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A Receptor Mechanism for Methamphetamine Action in Dopamine Transporter Regulation in Brain[S]

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ABSTRACT

This study reveals a novel receptor mechanism for methamphetamine action in dopamine transporter (DAT) regulation. Trace amine-associated receptor 1 (TAAR1) is expressed in brain dopaminergic nuclei and is activated by methamphetamine in vitro. Here, we show that methamphetamine interaction with TAAR1 inhibits [^{3}H]dopamine uptake, enhances or induces [^{3}H]dopamine efflux, and triggers DAT internalization. In time course assays in which methamphetamine and [^{3}H]dopamine were concurrently loaded into cells or synaptosomes or in pretreatment assays in which methamphetamine was washed away before [^{3}H]dopamine loading, methamphetamine caused a distinct inhibition in [^{3}H]dopamine uptake in TAAR1 + DAT-cotransfected cells and in wild-type mouse and rhesus monkey striatal synaptosomes. This distinct uptake inhibition was not observed in DAT-only transfected cells or in TAAR1 knockout mouse striatal synaptosomes. In [^{3}H]dopamine efflux assays using the same cell and synaptosome preparations, methamphetamine enhanced [^{3}H]dopamine efflux at a high loading concentration of [^{3}H]dopamine (1 μM) or induced [^{3}H]dopamine efflux at a low loading concentration of [^{3}H]dopamine (10 nM) in a TAAR1-dependent manner. In DAT biotinylation assays using the same cell and synaptosome preparations, we observed that 1 μM methamphetamine induced DAT internalization in a TAAR1-dependent manner. All these TAAR1-mediated effects of methamphetamine were blocked by the protein kinase inhibitors H89 [*N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline] and/or 2-{8-[(dimethylamino) methyl]-6,7,8,9-tetrahydropyrido[1,2-*a*]indol-3-yl}-3-(1-methylindol-3-yl)maleimide (Ro32-0432), suggesting that methamphetamine interaction with TAAR1 triggers cellular phosphorylation cascades and leads to the observed effects of methamphetamine on DAT. These findings demonstrate a mediatory role of TAAR1 in methamphetamine action in DAT regulation and implicate this receptor as a potential target of therapeutics drugs for methamphetamine addiction.

Methamphetamine is a widely abused and highly addictive psychostimulant and neurotoxic drug that dramatically interferes with cognition (memory and attention) and emotion (euphoria, surge in productivity, increase in self-esteem and impulsion) (Hart et al., 2001; Cretzmeyer et al., 2003). In the brain, a primary action of methamphetamine is to elevate the levels of extracellular monoamine neurotransmitters, especially dopamine, via interfering with their reuptake and promoting their release at the nerve endings (Rothman and Baumann, 2003; Sulzer et al., 2005; Fleckenstein et al., 2007), which is thought to be the major mechanism by which methamphetamine exerts its psychostimulant effects. Monoamine transporters, especially the dopamine transporter, are biological targets and conduits by which methamphetamine causes effects on transport of the monoamine neurotransmitters (Fischer and Cho, 1979; Sulzer et al., 1995; Sandoval et al., 2001). In addition to its ability to compete with neurotransmitters for reuptake at the transport sites (Rothman and Baumann, 2003; Han and Gu, 2006), methamphetamine also causes modulatory effects on the monoamine transporters, including phosphorylation, down-regulation of the transporters, and transport reversal (Cervinski et al., 2005; Johnson et al., 2005). However, the mechanisms by which methamphetamine triggers these effects are largely unknown.

It has been reported that TAAR1 is expressed in brain monoaminergic nuclei (Borowsky et al., 2001; Xie et al., 2007b; Lindemann et al., 2008) and colocalized with the dopamine transporter in a subset of dopamine neurons in the

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ABBREVIATIONS: TAAR1, trace amine-associated receptor 1; DAT, dopamine transporter; DMEM, Dulbecco's modified Eagle's medium; CRE, cAMP-response element; Luc, luciferase; PLB, passive lysis buffer; PK, protein kinase; PAGE, polyacrylamide gel electrophoresis; ANOVA, analysis of variance; HEK, human embryonic kidney; MPH, methylphenidate; Ro32-0432, 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-*a*]indol-3-yl}-3-(1-methylindol-3-yl)maleimide; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide.

rhesus monkey and mouse substantia nigra (Xie et al., 2007b). TAAR1 is activated by a spectrum of ligands (Bunzow et al., 2001; Hart et al., 2006; Liberles and Buck, 2006; Wainscott et al., 2007; Xie et al., 2007b), and methamphetamine is a potent agonist at this receptor in vitro (Bunzow et al., 2001; Reese et al., 2007; Xie and Miller, 2007; Xie et al., 2007b). Our laboratory previously reported that rhesus monkey TAAR1 is a modulator of the dopamine transporter in vitro (Xie and Miller, 2007), and others have shown that mouse TAAR1 modulates dopaminergic activity in the brain (Lindemann et al., 2008; Wolinsky et al., 2007). More recently, we further demonstrated that the trace amine β-phenylethylamine interacts with TAAR1 to alter monoamine transporter function and that TAAR1 signaling in response to the common biogenic amines integrates with activity of the monoamine autoreceptors to regulate transporter kinetics in brain synaptosomes (Xie and Miller, 2008; Xie et al., 2008b). These findings suggest that TAAR1 is a presynaptic monoaminergic modulator in the brain and led us to hypothesize that methamphetamine may cause regulatory effects on the dopamine transporter via its direct interaction with TAAR1.

In this study, we used transfected cells and rhesus monkey, wild-type (C57BL/6J), and TAAR1 knockout mouse striatal synaptosomes to investigate whether methamphetamine targets TAAR1 to regulate dopamine transporter function and trafficking in the brain. Our studies demonstrate that methamphetamine interacts with TAAR1 to inhibit dopamine uptake, induce dopamine efflux, and evoke dopamine transporter internalization in rhesus monkey and mouse brain striatal synaptosomes and in transfected cells. Furthermore, these TAAR1-mediated effects are linked to activation of protein kinases, suggesting that methamphetamine interaction with TAAR1 is a trigger for cellular phosphorylation cascades that lead to the observed effects of methamphetamine in DAT regulation. These findings reveal a novel receptor mechanism for methamphetamine action and may provide a new therapeutic target for methamphetamine addiction.

Materials and Methods

Materials. Dopamine, (+)-methamphetamine, methylphenidate, raclopride, forskolin, H89, Ro32-0432, and Protein Inhibitor Cocktail were purchased from Sigma-Aldrich (St. Louis, MO). All reagents for cell culture were obtained from Invitrogen (Carlsbad, CA). [^{3}H]Dopamine (60 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). ReadySafe cocktail was obtained from Beckman Coulter (Fullerton, CA). Trace amine-associated receptor 1 antibody (against the intracellular loop 3) and Rabbit Anti-Human Dopamine Transporter (against extracellular loop 2) antibody were purchased from Affinity Bioreagents (Golden, CO). Goat anti-rabbit IgG (H+L) was from Millipore Biosciences Research Reagents (Temecula, CA). A Cell Surface Protein Isolation Kit and a SuperSignal West Pico Chemiluminescent Substrate were obtained from Pierce Chemical (Rockford, IL).

Cell Culture and Transfection. Cells were grown in DMEM supplemented with fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and nonessential amino acids (0.1 mM) at 5% CO_2 and 37°C, and G-418 (Geneticin) was used for selection or maintenance of selection of stable cell lines. Calcium phosphate transfection was performed as described elsewhere (Xie et al., 2005, 2007a) to introduce the receptor and/or transporter into different cells for assays. For cotransfection, the ratio of constructs and the amount of total DNA were held constant with pcDNA3.1.

Animals and Synaptosome Preparation. All procedures with animals and animal tissues were conducted in accordance with the Animal Experimentation Protocol approved by the Harvard Medical Area Standing Committee on Animals. Monkey brains were obtained from juvenile rhesus (*Macaca mulatta*) monkeys that were sacrificed for other purposes, and the striatum (only putamen) was collected. TAAR1 knockout and wild-type mouse colonies were established at the New England Primate Research Center from six pairs of heterozygous mice given as a gift by Lundbeck Research USA, Inc. (Paramus, NJ). The mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation at 8 to 10 weeks of age, and the striatum was rapidly collected. Neuronal synaptosomes were prepared as described elsewhere (Xie and Miller, 2008; Xie et al., 2008b). In brief, striatal tissues were homogenized in 1.5-ml Eppendorf centrifuge tubes with a 10× volume of ice-cold unbuffered 0.32 M sucrose solution, pH 7.0. The homogenate was centrifuged (1000*g*, 10 min at 4°C) to yield a crude nuclear pellet and low-speed supernatant. The low-speed supernatant fraction was carefully transferred into another fresh tube and centrifuged at 10,000*g* and 4°C for 20 min to yield a synaptosome-containing pellet. This pellet was resuspended in an appropriate volume of ice-cold uptake buffer (a modified Krebs' buffer: 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 μM pargyline, 2 mg/ml glucose, 0.2 mg/ml ascorbic acid, pH 7.5) for further assays.

Dual Luciferase Reporter Assay. Cells were placed in 48-well plates (75,000 cells/well in 0.5 ml of medium). At 60 to 70% cell confluence, a luciferase reporter construct CRE-Luc (cAMP-sensitive) (Miller et al., 2005; Xie et al., 2007b) and a reporter control construct pGL4.73 (cAMP-irresponsive) were introduced into the cells along with the target receptor or transporter. After 12-h incubation under transfection condition, the cells were exposed to vehicle or 1 μM methamphetamine in serum-free DMEM for 18 h. Passive lysis buffer (PLB) and luciferase assay substrate reagents were prepared according to the manufacturer's protocol. Cell lysates were prepared by adding 100 μl of 1× PLB into each well to break the cells followed by incubation on a shaking platform at 25°C for 30 min. The lysate (20 μl) from each well was transferred into wells of an opaque 96-well microplate (PerkinElmer Life and Analytical Sciences). Luciferase substrate reagents (50 μl) were injected into each well, and after a 2-s delay, luciferase levels were measured as relative light units for 12 s on a Wallac 1420 multilabel counter, Victor 3V (PerkinElmer Life and Analytical Sciences).

[^{3}H]Dopamine Uptake Assay. Experiments were conducted as described elsewhere (Xie and Miller, 2008; Xie et al., 2008b). In brief, cells were suspended in serum-free DMEM at 5×10^6 cells/ml, and synaptosomes were prepared in uptake buffer at 1:200 (1 mg of striatal tissue/200 μl of buffer). For concurrent loading assays, cells and synaptosomes were loaded with 10 nM [^{3}H]dopamine alone or 10 nM [^{3}H]dopamine plus 100 nM methamphetamine for 1, 2, 3, 4, 5, 10, 20, or 30 min at 25°C. For pretreatment assays, cells and synaptosomes were pretreated with vehicle or methamphetamine for 10 min at 25°C, washed twice, and then loaded with 10 nM [^{3}H]dopamine for 5 min at 25°C. To block activation of protein kinases, the PKA inhibitor H89 (10 μM) or the PKC inhibitor Ro32-0432 (10 μM) was added into the reaction systems during drug treatment. Nonspecific uptake was defined in the presence of 10 μM methylphenidate. After [^{3}H]dopamine loading, cells or synaptosomes were washed twice and then lysed with 1× PLB buffer. The lysates were counted for disintegrations per minute values on a Beckman LS6000IC scintillation spectrophotometer. The [^{3}H]dopamine uptake at 30 min in the cells and synaptosomes exposed to 10 nM [^{3}H]dopamine alone was taken as maximal uptake (100%) in concurrent loading assays, and the [^{3}H]dopamine uptake in cells pretreated with serum-free DMEM or in synaptosomes pretreated with uptake buffer was taken as baseline (100%) in the pretreatment assays.

[^{3}H]Dopamine Efflux Assay. Experiments were conducted in 48-well plates for cells or in 1.5-ml Eppendorf centrifuge tubes for synaptosomes as described elsewhere (Xie and Miller, 2008; Xie et

al., 2008b). In brief, cells and synaptosomes were preloaded with [^{3}H]dopamine in serum-free DMEM for cells and in uptake buffer for synaptosomes at 25°C for 20 min, washed twice, and then exposed to vehicle or methamphetamine in serum-free DMEM for cells or in efflux buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 μM pargyline, 2 mg/ml glucose, 0.2 mg/ml ascorbic acid, pH 7.5) for synaptosomes at 25°C for 30 min. Nonspecific uptake was defined in the presence of 10 μM methylphenidate during [^{3}H]dopamine loading. For blockade of protein kinase activation, 10 μM H89 or 10 μM Ro32-0432 was added into the reaction systems during methamphetamine treatment. After methamphetamine treatment, cells or synaptosomes were washed twice and then lysed with 1× PLB buffer. The lysates were counted for disintegrations per minute values on a Beckman LS6000IC scintillation spectrophotometer. The retention of [^{3}H]dopamine in cells or synaptosomes treated with efflux buffer after [^{3}H]dopamine preloading was taken as baseline (100% retention).

Dopamine Transporter Trafficking. Biotinylation assays were used to determine whether TAAR1 signaling in response to methamphetamine alters the cell surface level of the dopamine transporter. Procedures were performed according to the manufacturer's protocol and described elsewhere (Bolan et al., 2007; Xie et al., 2008a). In brief, cells (1×10^7) or brain synaptosomes (generated from 10 mg of striatal tissue) were treated with drugs for different periods as indicated, washed twice with ice-cold PBS at the end of the treatment, and then incubated with 5 ml of biotin solution (sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate dissolved in ice-cold PBS) for 30 min at 4°C on a rocking platform. The reaction was quenched by the addition of 250 μl of quenching solution (provided). Then, the cells or synaptosomes were harvested by centrifugation at 1000*g* (for cells) or at 10,000*g* (for synaptosomes) for 3 min at 4°C, and the pellets were lysed in 250 μl of lysis buffer (provided) and homogenized with a motor-driven pellet pestle for 30 up-and-down strokes. The homogenate was incubated on ice for 30 min and then centrifuged at 10,000*g* for 2 min at 4°C to obtain the supernatant. The supernatant was incubated with Immobilized NeutrAvidin Gel, which was loaded into the column (provided), for 60 min at room temperature and then centrifuged at 1000*g* for 1 min at room temperature. The flow-through was collected for detection of unbiotinylated dopamine transporter levels. The gel-trapped proteins were rinsed twice with 500 μl of wash buffer (provided), which was supplemented with protease inhibitors and eluted by incubation with 250 μl of dithiothreitol-containing Laemmli sample buffer for 60 min at room temperature and centrifuged at 1000*g* for 2 min. The biotinylated proteins isolated by NeutrAvidin Gel and the unbiotinylated proteins in the flow-through were subjected to SDS-PAGE/Western blotting. The gray values of the blotting bands for the dopamine transporter were normalized to the level in untreated cells or synaptosomes.

SDS-PAGE and Western Blotting. Detailed procedures are described elsewhere (Xie et al., 2007b, 2008a). In brief, the prepared proteins were subjected to SDS-PAGE (10% acrylamide separating gel, 4% acrylamide stacking gel) and then electrotranslocated onto a polyvinylidene difluoride membrane (0.45 μm) presoaked in 100% methanol for 10 min. The membrane was then blocked with blocking buffer (10% nonfat milk, 10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) and incubated with the primary antibody at 1:1000 overnight at 4°C and secondary antibody (goat anti-rabbit IgG) at 1:5000 for 2 h at room temperature in blocking buffer. SuperSignal West Pico Chemiluminescent Substrate was used to visualize the blots under a luminescent image analyzer (LAS-1000; Fujifilm, Tokyo, Japan).

Data Analysis. [^{3}H]Dopamine uptake and efflux in cells and synaptosomes vary in radiation counting between cells and synaptosome preparations from different sources and, accordingly, are normalized to the maximum level or the baseline and expressed as percentage values in each set of assays. Results were finalized as mean ± S.E.M. of the indicated number of observations. Student's *t* test, one-way ANOVA/Turkey post hoc tests, or two-way ANOVA/Bonferroni post hoc tests were used for statistical analysis, and the results were considered significantly different at $p < 0.05$.

Results

Methamphetamine Induces CRE-Luc Expression via TAAR1 in Transfected Cells. We performed dual luciferase reporter assays and demonstrated that methamphetamine causes an increase in CRE-Luc expression, indicative of elevations in cAMP, via its interaction with rhesus monkey TAAR1. HEK293 and HEK293-derived stable rhesus monkey TAAR1 cells were transiently transfected with human DAT to generate cell lines labeled as DAT and TAAR1-DAT. Compared with vehicle treatment, methamphetamine increased CRE-Luc expression by 366.4 ± 42.6% in TAAR1-DAT cells ($p < 0.01$ by Student's *t* test) but did not alter the CRE-Luc expression in DAT cells (Fig. 1A). Western blotting showed that TAAR1 was expressed in TAAR1-DAT cells but not in DAT cells.

Methamphetamine Interaction with TAAR1 Inhibits [^{3}H]Dopamine Uptake. We have previously shown that methamphetamine-concurrent loading with [^{3}H]dopamine causes a distinct inhibition in [^{3}H]dopamine uptake that is TAAR1-dependent in transfected cells (Xie and Miller, 2007). Here, we performed similar concurrent loading assays to confirm the TAAR1-dependent effect in transfected cells and assessed whether methamphetamine causes a similar effect in brain striatal synaptosomes. To investigate the influence of TAAR1 activation by methamphetamine on [^{3}H]dopamine uptake, transfected cells and brain striatal synaptosomes were loaded with 10 nM [^{3}H]dopamine alone or 10 nM [^{3}H]dopamine plus 100 nM methamphetamine for different times. Methamphetamine inhibited [^{3}H]dopamine uptake in TAAR1-DAT and DAT cells, which is reflected by the right-and-downward shift of the uptake curves (Fig. 1B). It is notable that methamphetamine caused an additional and distinct inhibition of [^{3}H]dopamine uptake after 3 min in TAAR1-DAT cells that was not observed in DAT cells (Fig. 1B). Likewise, methamphetamine inhibited [^{3}H]dopamine uptake in rhesus monkey, wild-type mouse, and TAAR1 knockout mouse striatal synaptosomes, which is reflected by the right-and-downward shift of the uptake curves in each case (Fig. 1C). Similar to the TAAR1-dependent effect seen in the transfected cells, methamphetamine caused an additional and distinct inhibition of [^{3}H]dopamine uptake after 3 min in wild-type mouse and rhesus monkey striatal synaptosomes, which was not observed in TAAR1 knockout mouse striatal synaptosomes (Fig. 1C). Western blotting with a commercially available TAAR1 antibody indicated that TAAR1 was expressed in wild-type mouse and rhesus monkey striatal synaptosomes but not in untransfected HEK293 cells or TAAR1 knockout mouse striatal synaptosomes. Representative data of a full time course indicating the disintegrations per minute values of specific [^{3}H]dopamine uptake in the transfected cells and synaptosomes tested are shown (Supplemental Fig. S1). A statistical analysis demonstrating a significantly greater inhibition in [^{3}H]dopamine uptake at the time point of 10 min in TAAR1-DAT versus DAT cells ($p < 0.01$) and rhesus monkey or wild-type mouse versus TAAR1 knockout mouse synaptosomes ($p < 0.01$) is shown (Supplemental Fig. S2).

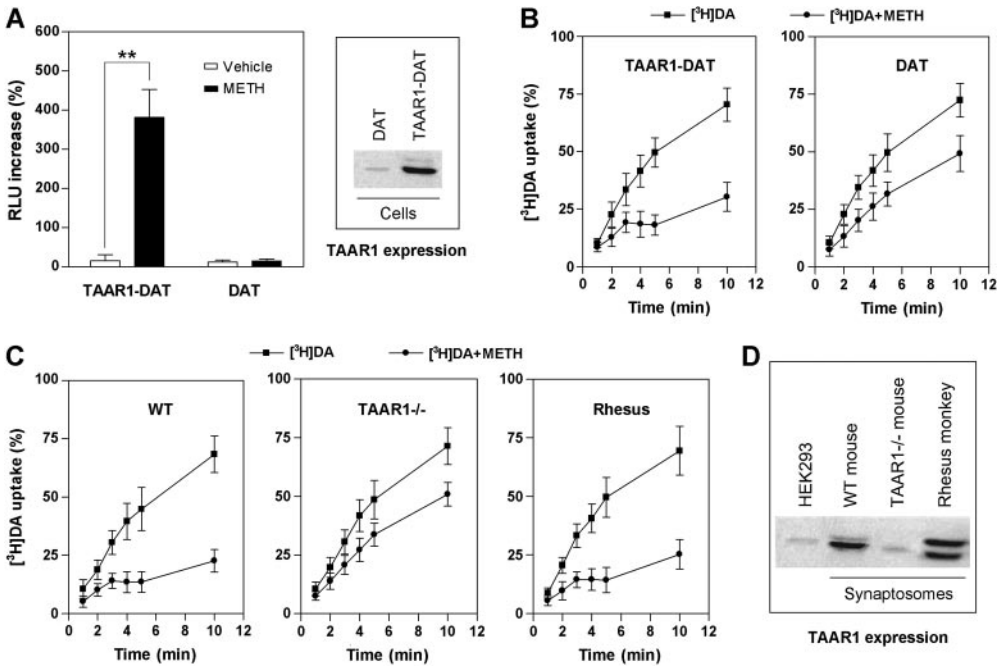

Fig. 1. TAAR1 response to methamphetamine and effects of concurrent loading of methamphetamine on [^{3}H]dopamine uptake in transfected cells and brain synaptosomes. A, TAAR1 expression and its activation by methamphetamine in transfected cells. HEK293 and HEK293-derived stable TAAR1 cells were transiently transfected with human DAT to generate cell lines labeled as DAT and TAAR1-DAT, respectively. CRE-Luc and pGL4.73 were simultaneously introduced into the cells to monitor cAMP variation, which was measured as percentage change in relative light units by luciferase assay after exposure to vehicle or 1 μM methamphetamine. Data are values of mean ± S.E.M. for three independent experiments performed in triplicate. **, $p < 0.01$ by Student's t test. Robust TAAR1 expression in TAAR1-DAT cells was detected by SDS-PAGE and Western bolting. B and C, role of TAAR1 in methamphetamine-induced inhibition of [^{3}H]dopamine uptake. Transfected cells and striatal synaptosomes were loaded with 10 nM [^{3}H]dopamine alone ([^{3}H]DA) or 10 nM [^{3}H]dopamine plus 100 nM methamphetamine ([^{3}H]DA + METH) for different time periods. Shown are the uptake curves over 10 min, generated in TAAR1-DAT and DAT cells (B), and in brain striatal synaptosomes generated from wild-type (WT) mouse, TAAR1 knockout (TAAR1−/−) mouse, and rhesus monkey (Rhesus) (C). Data are values of mean ± S.E.M. for three independent experiments performed in triplicate. D, TAAR1 expression levels detected by SDS-PAGE and Western blotting in the synaptosomes relative to untransfected HEK293 cells.

To further confirm the role of TAAR1 in methamphetamine inhibition of [^{3}H]dopamine uptake, we next evaluated effects of methamphetamine pretreatment on [^{3}H]dopamine uptake in the presence and absence of TAAR1. Transfected cells and mouse striatal synaptosomes were pretreated with methamphetamine (various concentrations or 1 μM) for 10 min, washed, and then uploaded with 10 nM [^{3}H]dopamine for 5 min. We chose a 10-min pretreatment interval based on the observed significant effects of methamphetamine at this time point. As shown in Fig. 2, A and B, methamphetamine dose-dependently inhibited [^{3}H]dopamine uptake in TAAR1-DAT cells and in wild-type mouse striatal synaptosomes but did not cause significant uptake inhibition in DAT cells or in TAAR1 knockout mouse striatal synaptosomes at the tested concentrations, indicating that the uptake inhibition caused by methamphetamine was mediated by TAAR1. Note that methamphetamine was washed away after pretreatment and consequently was not in competition with [^{3}H]dopamine during [^{3}H]dopamine loading under this condition.

Then, we assessed whether the TAAR1-mediated effects of methamphetamine on [^{3}H]dopamine uptake are phosphorylation-dependent. Because the dose-response evaluation showed that methamphetamine nearly reached a maximal effect in uptake inhibition at the concentration of 1 μM, we chose this single concentration for the assessment. TAAR1-DAT cells and wild-type mouse striatal synaptosomes were pretreated with 1 μM methamphetamine alone or 1 μM methamphetamine plus 10 μM of the PKA inhibitor H89 or 10 μM of the PKC inhibitor Ro32-0432 for 10 min, washed twice, and then loaded with 10 nM [^{3}H]dopamine for 5 min. As shown in Fig. 2, C and D, methamphetamine reduced [^{3}H]dopamine uptake in TAAR1-DAT cells and in wild-type mouse striatal synaptosomes by 37.4 ± 5.4 and 21.4 ± 3.8%, respectively, which significantly differed from vehicle treatment ($p < 0.01$). In contrast, methamphetamine did not cause significant uptake inhibition in DAT cells (3.4 ± 1.4%) or in TAAR1 knockout mouse striatal synaptosomes (2.2 ± 1.1%). Moreover, the methamphetamine-induced uptake inhibition in TAAR1-DAT cells and in wild-type mouse striatal synaptosomes was blocked by either H89 or Ro32-0432. Neither H89 (10 μM) nor Ro32-0432 (10 μM) alone significantly altered [^{3}H]dopamine uptake in TAAR1-DAT cells or wild-type mouse striatal synaptosomes (Supplemental Fig. S3), indicating that the TAAR1-dependent uptake inhibition caused by methamphetamine is phosphorylation dependent.

Methamphetamine Interaction with TAAR1 Induces [^{3}H]Dopamine Efflux. We first investigated methamphetamine-induced dopamine efflux in transfected cells. DAT cells were preloaded with various concentrations (1 nM~10 μM) of [^{3}H]dopamine for 20 min, washed twice, and then exposed to 1 μM methamphetamine for 30 min. As shown in Fig. 3A, methamphetamine induced [^{3}H]dopamine efflux in a [^{3}H]dopamine concentration-dependent manner. At the loading concentrations of 1 and 10 μM [^{3}H]dopamine, methamphetamine induced significant [^{3}H]dopamine efflux in DAT cells ($p < 0.01$). In contrast, vehicle treatment had no such

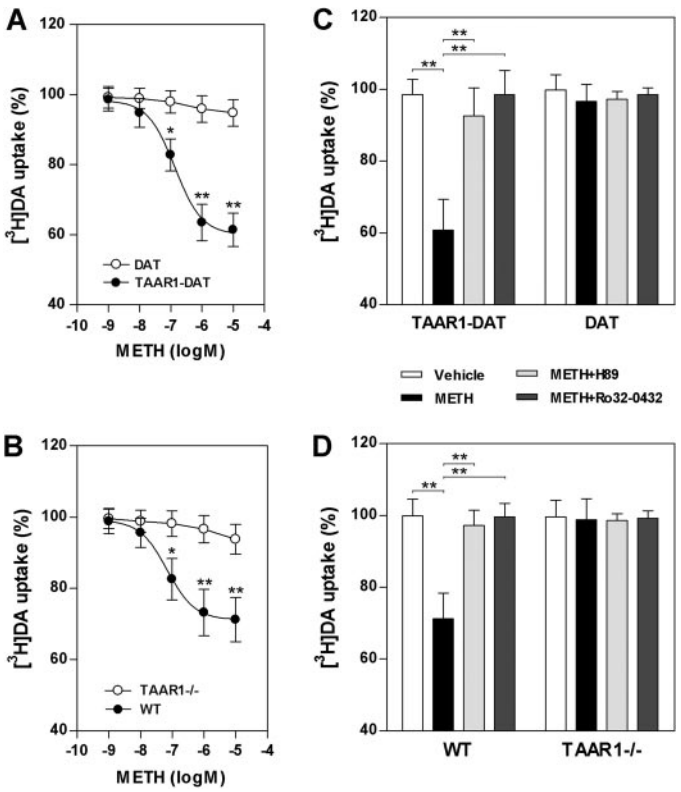

Fig. 2. Effects of methamphetamine pretreatment on [^{3}H]dopamine uptake in transfected cells and brain striatal synaptosomes. A and B, dose-response evaluation of the effect of methamphetamine on [^{3}H]dopamine uptake. TAAR1-DAT and DAT cells (A) or wild-type (WT) and TAAR1 knockout (TAAR1−/−) mouse striatal synaptosomes (B) were pretreated with methamphetamine (1 nM–10 μM) for 10 min, washed twice, and then loaded with 10 nM [^{3}H]dopamine for 5 min. *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA/Turkey post hoc test, compared with the baseline [DMEM (A) or buffer pretreatment (B)]. Note that methamphetamine was washed away after pretreatment and, consequently, is not in competition with [^{3}H]dopamine during [^{3}H]dopamine uploading. C and D, influence of phosphorylation inhibitors on the effects of methamphetamine. Cells and mouse striatal synaptosomes were pretreated with vehicle, 1 μM methamphetamine alone, or 1 μM methamphetamine plus 10 μM H89 or Ro32-0432 for 10 min, washed twice, and then loaded with 10 nM [^{3}H]dopamine for 5 min. **, $p < 0.01$ by one-way ANOVA/Turkey post hoc test. All data shown are values of mean ± S.E.M. for three independent experiments performed in triplicate.

effect. The data indicate that methamphetamine can only cause [^{3}H]dopamine efflux from DAT cells when they are loaded with high concentrations of [^{3}H]dopamine (1 μM or greater), in accordance with the lack of methamphetamine-induced [^{3}H]dopamine efflux in DAT cells loaded with 10 nM [^{3}H]dopamine that we reported previously (Xie and Miller, 2007).

Given the above findings, we chose a high (1 μM) and a low (10 nM) concentration of [^{3}H]dopamine to investigate TAAR1-mediated effects of methamphetamine on [^{3}H]dopamine efflux. Cells were preloaded with 1 μM or 10 nM [^{3}H]dopamine for 20 min, washed twice, and then treated with 1 μM methamphetamine for a time course. As shown in Fig. 3B, when the cells were preloaded with 1 μM [^{3}H]dopamine, methamphetamine caused [^{3}H]dopamine efflux in both DAT and TAAR1-DAT cells, but notably, it caused significantly greater [^{3}H]dopamine efflux in TAAR1-DAT cells than in DAT cells at the time points of 20 and 30 min ($p < 0.05$ at both points). As shown in Fig. 3C, when the cells were preloaded with 10 nM [^{3}H]dopamine, methamphetamine caused [^{3}H]dopamine efflux in TAAR1-DAT cells but not in DAT cells. The difference reached a significant level at 10 min ($p < 0.05$ at 10 min and $p < 0.01$ at 20 and 30 min). These results indicate that methamphetamine induces both TAAR1-dependent and -independent [^{3}H]dopamine efflux at the higher loading concentration of [^{3}H]dopamine but only induces a TAAR1-dependent [^{3}H]dopamine efflux at the lower loading concentration of [^{3}H]dopamine.

To further study the TAAR1-dependent and -independent effects of methamphetamine on [^{3}H]dopamine efflux at the high loading concentration of [^{3}H]dopamine, we evaluated the influence of the dopamine transporter blocker methylphenidate (MPH) and protein kinase inhibitors on [^{3}H]dopamine efflux. Transfected cells and mouse striatal synaptosomes were preloaded with 1 μM [^{3}H]dopamine for 20 min, washed twice, and then exposed to vehicle, 1 μM methamphetamine alone, or 1 μM methamphetamine plus 10 μM MPH, H89, or Ro32-0432 for 30 min. As shown in Fig. 4A, methamphetamine caused [^{3}H]dopamine efflux in both TAAR1-DAT and DAT cells, but greater [^{3}H]dopamine efflux was elicited in the TAAR1-DAT cells (retention level in TAAR1-DAT cells is 45.8 ± 6.2% and in DAT cells is 69.8 ± 5.5%, $p < 0.01$), which is similar to its effect at 30 min in Fig. 3B. MPH completely blocked the methamphetamine-induced [^{3}H]dopamine efflux in both TAAR1-DAT and DAT cells, indicating that the loss of the [^{3}H]dopamine signal is reflective of [^{3}H]dopamine efflux via the DAT. Although H89 did not alter methamphetamine-induced [^{3}H]dopamine efflux in either TAAR1-DAT or DAT cells, Ro32-0432 significantly attenuated [^{3}H]dopamine efflux caused by methamphetamine in TAAR1-DAT cells ($p < 0.01$) but did not alter its effect in the DAT cells.

Parallel to these observations in transfected cells were the results obtained in mouse striatal synaptosomes. As shown in Fig. 4B, methamphetamine caused [^{3}H]dopamine efflux in both wild-type and TAAR1 knockout mouse striatal synaptosomes, but greater [^{3}H]dopamine efflux was elicited in wild-type mouse striatal synaptosomes (retention level in wild-type synaptosomes is 52.8 ± 7.2% and in TAAR1 knockout synaptosomes is 78.2 ± 4.5%, $p < 0.01$). MPH completely blocked the methamphetamine-induced [^{3}H]dopamine efflux in both wild-type and TAAR1 knockout mouse striatal synaptosomes. H89 did not alter methamphetamine-induced [^{3}H]dopamine efflux in either wild-type or TAAR1 knockout mouse striatal synaptosomes, and Ro32-0432 significantly attenuated [^{3}H]dopamine efflux caused by methamphetamine in wild-type mouse striatal synaptosomes but did not alter methamphetamine-induced [^{3}H]dopamine efflux in TAAR1 knockout mouse striatal synaptosomes.

It is notable that we observed that Ro32-0432 only blocked the TAAR1-dependent effects caused by methamphetamine in TAAR1-DAT cells and in wild-type mouse striatal synaptosomes. This is indicated when one compares the effects of methamphetamine plus Ro32-0432 in TAAR1-DAT cells to the effects of methamphetamine alone in DAT cells in Fig. 4A or when one makes a similar comparison in Fig. 4B with the synaptosome data. Control studies confirmed that neither H89 (10 μM) nor Ro32-0432 (10 μM) alone alters [^{3}H]dopamine efflux in TAAR1-DAT cells or in wild-type mouse striatal synaptosomes (Supplemental Fig. S4).

We next performed dose-response assays to further investigate TAAR1-dependent effects of methamphetamine on

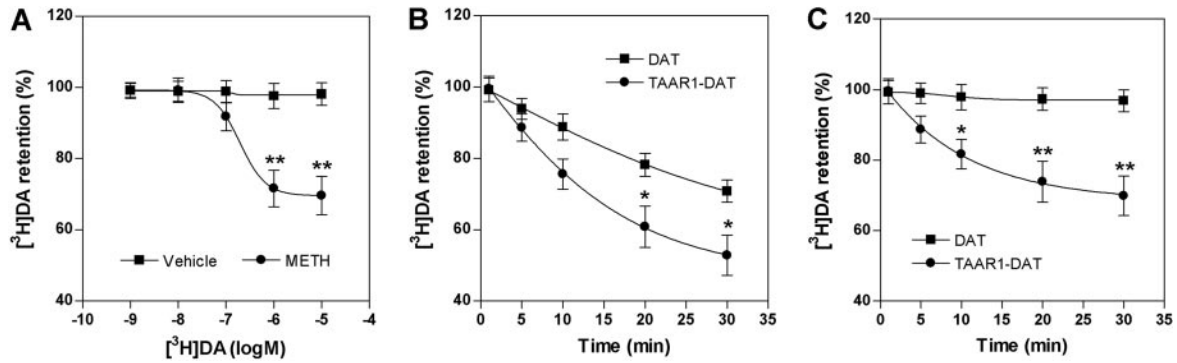

Fig. 3. Effects of methamphetamine on [^{3}H]dopamine efflux in transfected cells. A, DAT cells were preloaded with various concentrations of [^{3}H]dopamine for 20 min, washed twice, and then exposed to vehicle or 1 μM methamphetamine for 30 min. **, $p < 0.01$ by one-way ANNOVA/Turkey post hoc test, compared with baseline. B, DAT and TAAR-DAT cells were preloaded with 1 μM [^{3}H]dopamine for 20 min and then washed twice and treated with 1 μM methamphetamine for a time course of 1, 5, 10, 20, and 30 min. *, $p < 0.05$ by two-way ANOVA/Bonferroni post hoc test, compared with the level at the same time point in DAT cells. C, DAT and TAAR1-DAT cells were preloaded with 10 nM [^{3}H]dopamine for 20 min, washed twice, and then treated with 1 μM methamphetamine for a time course of 1, 5, 10, 20, and 30 min. *, $p < 0.05$; **, $p < 0.01$ by two-way ANOVA/Bonferroni post hoc test, compared with the level at the same time point in DAT cells. All data shown are values of mean ± S.E.M. for three independent experiments performed in triplicate.

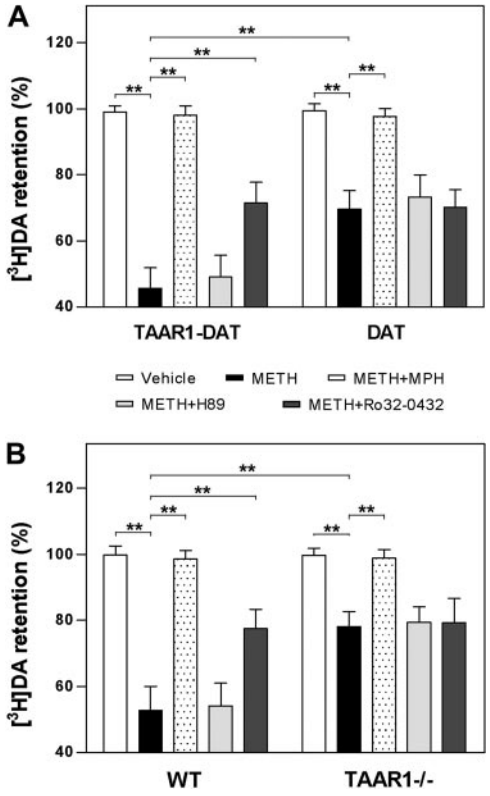

Fig. 4. Characterization of methamphetamine-induced [^{3}H]dopamine efflux at the preloading concentration of 1 μM [^{3}H]dopamine. TAAR-DAT and DAT cells (A) and wild-type (WT) and TAAR1 knockout (TAAR1−/−) mouse striatal synaptosomes (B) were preloaded with 1 μM [^{3}H]dopamine for 20 min, washed twice, and then exposed to vehicle, 1 μM methamphetamine alone, or 1 μM methamphetamine plus 10 μM MPH, H89, or Ro32-0432 for 30 min. **, $p < 0.01$ by two-way ANOVA/Bonferroni post hoc test. Data shown are values of mean ± S.E.M. for three independent experiments performed in triplicate.

[^{3}H]dopamine efflux in transfected cells and mouse striatal synaptosomes. In these experiments, the transfected cells and synaptosomes were preloaded with 10 nM [^{3}H]dopamine for 20 min, washed twice, and then exposed to various concentrations of methamphetamine for 30 min. As shown in Fig. 5, A and B, methamphetamine induced significant [^{3}H]dopamine efflux at concentrations at and above 100 nM in TAAR1-DAT cells ($p < 0.05$ at 100 nM; $p < 0.01$ at higher doses) and at and above 1 μM in wild-type synaptosomes ($p < 0.01$ at 1 μM and higher doses) but did not cause significant [^{3}H]dopamine efflux in DAT cells or in TAAR1 knockout mouse striatal synaptosomes across the test concentrations.

Further assays were performed to evaluate the influence of MPH and protein kinase inhibitors on [^{3}H]dopamine efflux in cells and synaptosomes that were preloaded with 10 nM [^{3}H]dopamine. After preloading for 20 min, cells or synaptosomes were washed twice and then exposed to vehicle, 1 μM methamphetamine alone, or 1 μM methamphetamine plus 10 μM MPH, H89, or Ro32-0432 for 30 min. As shown in Fig. 5, C and D, methamphetamine caused [^{3}H]dopamine efflux in TAAR1-DAT cells and in wild-type mouse striatal synaptosomes (similar to its effects at 1 μM in Fig. 5, A and B), and this effect was blocked by methylphenidate or Ro32-0432 but not by H89. In contrast, in DAT cells or in TAAR1 knockout mouse striatal synaptosomes, methamphetamine did not cause [^{3}H]dopamine efflux, and addition of MPH, H89, or Ro32-0432 had no effect. Similar to the data obtained in wild-type mouse striatal synaptosomes, methamphetamine caused [^{3}H]dopamine efflux in rhesus monkey striatal synaptosomes, and its effect was blocked by MPH or Ro32-0432 but not H89 (Fig. 5E).

Methamphetamine Interaction with TAAR1 Triggers Internalization of the Dopamine Transporter. The level of the dopamine transporter on the cell surface membrane was evaluated using biotinylation assays. Cells and synaptosomes were treated with 1 μM methamphetamine for different periods before the biotinylation reaction. Methamphetamine reduced cell surface dopamine transporter levels in TAAR1-DAT cells but not in DAT cells (Fig. 6A). The level of the biotinylated dopamine transporter in TAAR1-DAT cells significantly decreased at 5 min and reached a peak at 10 min and then retrieved at 30 and 60 min, whereas no significant change was observed in DAT cells ($p < 0.01$ at 5, 10, and 30 min). Likewise, methamphetamine reduced surface dopamine transporter levels in wild-type mouse striatal synaptosomes. The level of the biotinylated dopamine transporter decreased at 5 min and reached a peak at 10 min and then largely retrieved at 30 and 60 min ($p < 0.01$ at 5, 10, and 30 min), whereas only a small (yet significant) decrease was

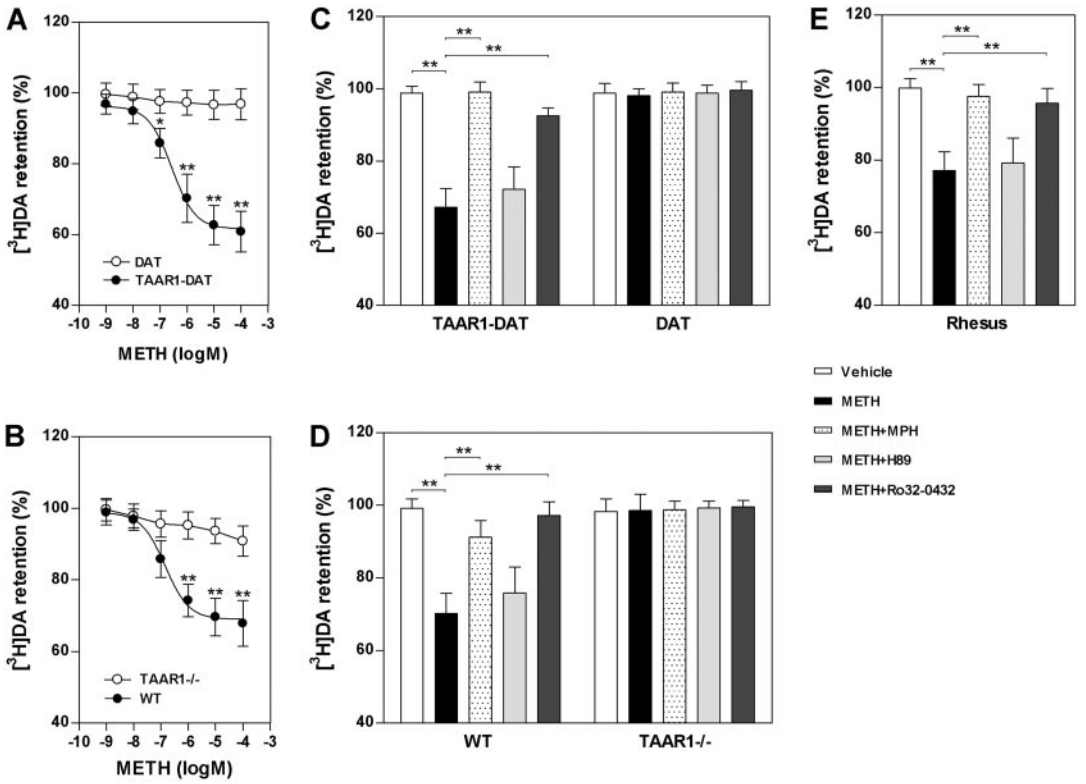

Fig. 5. Characterization of methamphetamine-induced [^{3}H]dopamine efflux at the preloading concentration of 10 nM [^{3}H]dopamine. A and B, dose-response evaluation of the effect of methamphetamine on [^{3}H]dopamine efflux in TAAR-DAT and DAT cells (A) and wild-type (WT) and TAAR1 knockout (TAAR1−/−) mouse striatal synaptosomes (B). Cells and synaptosomes were preloaded with 10 nM [^{3}H]dopamine for 20 min, washed twice, and then exposed to methamphetamine (1 nM∼100 μM) for 30 min. *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA/Turkey post hoc test, compared with baseline [DMEM (A) or buffer (B) treatment]. C to E, TAAR-DAT and DAT cells (C), wild-type (WT) and TAAR1 knockout (TAAR1−/−) mouse striatal synaptosomes (D), and rhesus monkey striatal synaptosomes (E) were preloaded with 10 nM [^{3}H]dopamine for 20 min, washed twice, and then exposed to vehicle, 1 μM methamphetamine alone, or 1 μM methamphetamine plus 10 μM MPH, H89, or Ro32-0432 for 30 min. **, $p < 0.01$ by two-way ANOVA/Bonferroni post hoc test (C and D), or by one-way ANOVA/Turkey post hoc test (E). All data shown are values of mean ± S.E.M. for three independent experiments performed in triplicate.

observed at 10 min ($p < 0.01$) in TAAR1 knockout mouse striatal synaptosomes (Fig. 6B). In a single rhesus monkey striatal synaptosome preparation, we observed that methamphetamine also caused similar changes in the surface dopamine transporter level as it did in wild-type mouse synaptosomes, whereas vehicle treatment had no effect (Fig. 6C). Furthermore, we found that although cell surface dopamine transporter levels decreased, the unbiotinylated dopamine transporter levels detected in the flow-through increased in TAAR1-DAT cells and in wild-type mouse striatal synaptosomes (Fig. 6D; representative of three similar observations). To determine whether the TAAR1-mediated dopamine transporter internalization was phosphorylation-dependent, TAAR1-DAT cells and wild-type mouse striatal synaptosomes were exposed to 1 μM methamphetamine for 10 min in the presence or absence of 10 μM H89 or 10 μM Ro32-0432 before the biotinylation reaction. As shown in Fig. 6E, methamphetamine reduced DAT levels on the cell surface in TAAR1-DAT cells and wild-type mouse striatal synaptosomes, relative to vehicle treatment, and this effect was blocked by the addition of Ro32-0432 but not H89.

Discussion

This study shows that methamphetamine interaction with TAAR1 results in inhibition of dopamine uptake, induction of dopamine efflux, and promotion of dopamine transporter internalization in rhesus monkey and mouse brain striatal synaptosomes. The TAAR1-dependent effects of methamphetamine demonstrated in this study reveal a novel mechanism by which methamphetamine exerts modulatory effects on the dopamine transporter in the brain. The data presented here along with our recent findings that common biogenic amines and β-phenylethylamine interact with TAAR1 to regulate monoamine transporter function in transfected cells and in monkey and mouse brain striatal synaptosomes (Xie and Miller, 2008; Xie et al., 2008b) provide strong evidence that TAAR1 is a monoaminergic modulator in the brain.

Methamphetamine concurrent loading along with [^{3}H]dopamine inhibited [^{3}H]dopamine uptake in transfected cells and synaptosomes in both the presence and absence of TAAR1 (the uptake curves shifted right and downward). In addition, methamphetamine caused a distinct, temporal inhibition of [^{3}H]dopamine uptake in TAAR1-DAT cells and rhesus monkey and wild-type mouse striatal synaptosomes that was not present in DAT cells or TAAR1 knockout mouse striatal synaptosomes. Similar to the effects of β-phenylethylamine (Xie and Miller, 2008), this effect of methamphetamine occurred after 3 min, which may reflect the time necessary for methamphetamine to activate the receptor. In our previous study, we demonstrated that TAAR1 was membrane-associated but did not reside on the extracellular

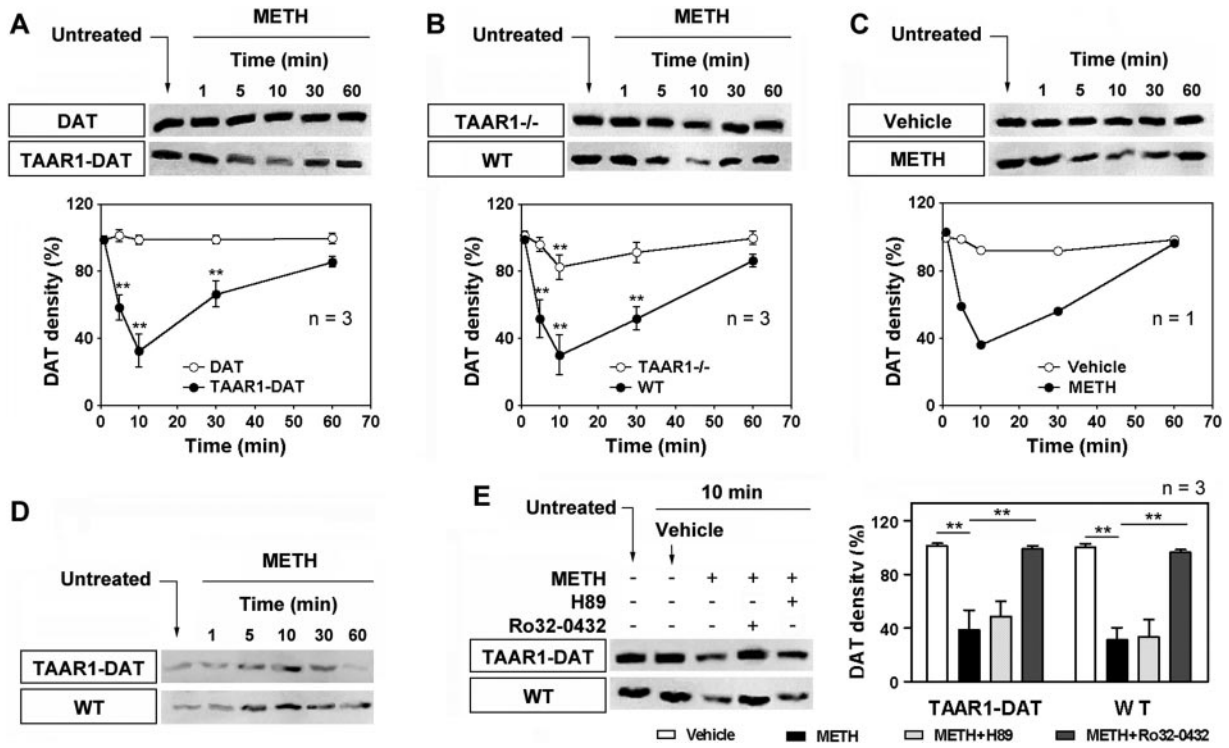

Fig. 6. Role of TAAR1 in methamphetamine-induced dopamine transporter internalization. A and B, transfected cells and synaptosomes were treated with 1 μM methamphetamine for different time periods before the biotinylation reaction. Shown are the changes in the level of biotinylated dopamine transporter in DAT versus TAAR1-DAT cells (A) and wild-type (WT) versus TAAR1 knockout (TAAR1−/−) mouse striatal synaptosomes (B) after methamphetamine exposure. *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA/Turkey post hoc test, compared with untreated cells or synaptosomes. Data shown are values of mean ± S.E.M. for three independent experiments performed in triplicate. C, shown are the changes in the level of biotinylated dopamine transporter rhesus monkey striatal synaptosomes after 1 μM methamphetamine treatment versus vehicle treatment. Data shown are values derived from a single experiment. D, representative data for the unbiotinylated dopamine transporter levels detected in the flow-through after 1 μM methamphetamine treatment in A and B. E, TAAR1-DAT cells and WT mouse striatal synaptosomes were exposed to vehicle, 1 μM methamphetamine, or 1 μM methamphetamine plus 10 μM H89 or 10 μM Ro32-0432 for 10 min before the biotinylation reaction. Data from a representative experiment (left) and analysis of mean ± S.E.M. for three independent experiments (right) is shown. **, $p < 0.01$ by one-way ANOVA/Turkey post hoc test.

plasma membrane in transfected cells (Xie et al., 2008a), which is supported by an observed intracellular distribution pattern of flag-tagged rat TAAR1 (Bunzow et al., 2001) and of GFP-tagged rhesus monkey TAAR1 (Miller et al., 2005) in transfected cells. Because the TAAR1-mediated uptake inhibition occurred at the same time in striatal synaptosomes and transfected cells, it is possible that TAAR1 is also located intracellularly in neurons, but further studies are needed to clarify this issue.

In the pretreatment assays, in which we exposed cells and synaptosomes to methamphetamine for 10 min and then washed it away before uploading of [^{3}H]dopamine, methamphetamine induced inhibition of [^{3}H]dopamine uptake only in the presence of TAAR1. In the concurrent loading assays, a shift in the uptake curves caused by methamphetamine was observed in both the presence and the absence of TAAR1. This shift most probably results from the competition of methamphetamine with [^{3}H]dopamine for uptake at the transport sites because no uptake inhibition was observed in the absence of TAAR1 (in DAT cells or TAAR1 knockout mouse striatal synaptosomes) in the pretreatment assays. In accordance, the competition effect on dopamine uptake is apparently TAAR1-independent, whereas the additional inhibition, or apparent "uptake halting" as we called this effect in our previous reports (Xie and Miller, 2007, 2008; Xie et al., 2008), is TAAR1-dependent.

Several previous studies reported methamphetamine-induced dopamine efflux in cells transfected with the dopamine transporter alone (Eshleman et al., 1994; Wilhelm et al., 2004; Johnson et al., 2005), suggesting that methamphetamine can cause dopamine efflux via direct interaction with the dopamine transporter. It is notable that in our previous study in which DAT cells were loaded with 10 nM [^{3}H]dopamine and then treated with 1 μM methamphetamine, we did not observe any [^{3}H]dopamine efflux (Xie and Miller, 2007). In this study, we demonstrated that methamphetamine only induced [^{3}H]dopamine efflux in DAT cells or in TAAR1 knockout mouse synaptosomes when the cells or synaptosomes were loaded with high concentrations of [^{3}H]dopamine (1 μM or higher), indicating that the direct effect of methamphetamine on the dopamine transporter to cause [^{3}H]dopamine efflux is dependent on the preloading concentration of [^{3}H]dopamine. Furthermore, we show that such effects caused by methamphetamine are not associated with either the PKA or the PKC phosphorylation pathways.

In contrast, TAAR1-mediated effects of methamphetamine on [^{3}H]dopamine efflux were observed at both lower and higher loading concentrations of [^{3}H]dopamine in striatal synaptosomes and in transfected cells and could be blocked not only by MPH but also by the PKC inhibitor Ro32-0432. These data indicate that the TAAR1-mediated effects of methamphetamine are dependent on PKC phosphorylation,

consistent with the role of PKC in substrate-induced efflux. Although it is also possible that the expression of TAAR1 could result in [^{3}H]dopamine being located in a more releasable pool that is more susceptible to the carrier-mediated exchange mechanism, we speculate that the TAAR1-mediated effects of methamphetamine on [^{3}H]dopamine efflux result from downstream PKC activation. PKC-dependent phosphorylation is known to play a key role in the functional regulation of the dopamine transporter by methamphetamine. Methamphetamine increases striatal PKC activity (Giambalvo, 2004), which in turn leads to down-regulation of dopamine transporter function (Zhang et al., 1997). Both methamphetamine-induced phosphorylation and down-regulation of the dopamine transporter are prevented by PKC inhibition in striatal synaptosomes (Sandoval et al., 2001). Moreover, PKC-dependent phosphorylation is also implicated in dopamine transporter internalization (Melikian and Buckley, 1999; Sandoval et al., 2001). Here, we found that TAAR1-mediated effects of methamphetamine on [^{3}H]dopamine uptake and [^{3}H]dopamine efflux were blocked by the PKC inhibitor Ro32-0432. We also found that TAAR1-mediated uptake inhibition by methamphetamine could also be blocked by the PKA inhibitor H89, which suggests that TAAR1 signaling is also involved in the regulation of DAT function via its activation of the PKA phosphorylation pathway. Our use of high (10 μM) concentrations of H89 and Ro32-0432 may call into question the drug specificity for PKA and PKC, respectively, but nevertheless, the present data demonstrate that TAAR1 activation by methamphetamine can serve as the trigger that initiates phosphorylation events that alter dopamine transporter function.

In the present study, we also demonstrated that 1 μM methamphetamine reduced cell surface levels of the dopamine transporter in TAAR1-DAT cells and in rhesus monkey and wild-type mouse striatal synaptosomes, whereas it did not internalize the dopamine transporter in DAT cells and had only a weak effect in TAAR1 knockout mouse striatal synaptosomes. These data indicate that methamphetamine interaction with TAAR1 can trigger dopamine transporter internalization. The blockade of this TAAR1-mediated effect of methamphetamine by Ro32-0432 suggests that activation of the PKC phosphorylation cascade, which is widely implicated in methamphetamine effects on dopamine transporter trafficking, can be triggered by TAAR1 signaling in response to methamphetamine. Dopamine transporter internalization may result in a lower transport capacity of the dopamine transporter, which is consistent with the observed TAAR1-mediated inhibition of [^{3}H]dopamine uptake. [^{3}H]Dopamine efflux that occurs at a similar time with the observed dopamine transporter internalization may be mediated by dopamine transporter molecules that apparently remain at the cell surface. Both in TAAR1-DAT cells and in wild-type mouse synaptosomes, over 30% of the detected (biotinylated) dopamine transporter apparently remains at the cell surface.

The present study reveals for the first time a receptor mechanism for methamphetamine action in brain. Adding to the complexity of the role of TAAR1 in mediating methamphetamine action in the brain are two recent reports that demonstrate an enhanced sensitivity of TAAR1 knockout mice to amphetamine and an amphetamine-induced increase in the release of catecholamine in the TAAR1 knockout mouse striatum (Wolinsky et al., 2007; Lindemann et al., 2008). Although methamphetamine was not tested by either group, it is predictable that methamphetamine would cause similar effects in TAAR1 knockout mice, and recent work in our lab reveals a greater sensitivity to methamphetamine in the knockout mice (presented at the Society for Neuroscience Annual Meeting, 2008; 59.13/DD19). Because neuroadaptive changes such as a dramatic increase of high-affinity D2 receptors occur in the TAAR1 knockout mice (Wolinsky et al., 2007), it is possible that their enhanced sensitivity to amphetamine may not be the direct consequence of the TAAR1 deficit but, rather, may be due to developmental adaptations. Alternatively, TAAR1 may have a much wider role in mediating methamphetamine action on brain dopamine neurons in addition to that revealed in our current data, which focuses solely on effects on the dopamine transporter that model presynaptic events. Our previous study demonstrated that TAAR1 is expressed in monoaminergic brain regions and colocalizes with the dopamine transporter in a subset of substantia nigra dopamine neurons (Xie et al., 2007b), supporting the current findings that functionally demonstrate TAAR1 and DAT colocalization in striatal synaptosomes. We also had found neurons scattered in the substantia nigra that stained positive for TAAR1 but negative for the dopamine transporter (Xie et al., 2007b). We hypothesize that these TAAR1-positive neurons may be inhibitory to dopamine neurons, thereby capable of indirectly influencing dopamine release (exocytosis) and transport (uptake or efflux) via interneuronal communication. If this were the case, then the deprivation of this TAAR1-mediated inhibition on dopamine neurons in response to amphetamine in TAAR1 knockout mice could result in the hypersensitivity to amphetamines.

Nonetheless, the present study demonstrates that TAAR1 mediates methamphetamine-induced regulation of dopamine transporter function and trafficking in brain striatal synaptosomes and in transfected cells and reveals a novel receptor mechanism for methamphetamine action in the brain for the first time. We also show that the TAAR1 receptor can function as a triggering mechanism for the phosphorylation-driven cellular events that hallmark dopamine transporter kinetic regulation and cellular translocation. In this regard, our findings implicate this receptor as a potential target of therapeutic drugs and open up new avenues of investigation.

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