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Dopamine D1 Receptor-induced Signaling through TrkB Receptors in Striatal Neurons*

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In addition to its role as a neurotransmitter, dopamine can stimulate neurite outgrowth and morphological effects upon primary neurons. To investigate the signal transduction mechanisms used by dopamine in developing striatal neurons, we focused upon the effects of activating the dopamine D1 receptor. Using the D1 receptor agonist SKF38393, we found that Trk neurotrophin receptors were activated in embryonic day 18 striatal neurons. K-252a, a Trk tyrosine kinase inhibitor, and a dopamine D1 receptor antagonist could block the effects of SKF38393. The increase in TrkB phosphorylation was not the result of increased neurotrophin production. Induction of TrkB activity by SKF38393 was accompanied by the phosphorylation of several Trk signaling proteins, including phospholipase Cγ, Akt, and MAPK. Biotinylation experiments followed by immunostaining by phospho-TrkB-specific antibodies indicated that the mechanism involved increased TrkB surface expression by dopamine D1 receptor activation. This increase in cell surface TrkB expression was dependent upon an increase in intracellular Ca^{2+}. These results indicate that stimulation of dopamine D1 receptors can be coupled to the neurotrophin receptor signaling to mediate the effects of dopamine upon striatal neurons.

Dopamine, the major neurotransmitter released from dopaminergic neurons, modulates neuronal activity (1–3) and influences key physiological functions related to locomotor activity, reward, and cognition (4, 5). Dopamine also appears to exert several developmental roles. In the lateral ganglionic eminence, dopamine receptors modulate the cell cycle of progenitor cells (6). Dopamine regulates neuronal differentiation and maturation, such as neurite extension and development of growth cones (7–9). However, the molecular mechanisms for these developmental activities have not yet been defined.

Dopamine receptors are classified as D1-like (D1 and D5) and D2-like (D2, D3, and D4) receptors (10). Activation of D1-like receptors enhances L-type calcium ion (Ca^{2+}) channel activity and increases intracellular Ca^{2+} concentration (11–13). Dopamine receptors are G protein-coupled receptors (GPCRs)[3] that regulate the signaling results in cyclic 3′-5′ AMP (cAMP) accumulation because of coupling with the heterotrimeric G protein subunits (14, 15). A number of GPCRs can transactivate receptor tyrosine kinases. This suggests that dopamine receptors may regulate trophic effects more broadly by using transactivation of other receptors.

Neurotrophins, such brain-derived neurotrophic factor (BDNF) and neurotrophin-3, are widely expressed in cortex, cerebellum, and hippocampus and have well established effects upon the differentiation and development of many neuronal populations in the central nervous system (16). In addition to neurotrophin binding to Trk receptors, it has been appreciated that Trk receptors can be transactivated by ligands that use GPCRs (17, 18). Transactivation of Trk receptors has been shown to account for neuroprotection and neuronal migration (19, 20).

In this article, we report that D1 receptor stimulation can lead to transactivation of TrkB receptor activity in rat striatal neurons *in vitro* and *in vivo*. The activation of D1 receptors results in the regulation of calcium influx and TrkB surface expression in primary striatal cultures. Our results suggest that D1 receptor mediates its signaling by transactivation of TrkB and imply that the development of striatal neurons can depend upon transactivation mechanisms.

EXPERIMENTAL PROCEDURES

Primary Culture—Striatal tissues were dissected from embryos of Sprague-Dawley rats (embryonic days 18–19, Charles River Laboratories, Inc., Wilmington, MA) and dissociated with 0.01% trypsin solution. The neurons were plated at a density of ~1.0 × 10^6 cells/ml in Dulbecco's modified Eagle's medium containing

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[3] The abbreviations used are: GPCR, G protein-coupled receptor; BDNF, brain-derived neurotrophic factor; DIV, day *in vitro*; NSE, neuron-specific enolase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA-AM, *O*,*O*′-bis(2-aminophenyl)ethylenglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester.

10% fetal bovine serum and grown for 5–6 days in serum-free conditions, as described previously (21). Glial cells were plated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and grown in 10% serum condition. On days *in vitro* (DIV) 5 or 6, the cultures were treated with dopamine agonists: SKF38393 (1 μM), quinpirole (1 μM; Sigma-Aldrich Corp.), ionomycin (10 μM; Calbiochem, San Diego, CA), or BDNF (5 ng/ml, Peprotech Inc., Rocky Hill, NJ). Prior to the dopamine agonist treatment, the cultures were preincubated with the dopamine D1 receptor antagonist SCH23390 (1 μM; Sigma) or K-252a (100 nM; Calbiochem), TrkB-Fc (10 μg; Chemicon, Temecula, CA), BAPTA (10 μM), or BAPTA-AM (10 μM, Calbiochem). All of the animal experiments were performed in accordance with the Division of Laboratory Animal Resources Guidelines of New York University School of Medicine.

Surface Biotinylation Assay—The cultures were treated with 1 μM SKF38393 for 3 h and then incubated with 1 mg/ml sulfo-NHS-LC-biotin (Pierce) in phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 min on ice (18). The cell lysates were incubated with immobilized streptoavidin-beaded agarose (ImmunoPure; Pierce) overnight at 4 °C. Biotinylated proteins were eluted with 2% SDS buffer at 100 °C and processed for Western blotting analysis.

Immunoprecipitation and Western Blot Analysis—Levels of phospho-TrkB, TrkB, phosphotyrosine were determined by immunoblot analysis using methods similar to those described previously (22). Protein samples for immunoprecipitation were prepared from culture cells or striatal tissues lysate by radioimmune precipitation assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS). For immunoprecipitation, the protein lysates were incubated with 2 μg of Trk antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or TrkB antibody (Cell Signaling Technology, Danvers, MA) overnight at 4 °C. The resulting immunocomplexes were precipitated with protein A-Sepharose beads (Amersham Biosciences). After denaturing with 2% SDS, the samples were separated by SDS-PAGE and transferred electrophoretically to polyvinylidine fluoride membranes (Immobilon-P®, Millipore, Bedford, MA). The membranes were probed with Trk antibody (1:1000; Santa Cruz), phosphotyrosine antibody (1:1000; Upstate Biotechnology Inc., Lake Placid, NY), TrkB antibody (1:1000; Cell Signaling), phospho-MAPK antibody (1:1000), MAPK antibody (1:1000), phospho-ARMS antibody (1:500), ARMS antibody (1:2000 (23), phospho-Akt antibody (1:1000), Akt antibody (1:1000; Cell Signaling), neuron-specific enolase (NSE) antibody (1:1000; Chemicon), GAD67 (glutamate decarboxylases of 67 kDa) antibody (1:500; Santa Cruz), glial fibrillary acidic protein antibody (1:1000; Chemicon), or actin antibody (1:5000; Chemicon). After extensive washing, the immunoreactivity on the membranes was detected with anti-rabbit/mouse immunoglobulins conjugated to horseradish peroxidase, followed by a chemiluminescence reaction (ECL kit; Amersham Biosciences). The band intensities were quantified by densitometry.

The specificity of phospho-TrkB antibody was confirmed by peptide competition assay (23). For the competition, we combined phospho-TrkB antibody with a 5-fold excess of TrkB peptide (LQNLAKSPVTLDIC) or phospho-TrkB peptide (LQNLAKSPVT(PO_3H_2)LDIC) in 500 μl of PBS and incubated overnight at 4 °C, and then we used them for Western blotting.

Immunocytochemistry—The cells were fixed with 4% paraformaldehyde in PBS or 4% paraformaldehyde + 0.1% glutaraldehyde in PBS for 20 min and then washed three times for 10 min with PBS. The fixed cultures were incubated overnight at 4 °C with phospho-TrkB antibody (1:1000), dopamine D1 receptor antibody (1:300; Chemicon), or TrkB antibody (N terminus, 1:300; Upstate) and incubated for 1 h at room temperature with Alexa Fluor® 568 goat anti-rabbit IgG secondary antibody (1:400), Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (1:200), donkey anti-goat IgG-Cy3 antibody (1:400; Molecular Probes, Eugene, OR), or goat anti-mouse IgG-biotin antibody (1:400; Vector Laboratories, Burlingame, CA). Following mouse IgG-biotin antibody, the cells were incubated with avidin-biotin horseradish peroxidase complexes (VECTASTAIN® Elite ABC kits, Vector Laboratories). The staining was proceeded with diaminobenzidine (0.5 mg/ml in 50 mM Tris, pH 7.5) in the presence of H_2O_2. The images of immunoreactive neurons were collected with a Nikon fluorescence microscope (ECLIPSE E800) and analyzed using AxioVision (Carl Zeiss, Göttingen, Germany).

Mouse Dopamine D1 Receptor Transfection—The expression vector of mouse D1 receptor (mD1R) was cloned in the mammalian expression vector pcDNA3.1/V5-His©TOPO©TA expression kit (Invitrogen). Expression of D1 receptor was detected by tritium-labeled dopamine binding assay described below.

Human TrkB transfected HEK293 (HEK293-TrkB) cells were prepared as described previously (24) and grown in 12-well plates at a density of 4×10^5 cells/well. The cells were transfected with mD1R plasmid with the calcium phosphate method. After 24 h, the cultured cells were challenged with chemical reagents above. The dopamine binding assay was assessed by incubating HEK293-TrkB cultures for 60 min at 4 °C in Tris Buffer (50 mM Tris, pH 7.4, 120 mM NaCl, 1 mM EDTA, 5 g of glucose, and 2 mM $CaCl_2$) containing 20 nM ^{3}H-dopamine (Amersham Biosciences) and 1 μM dopamine (Sigma). The cells were lysed, and their radioactive content was counted as described previously (21).

Dopamine D1 Receptor Agonist and Antagonist Challenge to Neonatal Rats—Sprague-Dawley rats (4 days old; Charles River Laboratories, Inc.) were housed on a 12-h light-dark cycle with free access to food and water. Different groups of rats were subcutaneously administered at the nape of the neck (25) with SKF38393 (Sigma; 1 mg/kg), SCH23390 (Sigma; 1 mg/kg), or control vehicle (0.25% ascorbic acid in distilled saline). SKF38393 and SCH23390 were dissolved in distilled saline with 0.25% ascorbic acid. The brain tissues of these animals used for immunoprecipitation and immunoblotting were harvested immediately following decapitation and dissected on ice. Peripherally administrated, these compounds penetrate the blood-brain barrier and affect neurochemical makers in the brain (26). The phosphorylation level of dopamine and cyclic AMP-regulated protein (relative molecular mass, 32,000; DARPP32) was increased in striatal tissues from SKF38393-administrated rats and decreased in those from SCH38393-administrated rats (data not shown).

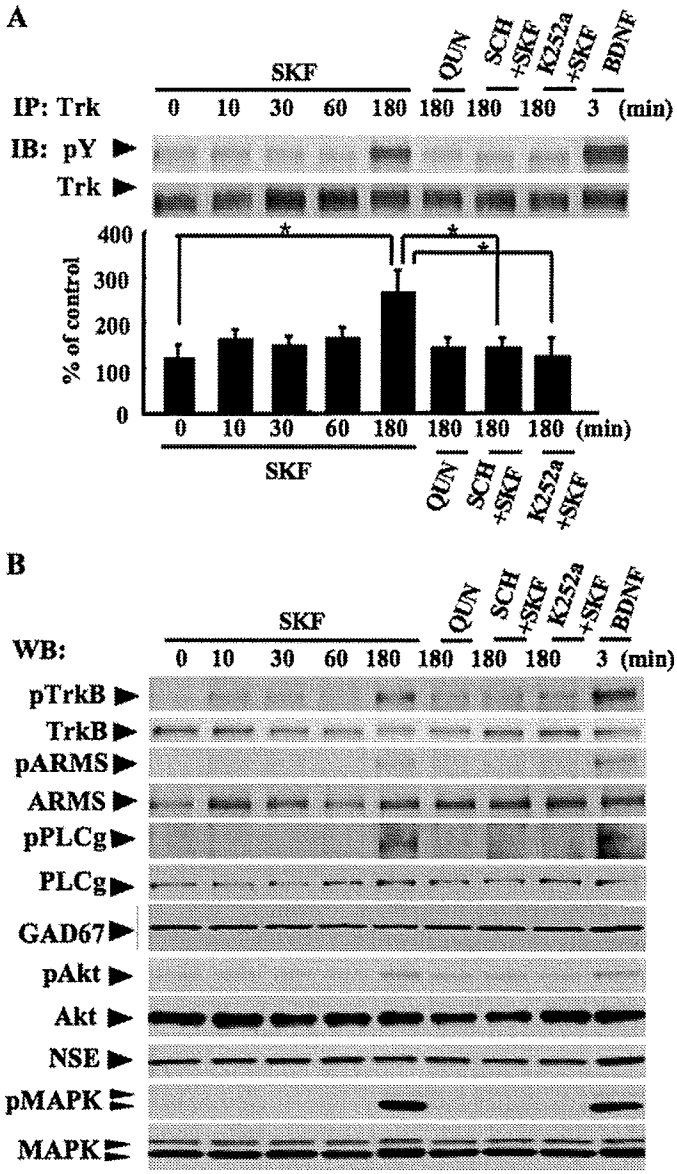

FIGURE 1. **Trk receptors phosphorylation by D1 receptor agonist in rat striatal cultures.** Neuronal striatal cultures were treated with control (PBS), SKF38393 (*SKF*; 1 μM, 10–180 min), quinpirole (*QUN*; 1 μM, 180min), and BDNF (5 ng/ml, 3 min) at 37 °C on DIV6. Some dishes were preincubated with K-252a (*K-252a+SKF*) or SCH23390 (*SCH+SKF*) prior to SKF38393 incubation. *A*, after immunoprecipitation (*IP*) with Trk antibody, the cell lysates were subjected to SDS-PAGE and Western blotting (*WB*) for phosphotyrosine (*pY*) and Trk. Typical immunoblots (*IB*) are shown. The protein levels were determined from independent wells ($n = 4$). *, $p < 0.05$. The *bars* indicate S.D. *B*, some of cell lysates for immunoprecipitation were also subjected to Western blotting. Representative immunoblots are shown. Note that treatment with SKF38393 increased the immunoreactivity of growth-associated protein 43 (a growth cone marker) in this culture (data not shown).

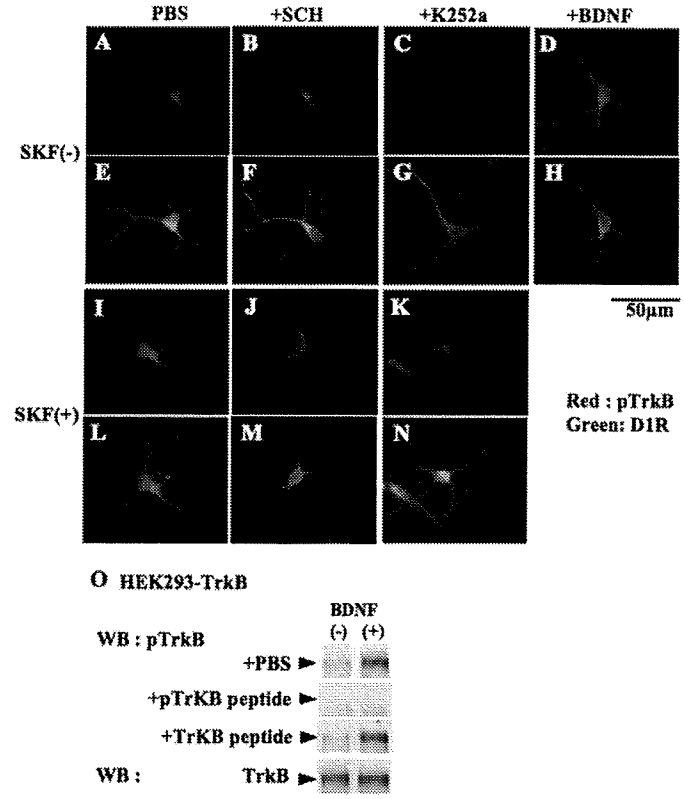

FIGURE 2. **Immunoreactivity of phospho-TrkB receptor induced by D1 receptor agonist in rat striatal neurons.** *A–N*, striatal cultures were treated with control (PBS), SKF38393 (*SKF*), or BDNF at 37 °C on DIV5. Some dishes were preincubated with K-252a or SCH23390 (*SCH*) prior to SKF38393 incubation. The cultures were fixed and immunostained with phospho-TrkB antibody (*red*) and dopamine D1 receptor antibody (*green*). Note that phospho-TrkB immunoreactivity was detected in 87 ± 11% of D1 receptor positive neurons (data not shown). *O*, specificity of phospho-TrkB antibody was measured by using HEK293-TrkB cells. To assess specificity of phospho-TrkB antibody, cell lysates (30 μg/each) obtained from HEK293-TrkB cells treated with control (PBS) or BDNF (10 ng/ml) for 5 min were probed with phospho-TrkB antibody as well as the antibody that was preincubated with either a phosphorylated or nonphosphorylated TrkB peptide competitor. *WB*, Western blotting.

Statistical Analysis—All of the values are presented as the means ± S.D. The pharmacological effects were analyzed using one-way analysis of variance followed by a Bonferroni test or a Mann-Whitney *U* test to evaluate the differences in immunoblots. A probability level of less than 0.05 was considered to be statistically significant.

RESULTS

Dopaminergic input from the substantia nigra plays an important role in the striatum (22, 27, 28). It has been reported that dopamine D1 receptor signaling can regulate neuronal morphology and expression of functional markers in developing striatal neurons (7, 8). We isolated striatal primary neurons from rat embryonic day 18 embryos and assessed the involvement of neurotrophic signals in the developing neurons.

TrkB, the receptor for BDNF and neurotrophin 4, is expressed in striatum neurons (29). In the striatum, BDNF/TrkB signaling is important in the proliferation, differentiation, and development of medium spiny neurons (30–33). To determine the identity and status of TrkB receptors in striatum neurons, we used a phospho-specific antibody against a C-terminal peptide of TrkB. First, we confirmed the specificity of the anti-phospho-TrkB antibody by a peptide competition assay (see Fig. 2*O*). Western blot analysis of HEK293-TrkB cells (24) resulted in activated TrkB protein after BDNF treatment. This signal was blocked after preincubation with the phosphorylated TrkB peptide and not with the nonphosphorylated TrkB peptide.

To establish whether Trk receptors are activated by dopamine receptor stimulation in striatum neurons, we prepared primary neuronal cultures from embryonic rat striatum and

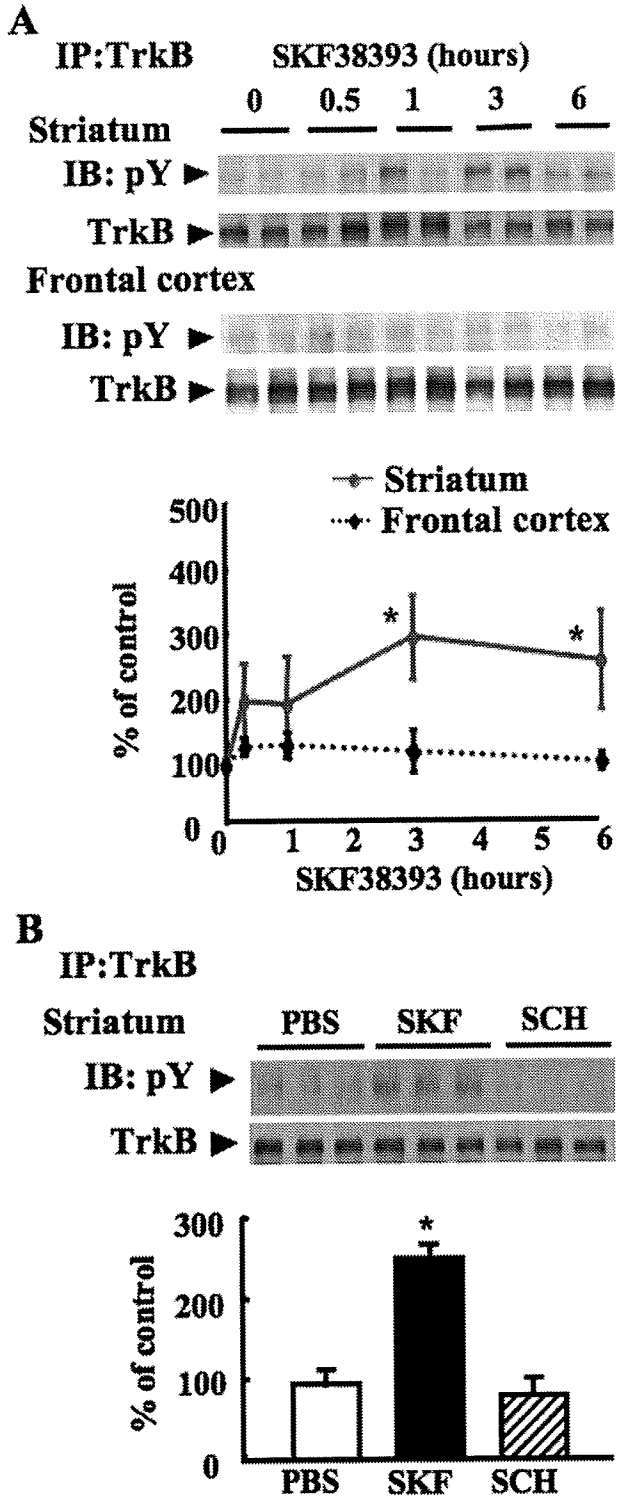

FIGURE 3. **Phospho-TrkB level regulated in the striatum of the rats administrated with D1 receptor agonist and antagonist.** Littermate rats were injected with control vehicle (PBS; 0.25% ascorbic acid-saline), SKF38393 (*A* and *B*, *SKF*; 1 mg/kg in 0.25% ascorbic acid-saline) and SCH23390 (*B*, *SCH*; 1 mg/kg in 0.25% ascorbic acid-saline) on postnatal day 4. *A*, the effects of D1 receptor stimulation at several time points on striatum and frontal cortex were estimated by determining phospho-TrkB. Duplicate samples are displayed. *B*, the effects of D1 receptor activation and inhibition after 3 h of administration on striatum were estimated by determining phospho-TrkB. Triplicate samples are displayed. Representative immunoblots (*IB*) are shown. Quantifications of phospho-TrkB immunoreactivity in striatum by NIH Image are shown. Note that there was no significant reduction of the neuron (NSE) marker in the striatum in this pharmacological paradigm (data not shown. control: 100 ± 12.9%, SKF38393: 100.7 ± 7.1%, SCH23390: 107.4 ± 14.8%, mean ± S.D.). $n = 4$; *, $p < 0.05$. The *bars* indicate S.D. *IP*, immunoprecipitation.

treated them with the D1 receptor agonist SKF38393 or the D2 receptor agonist quinpirole (34, 35). The neuronal cultures were examined on DIV6 and compared with neurons cultured in the presence of BDNF. Treatment with SKF38393 increased the level of phospho-Trk (Fig. 1*A*) and phospho-TrkB (Fig. 1*B*) in striatal neurons. However, the D2 receptor agonist, quinpirole, failed to stimulate the phosphorylation of TrkB (Fig. 1*B*). Pretreatment with D1 receptor-specific antagonist SCH23390 or tyrosine kinase inhibitor K-252a blocked the increase of TrkB phosphorylation by SKF38393 (Fig. 1*B*).

We also examined the activation of Trk signaling molecules, such as Akt, MAPK, phospholipase Cγ, and ARMS (36, 37). After incubation with SKF38393 for 3 h, the phosphorylation levels of several signaling molecules, such as phospholipase Cγ and MAPK, were increased, and they were also inhibited by the pretreatment with SCH23390 or K-252a (Fig. 1*B*). NSE antibody was used as a neuronal cell marker, and glutamate decarboxylase of 67 kDa (GAD67) was also used as a marker of medium size, spiny GABAergic neurons in striatum (28). The levels of NSE and GAD67 were both constant in our neuronal cultures.

An increase in phospho-TrkB immunoreactivity was also observed in striatal neurons after a 3-h stimulation with SKF38393 (Fig. 2). Phosphorylated TrkB was detected in the D1 receptor-positive neurons. The phospho-TrkB staining was reduced after treatment with SCH23390 or K-252a. These results indicated that TrkB receptors were phosphorylated in striatal neurons specifically by dopamine D1 receptor stimulation, leading to the phosphorylation of TrkB-dependent signaling *in vitro*.

To determine the effects of D1 receptor activation *in vivo*, we administered a subcutaneous injection of SKF38393 in postnatal rats (postnatal day 4; Fig. 3). Frontal cortex represents a target region of dopaminergic neurons in the ventral tegmental area. Therefore, frontal cortex and striatum from these animals were dissected after the injection, and the effect of SKF38393 was estimated by measuring activated phospho-TrkB protein level in the dopaminergic target region. In striatal lysates, an increase in phosphorylated TrkB was detected by immunoprecipitation at 3 and 6 h after the injection. No significant increases in phospho-TrkB levels in the frontal cortex were observed. These results suggest that D1 receptor-specific TrkB transactivation occurs in the striatum. We also administered SKF38393 or SCH23390 in postnatal rats (postnatal day 4; Fig. 3*B*). Three hours after injection, the level of phospho-TrkB was found to be reduced in the striatum of the rats with SCH23390 compared with the control littermates. This experiment confirmed that activation of D1 receptor can regulate TrkB activity in the striatum.

To confirm the activation of TrkB receptors in a heterologous cell system, we transfected a D1 receptor cDNA construct and assessed the phosphorylation of TrkB by SKF38393 in stably transfected TrkB-HEK293 cells (Fig. 4*B*). Phospho-TrkB levels were increased by SKF38393 and were reduced by prein-

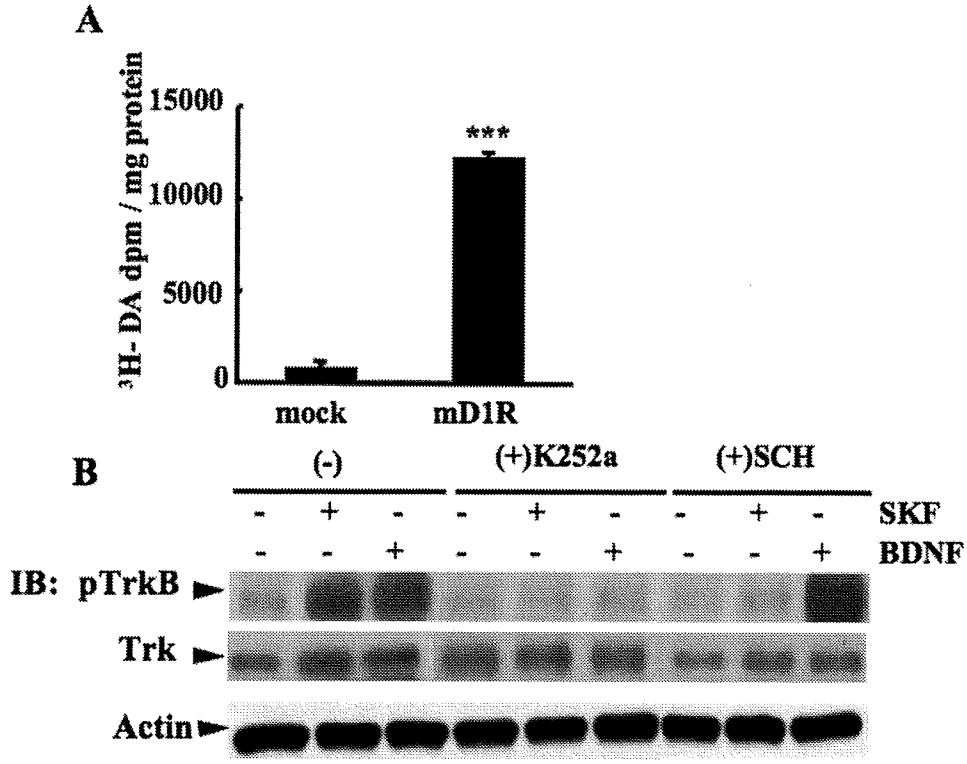

FIGURE 4. **Plasmid transfection to HEK293-TrkB cells.** *A*, HEK293-TrkB cells transfected with mouse D1 receptor expression vector (mD1R) or control vector (mock). After 60 min of incubation with ^{3}H-dopamine (*^{3}H-DA*)-containing buffer, radioactivity contents were determined from independent wells ($n = 4$). ***, $p < 0.001$. The *bars* indicate S.D. *B*, mD1R transfected HEK293-TrkB cells were treated with control (PBS), SKF38393 (*SKF*; 1 μM, 180 min), or BDNF (10 ng/ml, 3 min) at 37 °C. Some dishes were preincubated with K-252a (*(+)K252a*) or SCH23390 (*(+)SCH*) prior to SKF38393 incubation. The cell lysates were subjected to SDS-PAGE and Western blotting. Typical immunoblots (*IB*) are shown.

cubation of K252a or SCH23390, similar to primary neuronal cultures.

Dopamine can stimulate not only neurons but also non-neuronal cells (38, 39). Our striatal neuronal culture system contains glial cells, ~10% of total cell numbers (data not shown). Because glial cells increase postnatally, it is therefore questionable whether the TrkB phosphorylation in developing neurons reflects a direct action on these neurons or is mediated through non-neuronal cells reacting to dopamine.

To address the role of glial cells in transactivation, we compared the relative level of full-length TrkB in primary neuronal cultures and primary glial cultures (Fig. 5*A*). Full-length TrkB was not detectable in glial cell lysates. Next, conditioned medium from primary mixed glia culture was collected after the treatment with control (PBS), SKF38393, or SCH23390 prior to SKF38393 or control (PBS). The neuronal cultures were incubated with glial-conditioned media collected from each treatment, and the levels of phospho-TrkB in the cell lysates were detected by immunoprecipitation (Fig. 5*B*). No effects were observed in the neuronal cultures from either PBS- or SKF38393-conditioned medium. The results from these experiments suggest that TrkB activation in the striatal neurons by SKF38393 treatment was not mediated by glial cells.

Another potential explanation for the effect of D1 receptor stimulation is increased production of neurotrophins. Neurotrophins are synthesized as an immature form, which is cleaved and released as a mature form from neurons and non-neuronal cells (40, 41). We tested whether SKF38393-induced TrkB phosphorylation was mediated by the neurotrophin release by applying TrkB-Fc in the culture medium (Fig. 5, *C–I*). TrkB-Fc contains the extracellular domain of TrkB, which is effective in preventing the activation of native TrkB by sequestering BDNF (42). In striatal cultures, application of TrkB-Fc blocked the increase in phospho-TrkB immunoreactivity by exogenous BDNF but failed to inhibit TrkB phosphorylation after SKF38393 treatment (Fig. 5, *H* and *I*). These results provide evidence that the TrkB phosphorylation by D1 receptor stimulation was not mediated by the release of endogenous neurotrophin or from a secondary effect from glial cells.

Dopamine receptors can regulate concentration of intracellular calcium ions (Ca^{2+}) by modulating extracellular Ca^{2+} influx and intracellular Ca^{2+} pools (11–13). To assess whether Ca^{2+} contributed to TrkB phosphorylation by the D1 receptor agonist (Fig. 6), we treated striatal cultures with the calcium chelator BAPTA, the membrane-permeable chelator BAPTA-AM, and calcium ionophore ionomycin. The application of ionomycin induced TrkB phosphorylation, in a manner similar to SKF38393. Application of BAPTA or BAPTA-AM abolished the increase in phospho-TrkB levels observed in the presence of SKF38393 alone. In these experiments, we did not detect a significant difference between the effect of BAPTA and BAPTA-AM. Our data suggest that the intracellular concentrations of Ca^{2+} are regulated by D1 receptor, and increased Ca^{2+} may be involved in the transactivation of TrkB receptors by SKF38393.

It has been reported that TrkB surface expression is regulated by Ca^{2+} influx in hippocampal and cortical neurons (42–44). From our results, intracellular calcium is implicated in TrkB transactivation by D1 receptor agonists. To determine the level of surface TrkB expression after the treatment with D1 receptor agonist, we used a cell surface biotinylation assay on primary striatal cultures (Fig. 7, *A* and *B*). After a 3-h incubation with SKF38393, striatal neurons were subjected to treatment with NHS-LS-biotin. After immunoprecipitation with avidin-conjugated beads, more biotinylated TrkB was detected on the cell surface (Fig. 7*A*). Pretreatment with the D1 receptor antagonist, SCH23390, reduced the levels of biotinylated TrkB induced by SKF38393.

Similar inhibitory effects were obtained with BAPTA. Pretreatment with BAPTA also lowered the levels of TrkB surface expression. BAPTA-AM also showed the inhibitory effects, but no significant difference was observed compared

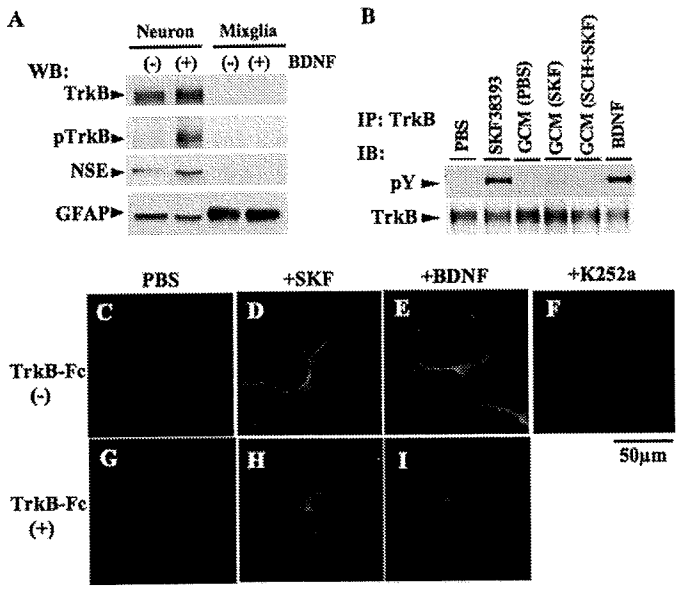

FIGURE 5. **Effects of glial cells or released factors from culture cells on TrkB transactivation in striatal culture.** *A*, striatal neuronal or glial cultures were treated with control (−) or BDNF (+; 10 ng/ml) for 3 min on DIV6. Cell lysates (30 μg each) were subjected to Western blotting (*WB*). Representative immunoblots (*IB*) are shown. *B*, striatal neuronal cultures were treated with conditioned medium from striatal glial cell culture (*GCM*) on DIV6. Primary glial cells were treated with control (PBS), SKF38393 (*SKF*; 1 μM, 3 h), or SCH23390 (1 μM, 1 h) prior to SKF38393 (*SCH+SKF*). After 3 h of incubation, the media were harvested and used as glial condition media for striatal neuronal culture. Neuronal cells were lysed after the treatment with SKF38393, each glial conditioned medium, or BDNF, and then cell samples were immunoprecipitated (*IP*) with anti-TrkB antibody. Representative immunoblots are shown. *C–I*, striatal cultures were treated with control (PBS), SKF38393 (+*SKF*), K-252a and BDNF on DIV6. Some of the cultures were preincubated with TrkB-Fc (10 μg/ml, for 20 min) in 37 °C prior to control (*G*), SKF38393 (*H*), or BDNF (*I*). The cultures were fixed and immunostained with anti-phospho-TrkB antibody.

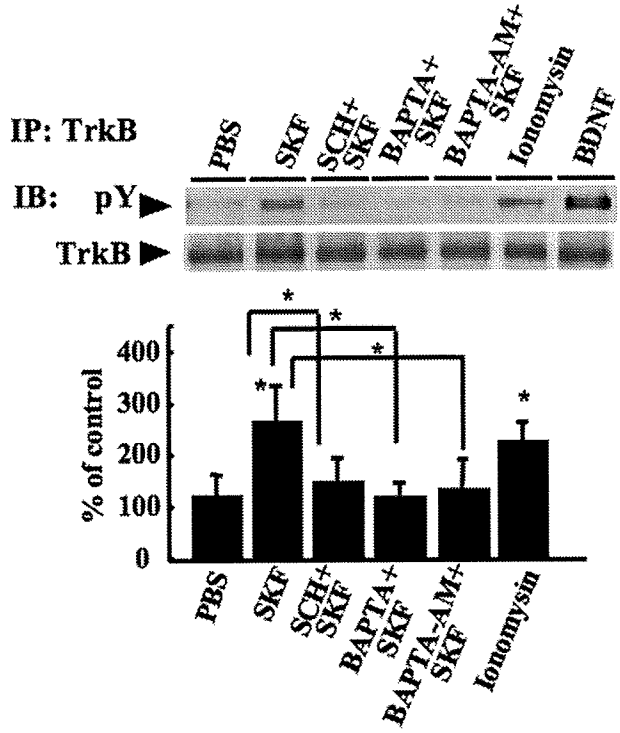

FIGURE 6. **Intracellular Ca^{2+} regulation is involved in the TrkB transactivation by D1 receptor agonist.** Striatal cultures were treated with control, SKF38393 (*SKF*), ionomycin (10 μM), and BDNF on DIV6. Some of the cultures were preincubated with SCH23390 (*SCH+SKF*), BAPTA (*BAPTA+SKF*; 10 μM for 30 min), or BAPTA-AM (*BAPTA-AM+SKF*; 10 μM for 30 min) in 37 °C prior to SKF38393. After immunoprecipitation (*IP*), the cell lysates were subjected to SDS-PAGE and Western blotting for phosphotyrosine (*pY*) and TrkB. Duplicate samples are displayed. Typical immunoblots (*IB*) are shown. The protein levels were determined from independent wells ($n = 4$). *, $p < 0.05$. The *bars* indicate S.D.

with SKF38393 treatment (Fig. 7*B*). In addition to the biotinylation assay, immunocytochemistry using an antibody against the N terminus of TrkB indicated that SKF38393 elevated TrkB surface immunoreactivity. The induction was also blocked by preincubation with SCH23390 or BAPTA. BAPTA-AM pretreatment also inhibited TrkB surface immunoreactivity but not to the same extent as BAPTA (Fig. 7, *C–H*). These data suggest that the D1 receptor stimulation increases Ca^{2+} influx and induces the transactivation and cell surface expression of TrkB.

DISCUSSION

Dopamine D1 receptors are linked to the G_s protein and activation of adenylate cyclase. G protein-coupled receptors have various functions from endocrine regulation to higher order behavior. In the central nervous system, GPCRs mediate neuronal functions not only as fast neurotransmitters but also as slow neuromodulators (15). The results presented here indicate that dopamine is capable of transactivating Trk receptors, in a time course similar to other GPCRs, such as adenosine and pituitary adenylate cyclase-activating polypeptide receptors (17, 18). Activation of TrkB through D1 GPCR signaling occurred without involvement of neurotrophins. This suggests that dopamine neurotransmitters are capable of communicating with neurotrophin signaling, which is responsible for many morphological changes in neurons. Trk receptors also regulate the expression and activity of ion channels and neurotransmitter receptors and therefore can modulate synaptic strength and plasticity (36). These studies indicate that mutual regulation between TrkB and dopamine D1 receptor signaling may contribute to normal brain function.

Surface expression levels of receptors are also important in modulating their signaling and function. Du *et al.* (43) reported that Ca^{2+} induces TrkB transport to plasma membrane in mature hippocampal neurons. The present data show that dopamine D1 receptor activation increases not only TrkB phosphorylation but also TrkB surface expression through the Ca^{2+} influx in developing striatal neuron. The findings in this work imply that receptor transactivation events by GPCRs such as D1 receptors may produce different physiological functions and signal transduction mechanisms than by direct ligand-receptor binding.

Dopamine is a predominant neurotransmitter that controls electrophysiological and motor activity. Dopamine has also been linked to neuronal development through its actions on axonal growth and growth cones. For instance, dopamine enhances outgrowth and arborization of processes and growth cones in embryonic striatal neurons (7, 8, 45, 46). Many of these effects have been ascribed to D1 dopamine receptor activity. We observed such enhancement of neuronal development (increasing of neurite extension and growth cone number) by D1 receptor agonist in our culture system (data not shown).

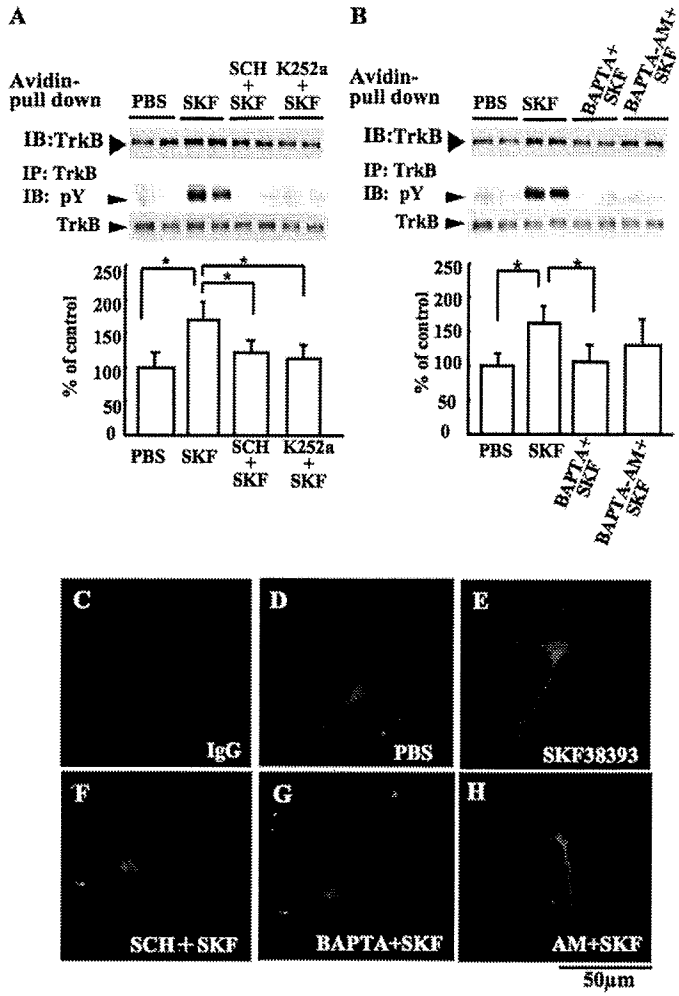

FIGURE 7. **Surface TrkB expression regulated by D1 receptor agonist.** Striatal cultures were treated with control vehicle (PBS), SKF38393 (*SKF*), and BDNF on DIV6. Some of the cultures were preincubated with SCH23390 (*A, SCH+SKF*), K-252a (*A*), BAPTA (*B, BAPTA+SKF*), or BAPTA-AM (*B, BAPTA-AM+SKF*) in 37 °C prior to SKF38393. The cultures were incubated with biotin-PBS for 30 min on ice and then lysed with radioimmune precipitation assay buffer. Half of biotinylated proteins were precipitated by avidin-conjugated beads and then subjected to SDS-PAGE and Western blotting for TrkB. The other samples were used for immunoprecipitation (*IP*) with TrkB. Duplicate samples are displayed. Typical immunoblots (*IB*) are shown. Protein levels were determined from independent wells ($n = 4$). Quantifications of surface biotinylated TrkB levels by NIH Image are shown. *, $p < 0.05$. The *bars* indicate S.D. *C–H*, striatal cultures were treated with control (PBS) and SKF38393 on DIV6. Some of the cultures were preincubated with SCH23390 (*SCH+SKF*), BAPTA (*BAPTA+SKF*), or BAPTA-AM (*AM+SKF*) in 37 °C prior to SKF38393. The cultures were fixed and immunostained with rabbit control IgG (*C*) or TrkB (N terminus) antibody (*D–H*) without detergent. Representative pictures are provided.

These studies suggest that the D1 receptor is crucial for the normal development of striatal neurons during development.

Jung and Bennett (47) observed that acute cocaine treatment increased TrkB mRNA in neonatal striatum. The induction of TrkB mRNA by cocaine was suppressed by SCH23390, a specific antagonist of D1 receptor. Our results did not show a significant enhancement of TrkB protein level *in vitro* (Fig. 1) or *in vivo* (Fig. 3) by the D1 receptor-specific agonist SKF38393. Rather we found the transactivation of TrkB was inhibited by SCH23390. It is formally possible that acute stimulation of D1 receptor and subsequent TrkB transactivation might be involved in prolonged transcription and/or translation of TrkB.

Mice lacking D1 receptors displayed growth retardation, but the general anatomy of the brain was normal. The size of the striatum in adult mutant mice was reduced compared with wild type. These mutant mice displayed selective electrophysiological and behavioral alterations (48, 49). These studies indicate that the lack of D1 receptor in the developing brain may alter neurotransmission and behavior in adults. In our experiments, TrkB and phospho-TrkB levels in striatum from D1 receptor null mice were altered compared with wild type mice.[4] Therefore, dopamine/dopamine receptor signaling in the knock-out mice differed from wild type animals. However, preliminary analysis made it difficult to establish a causal relationship between dopamine D1 receptor and TrkB signaling. Our results here indicate that dopamine/D1 receptor signaling can contribute to BDNF/TrkB activity. It has been observed that acute BDNF stimulation increased D1R mRNA *in vitro*, and the D1 receptor mRNA level is decreased in the TrkB null mice (50). Collectively, these studies suggest that BDNF-TrkB and dopamine-D1 receptor signaling are intertwined and involved in the neuronal development in striatum through modulation of neurotrophic responsiveness. However, further work will be needed to fully elucidate the *in vivo* effects of dopamine on TrkB.

Neurotrophic factors regulate numerous neuronal functions in development and adult life and in response to neuronal injury. As a result, neurotrophins have been implicated in the pathophysiology of a wide variety of neurodegenerative and psychiatric disorders and have been considered to be a therapeutic strategy for neuropsychiatric disorders. Dopaminergic systems also have been studied in relation to Parkinson disease (22, 51) and Huntington disease (52). These parallels suggest that the misbalance in the signaling between neurotrophins and dopamine may be important in neurodegenerative disorders.

Activation of neurotrophin signaling pathways through other receptor systems offers an alternative mechanism of communication in the nervous system. For example, antidepressant agents acting via monoamine G protein-coupled receptors can lead to increased neurotrophin signaling (53). The results with dopamine D1 receptors suggest that agonists of dopamine receptors may be identified with neurotrophic effects for the treatment of neurodegenerative diseases. This approach would involve selective targeting of neurons that express specific GPCRs and trophic factor receptors.

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Hyperthermic and lethal effects of methamphetamine: Roles of dopamine D1 and D2 receptors

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Both human and animal studies suggest that hyperthermia contributes to the lethal effects of methamphetamine. To elucidate the roles of dopamine D1 and D2 receptors in methamphetamine-induced hyperthermia and lethal effects, we used D1 knockout (D1KO) mice, D2 knockout (D2KO) mice, and wild-type littermates. After the administration (i.p.) of a single dose of 30 mg/kg methamphetamine, no hyperthermic effect on body temperature was observed in D2KO mice, though there was a slight elevation in D1KO mice and a marked elevation in wild-type mice. Approximately 27% of the wild-type mice died after the administration, compared to only 7% of D1KO mice and 4% of D2KO mice. In conclusion, both D1 and D2 receptors play roles in the lethal toxic effects of methamphetamine, and mainly the D2 receptor is involved in the elevation of body temperature.

Methamphetamine, a drug of abuse known worldwide for its addictive effects and neurotoxicity, causes somatic and psychiatric disorders. Methamphetamine can cause death in humans, non-human primates, and rodents [6,12,19]. As one of its acute symptoms, methamphetamine-induced hyperthermia which is observed in both human and animals, could contribute to the lethal effects of methamphetamine.

Methamphetamine enters terminals/neurons via monoamine transporters, displaces both vesicular and intracellular monoamines, and facilitates the release of monoamines into the extraneuronal space through synaptic transport via the monoamine transporters [15]. Studies in animals suggest that the activation of dopamine receptors is crucial for methamphetamine-induced hyperthermia [1,4,9,10] and death [5,16]. However, the protective roles of dopamine receptor antagonists against methamphetamine-induced hyperthermia and lethal effects are affected by doses of antagonists and/or methamphetamine, and ambient temperature.

The lethal effect of methamphetamine was diminished by pretreatment with the neuroleptic agent haloperidol [17], the selective D1 receptor antagonist SCH23390 [11], or the D2 receptor antagonist spiperone [5]. By contrast, some have reported that no alteration was demonstrated on pretreatment with SCH23390 [8] or haloperidol [7], and the lethal effect was actually enhanced by pretreatment with sulpiride, a D2 receptor antagonist [5].

Similarly, there are discrepancies in results regarding the prevention of methamphetamine-induced hyperthermia through pretreatment with antagonists; some studies reported preventive roles of haloperidol [9], SCH23390 [4], and eticlopride, a selective D2 antagonist [4] while others showed no effect of SCH23390, and raclopride, selective D2 antagonists [5,18] In this study, we examined the roles of D1 and D2 receptors in the hyperthermic and lethal effects of methamphetamine using D1 knockout (D1KO) and D2 knockout (D2KO) mice.

D1KO mice (The Jackson Laboratory, Maine, USA), D2KO mice (The Jackson Laboratory), and wild-type littermates, 15 ± 2 (average ± S.D.) weeks old, were maintained under conditions of controlled temperature and lighting, with food and water provided ad libitum (genetic background was C57BL/129Sv for all genotypes). The mice were habituated one day prior to the experiments in a temperature-controlled chamber (22 ± 1 °C; light 8:00–20:00; LP-30CCFL-8AR, Nippon Medical and Chemical Instruments CO., LTD., Osaka, Japan), and all the experiments were performed with this chamber. Seventy-one wild-type mice, 21 D1KO mice, and 23 D2KO mice were intraperitoneally (i.p.) administered 30 mg/kg of d-methamphetamine (Dainippon Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan), while 47 wild-type mice, 27 D1KO mice, and 12 D2KO mice were treated saline (i.p.). To exclude effects of circadian rhythm, mice were administered either saline or methamphetamine from 16:00 to 17:30. The maintenance and testing of animals were carried out in accordance with the guidelines of the animal ethics committee at Tohoku University Graduate School of Medicine, Japan.

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Table 1
Lethality and highest temperature in the three genotypes

Genotype	Lethality%(died/total)		Highest temperature °C (n)
D1KO	7* (2/29)	Survived	38.8 ± 0.21 (27)
		Died	40.3 ± 0.75 (2)
D2KO	4* (1/23)	Survived	38.2 ± 0.27 (22)
		Died	39.2 (1)
Wild-type	27 (19/71)	Survived	39.9 ± 0.12 (52)
		Died	40.7 ± 0.32 (19)**

Mice were given 30 mg/kg of methamphetamine (i.p.). Rectal temperature was measured just before (0 min), and 30 and 60 min after the administration. Lethality was significantly lower in D1KO and D2KO mice than wild-type mice (Fisher's exact test, *$p < 0.05$). In all genotypes, the highest temperatures were higher in dead mice, especially wild-type mice (Student's t-test, **$p < 0.05$).

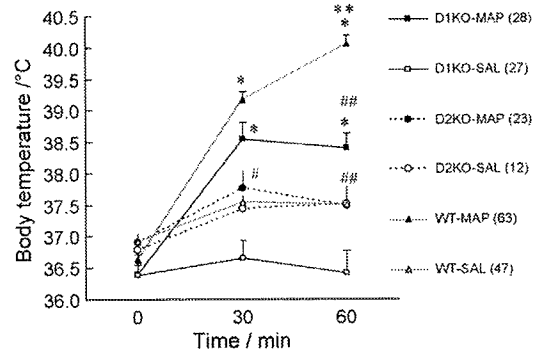

Fig. 1. Effect of a single dose of methamphetamine (MAP) at 30 mg/kg on temperature in mice. MAP extensively increased temperature through three-time points in wild-type mice (*$p < 0.001$ compared to saline-treated wild-type mice, **$p < 0.001$ compared to MAP-treated wild-type mice at 30 min). MAP increased temperature in D1KO mice (*$p < 0.001$ compared to saline-treated D1KO mice), although it leveled off after 30 min. There was no significant difference between saline and MAP treatments in D2KO mice. At 30 min, the temperature of MAP-treated D2KO mice was significantly lower than that of MAP-treated wild-type mice (#$p < 0.001$). At 60 min, temperature was significantly lower in both D1KO and D2KO mice treated with MAP than in wild-type mice (##$p < 0.001$). Figures in parentheses represent numbers of mice. Values represent the mean ± S.E.M.

Core body temperature was determined using an electronic thermometer (BAT-10, Physitemp Instruments, New Jersey, USA) with a rectal probe (RET-3, Physitemp Instruments) three times just before (0 min) and at 30 and 60 min after drug administration. We chose 60 min time period based on our preliminary experiments (unpublished) and previous report [18]. To evaluate lethality, we counted the number of mice that died within 24 h after methamphetamine treatment. The data on temperature from mice which died within 60 min were excluded.

The data represent means ± S.E.M. Statistical analyses were performed using a one-way analysis of variance (ANOVA) for rectal temperature. For post hoc comparisons, Tukey's HSD test was used for comparison of methamphetamine-treated with saline-treated controls of same genotype, comparison of time condition in same treatment and genotype, and comparison among genotypes. Fisher's exact test was applied for lethality. The criterion of statistical significance was set at $p < 0.05$.

At 30 mg/kg, methamphetamine was significantly less lethal in D1KO mice (7%) and D2KO mice (4%) than in the wild-type mice (27%). Two wild-type mice died within 30 min followed by six wild-type mice within 60 min, and 11 wild-type mice within 24 h. On the other hand, one D1KO mouse died within 30 min, one D1KO mouse died within 60 min, and one D2KO mouse died within 24 h. Means of highest temperatures of the dead mice were higher than those of the surviving mice (Table 1).

Body temperature in wild-type mice hit 39.2 °C at 30 min and kept rising and exceeded 40.1 °C at 60 min after methamphetamine's administration. Both temperatures are significantly higher than that of saline-treated wild-type mice ($p < 0.001$), and the temperature at 60 min was higher than that of at 30 min ($p < 0.001$). Body temperature in D1KO mice also rose to 38.5 °C at 30 min by methamphetamine, although it leveled off after 30 min and ended with 38.4 °C at 60 min. While in D2KO mice, there was no significant increase of body temperature induced by methamphetamine and the temperatures at 30 min (37.8 °C) and 60 min (37.5 °C) were significantly lower than that of wild-type mice (Fig. 1). Although D1KO mice showed methamphetamine-induced rise in body temperature, its absolute temperature was not significantly different from that of D2KO at any time point, and body temperatures at 60 min was significantly lower than that of wild-type mice ($p < 0.001$). Overall, methamphetamine-induced net increase of body temperature was highest in wild-type mice (3.5 °C), followed by D1KO mice (2.1 °C).

The body temperature of the wild-type mice which died from methamphetamine treatment was significantly higher than that of the survivors (Table 1). The body temperature of the D1KO or D2KO mice which died was also higher than that of surviving mice. The difference in body temperature between the dead and surviving D1KO or D2KO mice did not reach a level of statistical significance however, the number of dead mice being too small.

The present results indicate that these subtypes of dopamine receptors work similarly in lethal effects and differently in the hyperthermic effects of methamphetamine. Our results showed that methamphetamine exerts a lethal effect via D1 and D2 receptors, because deletion of these receptors in mice significantly decreased the toxicity of methamphetamine within 24 h after the drug treatment. A previous paper stated that the activation of the D1 receptor is an important event in the lethality caused by methamphetamine [5]. Our study suggested the importance of the D2 receptor, along with the D1 receptor, in the lethal toxicity of methamphetamine.

The absence of methamphetamine-induced hyperthermia in our study with D2KO mice may suggest that the D2 receptor plays a major role in inhibiting methamphetamine-induced hyperthermia. The lower basal body temperature of D1KO mice seemed to attenuate the hyperthermic effect induced by methamphetamine, although there was a notable difference in the net increase in body temperature between the wild-type and D1KO mice. Moreover, the result that D1KO mice did not continuously rise its temperature and settled at lower temperature than wild-type mice implies that D1 receptor takes roles in causing latter and serious hyperthermia by methamphetamine differently from D2 receptor. There are also possibilities that levels of D2 or D1 receptor expression or function, especially in hypothalamus, might be affected in D1KO mice or D2KO mice, respectively.

The body temperature of the wild-type mice which died following methamphetamine treatment was significantly higher than that of the mice which survived in our study. The body temperature of the average of the two died D1KO mice or the one died D2KO mouse was also higher than that of the mice which survived in respective genotypes. Note that the number of dead mice was too small to apply statistic analysis. The body temperature of rats treated with methamphetamine exceeded 41 °C [2], whereas animals placed on ice survived from hyperthermia caused by methamphetamine or the coadministration of methamphetamine and morphine [3,13,14]. The lethal effect of methamphetamine could be prevented by controlling body temperature.

In summary, the results of the present study suggest both D1 receptor and D2 receptor take roles in the lethal effect of metham-

phetamine. These two subtypes work differently in hyperthermic effects of methamphetamine. Since D2KO mice completely eliminated methamphetamine-induced hyperthermia, D2 receptor may take acute and major roles in methamphetamine-induced hyperthermia. While D1KO mice did not let the temperature hit the serious level, D1 receptor may take roles in later stage in 60 min and crucial hyperthermia. Elucidating the roles of these receptors in the toxicity of methamphetamine should help in the development of novel medications and treatments.

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シンポジウム

薬物依存とメチルフェニデート

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はじめに

本邦では昨年末，AD/HD治療薬物として精神刺激薬であるメチルフェニデートの徐放剤（商品名：コンサータ®）が認可され，適正流通管理委員会の登録の下での医師・管理薬剤師のみに取り扱いが限定されることとなった．従来，ナルコレプシーとうつ病治療への適応が認められていたリタリン®は，その乱用・依存が社会的な問題となったため，うつ病への適応が取り消され，現在はナルコレプシーにのみ適応が認められている．

本稿ではまず，薬物依存の病態について依存性薬物が脳内報酬系に働き，ドーパミン神経伝達を変化させることを中心に述べる．次に，メチルフェニデートの標的分子であるモノアミン輸送体（トランスポーター）への作用機序について述べる．さらに，精神刺激薬の反復投与による行動感作，AD/HD患者におけるメチルフェニデートの逆説的治療効果に関連する動物モデルについて紹介する．また，AD/HD患者の薬物依存のリスクと，精神刺激薬を用いて治療を行う際の依存リスクについても論じたい．

本題に入る前に留意すべき点として，メチルフェニデートの依存リスクを論ずる際に，健常者を含むAD/HD患者以外の人がメチルフェニデートを乱用あるいは服用する場合と，AD/HD患者にメチルフェニデートが治療薬として用いられる場合の依存リスクを同列に論じることは適切ではないことを強調しておきたい．

1. 依存性薬物と脳内報酬系

食物摂取などの生命維持に必要な活動が快情動を伴うように，脳は快情動を報酬として，その行動を何度も繰り返すように働きかける仕組みを有する．依存性薬物は，生理的な報酬と同じ神経回路に作用し薬物を摂取する行動を引き起こすが，食物の摂取など日常の行動によって得られる生理的な効果よりもはるかに強力な報酬をもたらす．この強力な報酬効果を求めて，依存薬物を摂取するよう強く駆り立てる「渇望」が現れ，依存性薬物の「探索行動」を伴うようになる．

薬物依存は再発しやすい慢性疾患であり，(1)精神刺激薬や麻薬などの依存性薬物がもたらす快情動，つまり「報酬効果」を得るために連続的，あるいは周期的に摂取してしまう強迫的な状態，(2)薬物摂取を制限することが困難な状態，(3)摂取が制限されると不安や焦燥などの不快感が出現する．依存を形成する薬物群は「依存性薬物」と呼ばれ，その再体験を求めるようになる強化効果を示す[28)]．条件付けの強さ，反応の確率を増強する性質を持つ刺激や物質は「強化因子」と呼ばれ，依存性薬物は薬物摂取を増強する正の強化因子として働いていると言える[10)]．

依存性薬物は，摂取時の自覚効果から，刺激・覚醒効果が強い興奮系と鎮静・酩酊効果が強い抑制系に大別される．しかし，興奮系と抑制系は自覚効果が異なるにもかかわらず，ともに強い精神依存を形成する．また，抑制系は精神依存に加え

て身体依存も形成する．依存性薬物の中でメタンフェタミンやメチルフェニデートは，主に中枢神経系を興奮させることから精神刺激薬と呼ばれる．一方で，オピオイド類の麻薬であるヘロイン（ジアセチルモルヒネ）は主に抑制効果を示す[26]．

精神刺激薬やオピオイドなどの依存性薬物は，極めて強い快情動を起こし強い報酬効果を持つ．依存性薬物により引き起こされる報酬効果は，可塑的に脳内神経伝達を大きく変化させる．この報酬効果に関与する神経伝達系として，ドーパミン系が大きな役割を担うと考えられてきた[13,23,25]．ドーパミン系の中でも特に，脳幹の腹側被蓋野（ventral tegmental area, VTA）から側坐核（nucleus accumbens, NAc）に投射する中脳辺縁系ドーパミン経路の破壊は，精神刺激薬の報酬を著しく減少させたことから，依存形成に重要な役割を果たしていると考えられる[17]．

2．メチルフェニデートの標的分子としてのモノアミン輸送体

モノアミン輸送体（トランスポーター）は，神経終末の細胞膜に存在しNa^+/Cl^-依存性にモノアミンを神経終末内に取り込む膜蛋白質であり，アミノ酸トランスポーターなどとともにSLC 6（solute carrier 6）と呼ばれる遺伝子ファミリーを形成している[8]．神経終末から放出されたドーパミン（DA），ノルエピネフリン（NE），セロトニン（5-HT）は，細胞膜モノアミントランスポーターにより神経終末に再取り込みされ，神経伝達が終了する．細胞膜モノアミントランスポーターは，DA，NE，5-HTそれぞれの基質ごとに，対応する基質の作動性ニューロンの前シナプス神経終末の細胞膜に主に発現している[24]．

メチルフェニデートやコカインは細胞膜モノアミントランスポーターに結合し，再取り込みを阻害，シナプス間隙に放出されたモノアミンの濃度を増加させることにより効果を示す．コカインの類似化合物質の場合，モノアミントランスポーターの一つであるドーパミントランスポーター（DAT）への結合親和性が強いほど，報酬効果もより強いことが知られている[12]．一方，抗うつ剤の多くも細胞膜モノアミントランスポーターの阻害効果を持つが，主にノルエピネフリントランスポーター（NET），あるいはセロトニントランスポーター（SERT）に結合し，ドーパミントランスポーターへの結合親和性は極めて低い点が特徴であり，報酬効果を与えることはないものと考えられる．

メチルフェニデートは，コカインと同様にDATに対する阻害効果を有するため，報酬効果を持つ依存性薬物となりうる．コカインは非特異的なモノアミントランスポーターの阻害剤であり，DATに加えSERTとNETへの阻害効果を有する．一方，メチルフェニデートはDAT，NETへの阻害効果を有するが，SERTに対する親和性はほとんど持たない．

3．精神刺激薬と行動感作

メタンフェタミンやコカインなどの精神刺激薬を実験動物に反復投与すると，異常行動，多動が進行性に増大する現象が出現し，この薬物反応の増強を行動感作（逆耐性現象）と呼んでいる[22]．行動感作は，一旦形成されると長期の断薬期間を経た後にも，薬物再投与で容易に再現されることから，神経の可塑的変化が大きな因子であると示唆されている．依存性薬物は，直接，または間接的にドーパミン神経伝達を増強させることにより報酬効果を示すことから，薬物依存が形成される条件としてドーパミン神経伝達の増強が必要であると言える．しかし，依存が形成された後には，ドーパミン系を制御する神経伝達が可塑的に変化していると考えられる．つまり，薬物依存の初期と，強化・維持期では，その神経機序は異なると予想される．中脳辺縁系ドーパミン経路の活性化は依存の初期に重要であり，強化・維持期にはドーパミン経路に加え，前頭前野，扁桃核，海馬から側坐核へのグルタミン酸経路の活性化が重要であると考えられている．

実際に，メチルフェニデートを反復投与した実験動物で行動感作の形成が確認された報告があ

る[5,7,11]．しかし，メチルフェニデートによる治療を長期間受けているAD/HD患者に，必ずしも動物実験で観察された行動感作が形成されない理由として，小児期での投与が多いことが挙げられる．小児期は，思春期以降とドーパミン神経伝達の機序が異なるため，メチルフェニデートへの反応性も小児期と思春期以降とでは異なるものと考えられる．

4. AD/HD患者におけるメチルフェニデートの逆説的治療効果

DAT欠損マウスは，新しい環境に置かれた際，野生型マウスと比較して極めて高い移所運動量を示す[16]．野生型マウスにメチルフェニデートを投与すると，活動量が顕著に増加するが，DAT欠損マウスにメチルフェニデートを投与すると活動量が劇的に減少する．これは，健常者への覚せい剤の投与が興奮や過活動を引き起こすにもかかわらず，AD/HD患者へは治療効果をもたらすことと一致している．これらの類似性から，DAT欠損マウスはAD/HDの動物モデルの一つと考えられている[18,27]．

脳内微少透析法を用いてDAT欠損マウスの細胞外DA量を測定すると，大脳基底核の細胞外DA量は野生型の約10倍に増加していたが，前頭前野皮質では野生型と同程度のDA濃度を示した[15]．また，野生型マウスではメチルフェニデート投与後，線条体で細胞外DA量が顕著に増加していたが，DAT欠損マウスでは変化は見られなかった．これに対して，前頭前野皮質では，野生型マウス，DAT欠損マウスともにメチルフェニデートによる細胞外DA量の顕著な上昇が確認された．この差異は，大脳基底核と前頭前野皮質でDA神経の制御機構が異なることに起因すると考えられる．大脳基低核へ投射している黒質から線条体を含むDA神経線維には，DATが多数存在するため，線条体でのDAの再取り込みはDATによってのみ行われている．一方，前頭前野皮質のDA神経終末上には，DATが少ないために[14]，DAの再取り込みの役割をNETが代わりに果たしていると考えられている[3,4]．

メチルフェニデートは，非特異的なモノアミントランスポーター阻害剤である．DAT欠損マウスには，SERTとNETが存在する．しかし，メチルフェニデートのSERTに対する親和性が低いことから[6]，メチルフェニデートは前頭前野皮質のNETに作用し，NETによるNE，およびDAの再取り込みを阻害するため，NEとともにDA濃度が上昇すると考えられる．AD/HDにおいては，辺縁系でDA神経伝達が亢進しているのに対し，前頭前野皮質ではむしろ低下しているという仮説もあることから，筆者らはこの前頭前野皮質におけるDA濃度の上昇が，メチルフェニデートのDAT欠損マウスの運動量低下作用に関与しているのではないかと考えている．

5. AD/HD患者における薬物依存のリスク

思春期以降，AD/HDと薬物依存の併発は30％以上の高率と報告されている[9,19]．さらに思春期以降のAD/HD患者群では，加齢に伴い乱用の発症リスクが健常者よりも高くなり，薬物乱用から依存へ移行する割合も高いことが確認されている[2]．

精神刺激薬の投与がAD/HD患者の予後に与える影響を追跡調査したところ，薬物乱用/依存の発症頻度は，未治療群75％，治療群25％，対照群18％と，未治療群の乱用/依存発症頻度が有意に高く，小児期に精神刺激薬治療を行った場合は治療を行わなかった場合よりも，将来の依存発症のリスクが軽減されるという結果が報告された[1]．さらに，小児のAD/HD患者に対する精神刺激薬治療の有無により，将来の依存リスクを検討した報告をメタ解析した研究では，小児期に精神刺激薬治療を行った場合，治療を行わなかった場合と比べ，将来の依存発症リスクが半減する結果が示された[21]．

未治療のAD/HD患者の薬物乱用・依存の発症リスクが高い理由の一つとして，AD/HD症状による機能不全を補う目的で精神刺激薬などを自己投与している可能性がある．これは，思春期前

の精神刺激薬治療が，AD/HD患者の思春期以降の薬物依存リスクを軽減する効果があるとする報告と一致する．思春期に達したAD/HD患者に対し，精神刺激薬をいつまで投与すべきかについて，未だガイドラインが示されていない[20]．そのため，思春期後の精神刺激薬治療には，薬物依存のリスクを伴うことを踏まえる必要がある．思春期以降のAD/HD患者にメチルフェニデートの投与による快情動が出現する場合には，乱用・依存の発症のサインの一つと認識される．さらに思春期以降には，統合失調症や気分障害を始めとする様々な精神疾患の発症リスクも高まることから，それらの精神疾患の兆候が現れた際にも，メチルフェニデートの使用を控え，別の治療法を検討する必要があると言える．

ま と め

メチルフェニデートは，コカインやメタンフェタミンと薬理学的に類似した精神刺激薬であり，健常者や野生型実験動物への投与は報酬効果をもたらし，乱用・依存を引き起こす．報酬効果に大きく寄与し，報酬系と呼ばれる脳幹の腹側被蓋野から側坐核に投射する中脳辺縁系DA経路が薬物依存の病態理解に重要であり，メチルフェニデートは他の精神刺激薬と同様にモノアミントランスポーター，ことにDATを標的分子とする．

DAT欠損マウスは，精神刺激薬によってその多動が抑制されることから，AD/HDの動物モデルの一つと考えられる．メチルフェニデートは，NETを介してDAT密度の少ない前頭前野皮質のDA神経伝達を亢進することで，AD/HDへの治療効果をもたらしていると考えられる．

健常者には興奮，快情動をもたらすメチルフェニデートだが，AD/HD患者の多動には逆説的に治療効果を示す．小児期に精神刺激薬の治療を行う場合，治療を行わない場合よりも将来の依存のリスクが軽減されると報告されている．しかし，健常者に比べてAD/HD患者の薬物乱用/依存の発症頻度は高いことから，思春期以降のAD/HD患者へのメチルフェニデートの投与に際して，快情動などの報酬効果を示唆する症状を伴う場合には慎重な対応が必要と考えられる．

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分担研究報告書

覚せい剤精神障害に対する治療法の開発

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[研究要旨]
これまで我々は、第二世代抗生物質ミノサイクリンが覚せい剤の投与によって引き起こされる様々な障害に対して治療効果を示すことを報告した。今回我々は覚せい剤投与による薬物依存形成におけるミノサイクリンの効果を検討した。場所嗜好性試験より、覚せい剤投与による場所嗜好性の増加が観察され、ミノサイクリンの前投与は、覚せい剤投与による場所嗜好性の増加を有意に抑制した。一方、ミノサイクリン単独投与は、生理食塩水投与群と同等であった。本研究の結果より、ミノサイクリンは覚せい剤依存の治療薬となる可能性が示唆された。

A. 研究目的

覚せい剤(Methamphetamine: METH)の乱用により依存が形成されるが、現在のところ、根本的に治療する薬剤は無い。これまで私たちは、第二世代抗生物質の一つであるミノサイクリンが覚せい剤や合成麻薬 MDMA などの投与によって引き起こされる様々な障害に対して治療効果を示すことを報告した（1-6)。今回我々は METH 投与による薬物依存におけるミノサイクリンの効果を検討した。

B. 研究方法

1．実験動物

実験には9～12 週齢の C57BL/6J 雄性マウス（日本エスエルシー株式会社）を使用した。場所嗜好性試験（CPP）の測定は、Saline (10 ml/kg)もしくはミノサイクリン(40 mg/kg)を投与し 30 分後に METH (1 mg/kg)を投与し、その後、白または黒のボックスに 30 分間閉じ込め、条件付けを行った。薬物処置側ボックスの滞在時間から溶媒処置側ボックスの滞在時間を差し引いた値を CPP スコアとした。

C. 研究結果

場所嗜好性試験（CPP）より、METH 投与による場所嗜好性の増加が観察され、ミノサイクリン（40 mg/kg）の前投与は、METH 投与による場所嗜好性の増加を有意に抑制した。一方、ミノサイクリン単独投与は、生理食塩水投与群と同等であった。

D. 考察

本研究の結果より、ミノサイクリンは覚せい剤乱用によって引き起こされる覚せい剤依存の治療薬となる可能性が示唆された。

E. 結論

本研究の結果より、ミノサイクリンは覚せい剤依存の治療薬となる可能性が示唆された。

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G. 知的財産権の出願・登録状況（予定も含む）

１．特許取得

なし

２．実用新案登録

なし

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Association Study Between Polymorphisms in Glutathione-Related Genes and Methamphetamine Use Disorder in a Japanese Population

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Accumulating evidence suggests that oxidative stress plays a role in the mechanisms of action of methamphetamine (METH) in the brain. In the present study, we investigated the association between the genetic polymorphisms among glutathione (GSH)-related enzymes; glutathione *S*-transferases (GSTs) such as GSTT1 (Non-deletion/Null), GSTT2 (Met139Ile), GSTA1 (−69C/T), and GSTO1 (Ala140Asp); glutathione peroxidase 1 (GPX1) (Pro198Leu); and glutamate-cysteine ligase modifier (GCLM) subunit and METH use disorder in a Japanese population. Two hundred eighteen METH abusers and 233 healthy controls were enrolled in the study. There was a significant difference in GSTT1 genotype frequency between patients with METH psychosis and controls ($P = 0.039$, odds ratio: 1.52, 95% CI 1.03–2.24). Furthermore, the frequency (66.0%) of the GSTT1 null genotype among prolonged-type METH psychotic patients with spontaneous relapse was significantly higher ($P = 0.025$, odds ratio: 2.43, 95% CI 1.13–5.23) than that (44.4%) of transient-type METH psychotic patients without spontaneous relapse. However, there were no associations between the polymorphisms of other genes and METH abuse. The present study suggests that the polymorphism of the GSTT1 gene might be a genetic risk factor of the development of METH psychosis in a Japanese population. © 2008 Wiley-Liss, Inc.

KEY WORDS: methamphetamine; psychosis; drug abuse; oxidative stress; polymorphism

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INTRODUCTION

Methamphetamine (METH) is one of the most widely used illicit drugs, and its abuse continues to be a growing problem worldwide. Accumulating evidence has suggested that genetic factors play a role in vulnerability to METH abuse and in the psychiatric symptoms related to METH abuse [Kosten et al., 1998; Merikangas et al., 1998; Kendler et al., 2000; Uhl et al., 2002; Goldman et al., 2005; Hashimoto, 2007; Matsuzawa et al., 2007].

Several lines of evidence suggest that oxidative stress may be involved in the METH-induced neuronal damage in the brain, and that antioxidants including glutathione (GSH) and *N*-acetyl-L-cysteine could be potential therapeutic drugs for METH psychosis and addiction [Cubells et al., 1994; Choi et al., 2002; Cadet et al., 2003; Fukami et al., 2004; Hashimoto et al., 2004; Miyazaki et al., 2006; Achat-Mendes et al., 2007;

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