith-Roe, S.L. & Holahan, M.R. (1997) Response-reinforcement learning is dependent on N-methyl-D-aspartate receptor activation in dopamine release induced by repeated treatment with methamphetamine and morphine and/or subsequent glutamate-dependent neuronal adaptations related to acquisition of their conditioned rewarding effects. Alternatively, it is possible that local overexpression of GLT-1 within the NAc shell might suppress the elevation of the extracellular glutamate levels at the synaptic cleft during the test session, resulting in reduced expression of their conditioned rewarding effects.

However, it has been shown that several types of glutamate receptor antagonists attenuate the development of physical dependence on morphine and/or the expression of morphine withdrawal-induced somatic signs (Trujillo & Akil, 1991; Fundytus & Codere, 1994; Rasmussen et al., 1996). However, the role of the glutamatergic system in the NAc remains controversial. It has been reported that microinjection of opioid antagonists into the NAc of morphine-dependent rats elicited only weak morphine withdrawal-induced somatic signs (Maldonado et al., 1992) and electrolytic lesions of the NAc failed to alter them (Kelsey & Arnold, 1994), while repeated microinjections of ketamine, a non-competitive N-methyl-D-aspartate receptor antagonist, into the NAc attenuated the expression of somatic signs (Ji et al., 2004).

In the present study, overexpression of GLT-1 within the bilateral NAc shell and its surroundings, including the NAc core (see Fig. 2C), did not affect them, although we previously reported that overexpression of GLT-1 within the bilateral locus coeruleus did attenuate them (Ozawa et al., 2004). Taken together, we consider that, at least, the glutamate uptake system involving GLT-1 within the NAc shell is not likely to play an essential role in physical dependence on morphine.

In conclusion, we found that gene transfer of a glial glutamate transporter, GLT-1, within the bilateral NAc shell mediated by recombinant adenosine significantly attenuated the methamphetamine- and morphine-induced CPP, while it had no effect on the naloxone-precipitated morphine withdrawal-induced somatic signs. These results suggest that GLT-1 within the NAc shell plays an inhibitory role in the methamphetamine- and morphine-induced CPP but not physical dependence on morphine. The present study may provide a new strategy for preventing drug dependence by regulating the expression and/or activity of GLT-1.

Acknowledgements

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Abbreviations

CPP, conditioned place preference; EGFP, enhanced green fluorescence protein; NAc, nucleus accumbens.

References


