Membrane-permeable C-terminal Dopamine Transporter Peptides Attenuate Amphetamine-evoked Dopamine Release**

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Background: The significance of dopamine transporter (DAT) C-terminal protein-protein interactions for amphetamine-induced dopamine efflux is unsettled.

Results: Cell-permeable C-terminal DAT peptides attenuate amphetamine-induced dopamine efflux and locomotor activity in mice.

Conclusion: DAT C-terminal protein-protein interactions are critical for the effects of amphetamine in vivo.

Significance: Targeting protein-protein interactions might be a way of inhibiting the effects of psychostimulants.

The dopamine transporter (DAT) is responsible for sequestration of extracellular dopamine (DA). The psychostimulant amphetamine (AMPH) is a DAT substrate, which is actively transported into the nerve terminal, eliciting vesicular depletion and reversal of DA transport via DAT. Here, we investigate the role of the DAT C terminus in AMPH-evoked DA efflux using cell-permeant dominant-negative peptides. A peptide, which corresponded to the last 24 C-terminal residues of DAT (TAT-C24 DAT) and thereby contained the Ca2+-calmodulin-dependent protein kinase IIα (CaMKIIα) binding domain and the PSD-95/Discs-large/ZO-1 (PDZ)-binding sequence of DAT, was made membrane-permeable by fusing it to the cell membrane transduction domain of the HIV-1 Tat protein (TAT-C24WT). The ability of TAT-C24WT but not a scrambled peptide (TAT-C24Scr) to block the CaMKIIα-DAT interaction was supported by co-immunoprecipitation experiments in heterologous cells. In heterologous cells, we also found that TAT-C24WT, but not TAT-C24Scr, decreased AMPH-evoked 1-methyl-4-phenylpyridinium efflux. Moreover, chronoamperometric recordings in striatum revealed diminished AMPH-evoked DA efflux in mice preinjected with TAT-C24WT. Both in heterologous cells and in striatum, the peptide did not further inhibit efflux upon KN-93-mediated inhibition of CaMKIIα activity, consistent with a dominant-negative action preventing binding of CaMKIIα to the DAT C terminus. This was further supported by the ability of a peptide with perturbed PDZ-binding sequence, but preserved CaMKIIα binding (TAT-C24AAA), to diminish AMPH-evoked DA efflux in vivo to the same extent as TAT-C24WT. Finally, AMPH-induced locomotor hyperactivity was attenuated following systemic administration of TAT-C24WT but not TAT-C24Scr. Summarized, our findings substantiate that DAT C-terminal protein-protein interactions are critical for AMPH-evoked DA efflux and suggest that it may be possible to target protein-protein interactions to modulate transporter function and interfere with psychostimulant effects.

Dopamine (DA)2 plays a pivotal role as a modulatory neurotransmitter controlling motor function, cognitive processes, reward mechanisms, as well as neuroendocrine secretion. Aberrant DA signaling contributes to the development of neuropsychiatric diseases such as schizophrenia, drug addiction, and Parkinson disease (1, 2). DA homeostasis in the brain is regulated by the presynaptic DA transporter (DAT), which removes extracellular DA from the synaptic cleft and thereby maintains homeostatic transmitter levels. DAT belongs to the solute carrier 6 (SLC6) gene family of sodium/chloride-coupled solute carrier transporters (also referred to as the family of neurotransmitter/solute carrier 6 (SLC6) gene family of sodium/chloride-coupled transporters (also referred to as the family of neurotransmitter/solute carrier transporters) characterized by 12 transmembrane domains with both the N and C termini located within the

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2 The abbreviations used are: DA, dopamine; CaMKIIα, Ca2+-calmodulin dependent protein kinase IIα; DAT, dopamine transporter; AMPH, amphetamine; AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid; IP, immunoprecipitation; aCSF, artificial CSF; ANOVA, analysis of variance; SA, saline; MPP+, 1-methyl-4-phenylpyridinium.

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Deletion of DAT in genetically modified mouse models has shown that DAT is fundamental for regulating spatio-temporal availability of DA (1, 5). DAT is also a molecular substrate for the psychostimulants cocaine and amphetamine (AMPH). Whereas cocaine acts as a competitive DAT inhibitor (6), AMPH is a substrate, which is actively transported into the presynaptic nerve terminal, leading to vesicular depletion of DA and reverse transport of DA via DAT (7, 8). This DA efflux dramatically elevates extracellular DA levels and is believed to contribute substantially to the addictive properties of AMPH (9). AMPH also causes DAT to move away from the plasma membrane to the intracellular compartment, thereby decreasing DAT surface levels (10–12). Interestingly, AMPH-induced endocytosis of DAT has been suggested to occur through a mechanism requiring stimulation of Ca\(^{2+}\)-calmodulin-dependent protein kinase II\(\alpha\) (CaMKII\(\alpha\)) (13).

Several DAT-interacting proteins have been identified and suggested to be involved in regulating trafficking or the transport activity (3, 4, 14, 15). Both CaMKII\(\alpha\) and the PSD-95/Discs-large/ZO-1 homology (PDZ) domain protein PICK1 (protein interacting with C kinase 1) have been shown to associate with the distal C terminus of DAT (16). Whereas the last 3–4 C-terminal residues of DAT constitute a prototypical PDZ-binding sequence that interacts with the PDZ domain of Discs-large/ZO-1 homology (PDZ) domain protein PICK1 port activity (3, 4, 14, 15). Both CaMKII\(\alpha\) and AMPH are suggested to be involved in regulating trafficking or the transport activity has provided further indirect support for the model (26). Moreover, the model suggests, in agreement with several earlier observations (8, 27), that AMPH-induced DA efflux is not directly coupled to uptake and that inward and outward transport represents different functional modes of the transporter.

Despite the presently available data, the significance of CaMKII\(\alpha\) association to the DAT C terminus remains uncertain. Moreover, it remains to be determined whether disrupting such C-terminal protein–protein interactions might represent a possible pharmacological target for blunting the psychostimulatory effects of AMPH. To address these questions, we designed cell-permeable peptides corresponding to the DAT C terminus and tested their effect in DAT-expressing heterologous cells as well as in vivo. Our data demonstrate that a peptide corresponding to the last 24 C-terminal residues of DAT, which constitutes the CaMKII\(\alpha\) binding domain, can act in a dominant-negative fashion causing impaired AMPH-induced MPP\(^+\) efflux in heterologous cells and decreased AMPH-induced DA efflux in the striatum of living mice. A similar effect was observed for a peptide with perturbed PDZ binding sequence suggesting that binding of PDZ domain protein to DAT is less important for efflux than CaMKII\(\alpha\). Co-immunoprecipitation experiments validated the interaction between DAT and CaMKII\(\alpha\) and we further showed that the cell-permeable DAT C-terminal peptide disrupted the interaction in heterologous cells. Furthermore, systemic administration of the C-terminal WT DAT peptide, but not a scrambled peptide, attenuated AMPH-stimulated locomotor activity in mice.

**EXPERIMENTAL PROCEDURES**

**Immunocytochemistry**—Synthetic peptides were purchased from Schafer N (Copenhagen, Denmark). The amino acid sequence was H-YGRKKRRQRRRPEKDELVRDGEVRQFTLRHWLKVOH for TAT-C24WT, H-YGRKKRRQRRRPEKDRELVRDGEVRQFRLRHWAAA-OH for TAT-C24AAA, and H-CYGRKKRRQRRRPTLFLLFQVEVDWRDLKVGHFER-OH for TAT-C24Scr peptide. Biotinylated TAT peptide variants (TAT-C24WT and TAT-C24Scr) were dissolved in 0.9% saline and immediately frozen in aliquots (10 mg/ml; corresponds to TAT-C24WT 2.1 mm and TAT-C24Scr 2.2 mm). The TAT peptide aliquots were thawed on ice right before use. HEK293 cells were seeded in 6-well plates, and biotinylated peptides (TAT-C24WT and TAT-C24Scr; final concentration 1 \(\mu\)M) were added to the respective wells. Following a 1-h incubation with TAT peptides at 37 °C, cells were fixed in 4% paraformaldehyde and rinsed in phosphate-buffered saline (PBS). Fluorescein streptavidin (1:50, Vector Laboratories Inc.) was incubated with cells for 3 h in PBS containing 0.1% Triton X-100 at room temperature. This was followed by additional rinsing in PBS, and cells were finally mounted on glass slides with Prolong\textsuperscript{\textregistered}Gold antifade reagent (Invitrogen). TAT peptides were visualized using a Zeiss LSM 510 confocal laser-scanning microscope with an oil immersion 63 × 1.4 numerical aperture objective (Carl Zeiss). Streptavidin fluorescein was excised with a 488 nm laser line from an argon-krypton laser, and detection of the emitted light was done using a 505–530-nm bandpass filter. Images were analyzed using the ImageJ software.

**Co-immunoprecipitation**—T-Rex HEK293 cells (human embryonic kidney) transfected to stably express CaMKII\(\alpha\) in a tetracycline-dependent manner (based on stable expression of Tet repressor from pcDNA6/TR) and with stable expression of human DAT (hDAT, N-terminally tagged with YFP) were cultivated as described previously (T-Rex 293 CaMKII\(\alpha\) YFP-hDAT cells) (17). Cells were maintained at 37 °C in a humidified 5% CO\(_2\) atmosphere. Confluent cells in 75-cm\(^2\) flasks were preincubated with TAT-C24WT (1 \(\mu\)M), TAT-C24Scr (1 \(\mu\)M), or vehicle for 1 h before the cells were rinsed in Tris-buffered saline (TBS) followed by solubilization in ice-cold lysis buffer containing 1% (v/v) Triton X-100, 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM sodium orthovanadate, 5 mM NaF, 5 mM sodium pyrophosphate, and a protease inhibitor mixture.
DAT C-terminal Interactions and AMPH-evoked DA Release

(Roche Diagnostics). Cell lysates were centrifuged at 16,000 \( \times g \) for 15 min at 4 °C to remove cell debris and supernatant was then diluted to 1 mg/mL. The supernatant was incubated with rat anti-DAT antibody (MAB369 1:1000, Millipore) for 1 h at 4 °C during constant rotation. 25 \( \mu l \) of protein-G agarose beads (Roche Diagnostics) were washed once with lysis buffer and then incubated with supernatant for 2 h at 4 °C during constant rotation. As control for these experiments, pulldown without primary antibody and cell lysates without TAT-C24WT/Scr preincubation were performed in parallel. Subsequently, beads were washed twice in TBS containing 0.1% (v/v) Triton X-100 and then eluted using 5\( \times \) loading buffer. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes. Membranes were blocked in PBS containing 0.05% Tween 20 and 5% dry milk and incubated overnight with antibodies against rabbit phosphorylated CaMKII (Thr-286), (1:1000, Santa Cruz Biotechnology). Following incubation with HRP-conjugated anti-rabbit antibodies, the blots were visualized by chemiluminescence (ECL kit, Amersham Biosciences). To normalize pulldown of pCaMKII, blots were stripped and re-probed for rat anti-DAT (MAB369, 1:1000, Millipore) to determine DAT immunoprecipitation for each condition. CaMKII\( \alpha \) co-IP were normalized to TAT-C24Scr for each experiment, and then a one-sample t test was used for statistical analysis (\( p < 0.05 \)).

Biotinylation of Surface Proteins—T-Rex 293 CaMKII\( \alpha \) YFP-hDAT cells were seeded 2 days before the experiment in precoated 6-well plates at a density of 500,000 cells/well. On the day of the experiment, cells were preincubated with 1 \( \mu M \) TAT-C24WT, TAT-C24Scr, or vehicle (control) (without peptide treatment) for 1 h. Cells were rinsed twice in cold PBS and then incubated with sulfo-NHS-biotin (Thermo Scientific) for 40 min on ice. Rinsing twice in 100 mM glycine was used to quench excess biotin, and this was followed by additional washing. Cells were lysed in 500 \( \mu l \) of solubilization buffer containing 1% (v/v) Triton X-100, 25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 5 mM N-ethylmaleimide, and a protease inhibitor mixture (Roche Diagnostics). Cell lysates were centrifuged at 16,000 \( \times g \) for 15 min at 4 °C to remove cell debris, and supernatant was collected. An aliquot was removed to determine protein concentration using BCA assay. 175 \( \mu l \) of avidin beads (Thermo Scientific) were washed once in 0.8 ml of lysis buffer, and 300-\( \mu l \) protein extracts were then incubated with the avidin beads overnight at 4 °C during constant rotation. The beads were washed four times in lysis buffer and then eluted using SDS sample buffer containing 100 mM DTT. Total protein extracts and eluted proteins were separated on an SDS-PAGE and transferred to Immobilon-P membranes. Membranes were blocked in PBS containing 0.05% Tween 20 and 5% dry milk and incubated overnight with a monoclonal rat anti-DAT antibody (MAB369, Millipore). Following incubation with an anti-rat horseradish peroxidase-conjugated secondary antibody, the blots were visualized by chemiluminescence (ECL-kit, Amersham Biosciences) and quantified using ImageJ.

DA Uptake—T-Rex 293 CaMKII\( \alpha \) YFP-hDAT cells were seeded in 24-well plates at 100,000 cells/well on the day prior to the assay. On the day of the experiment, cells were preincubated with TAT-C24WT and Tat-C24Scr at a final concentration of 1 \( \mu M \) for 30 min and then rinsed once in uptake buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 1 mM L-ascorbic acid, 5 mM D-glucose, and 10 nM catechol-O-methyltransferase inhibitor (RO-41-0960), pH 7.4). After addition of prewarmed uptake buffer and unlabeled DA or AMPH at given concentrations, uptake was initiated by addition of labeled DA (91.1 Ci/mmol) (PerkinElmer Life Sciences). Following 5 min of incubation at 37 °C, reaction was terminated by washing the cells twice with ice-cold uptake buffer followed by cell lysis in 1% SDS. All samples were transferred to 24-well counting plates, followed by addition of Optiphase Hisafe scintillation fluid (PerkinElmer Life Sciences). Samples were finally counted in a Wallac Tri-Lux \( \beta \)-scintillation counter (PerkinElmer Life Sciences). Uptake data were analyzed by nonlinear regression analysis using GraphPad Prism. The IC\(_{50}\) values used in the estimation of \( K_m \) and \( V_{max} \) values for uptake were calculated from the means of IC\(_{50}\) values and the S.E. interval of the IC\(_{50}\) ± S.E.

Cell Culture and MPP\(^+\) Efflux—In vitro experiments were carried out using the T-Rex 293 cells with stable expression of human DAT and CaMKII\( \alpha \) (in a tetracycline-dependent manner) (T-Rex 293 CaMKII\( \alpha \) FLAG-hDAT cells) as described previously (17). Efflux experiments were performed in a superfusion system as described using MPP\(^+\) as substrate (27). Cells (20 \( \times 10^5 \)) were seeded on glass coverslips (5 mm in diameter) and coated with poly-d-lysine (Sigma), and at this time point (1 day before the experiment), tetracycline was added to induce CaMKII\( \alpha \) expression (17). Prior to the experiment, 0.3 \( \mu M \) of the radioactive labeled DAT substrate [\(^3\)H]MPP\(^+\) (PerkinElmer Life Sciences) was added to the cells and incubated for 30 min at 37 °C. Some coverslips received freshly dissolved TAT peptides (TAT-C24WT, TAT-C24Scr, and TAT-C24AAA) at a final concentration of 50 nM. The coverslips were transferred to small superfusion chambers (volume = 0.2 ml) and superfused with Krebs-Ringer HEPES buffer at room temperature (25 °C) at a rate of 0.7 ml/min for 45 min to establish a stable efflux line (= basal efflux). After basal efflux was stabilized, the experiment was initiated with the collection of fractions every 2 min. After 6 min, 15 \( \mu M \) KN-93 (Calbiochem), a CaMKII-inhibitor, was added to the buffer of the channels indicated; the remaining channels received buffer. After 10 min, AMPH (3 \( \mu M \), Sigma) was added to all buffers. Finally, the remaining radioactivity was recovered by superfusing the cells for 6 min with 1% SDS. AMPH-induced release of radioactivity was calculated as percentage of radioactivity present in the cell at any given time point.

Chronoamperometry—In vivo high speed chronoamperometry was conducted using the FAST-12 system (Quanteon LLC) as described previously with some modifications (28, 29). Recording electrode/micropipette assemblies were constructed using a single carbon fiber (30 \( \mu M \) diameter (Specialty Materials), which was sealed inside using fused silica tubing (Schott, North America). The exposed tip of the carbon fiber (150 \( \mu M \) in length) was coated with 5% Nafion (Aldrich) (3–4 coats baked at 200 °C for 5 min per coat) to provide a 1000-fold selectivity of DA over its metabolite dihydroxyphenylacetic acid. Under these conditions, microelectrodes displayed linear amperometric responses to...
0.25–10 μM DA during in vitro calibration in 100 mM phosphate-buffered saline, pH 7.4.

Male C57Bl/6 mice weighing between 25 and 30 g were anesthetized by intraperitoneal injection (10 ml/kg body weight) of a mixture of urethane (70 mg/ml) and α-chloralose (7 mg/ml), then fitted with an endotracheal tube to facilitate breathing, and placed into a stereotaxic frame (David Kopf Instruments). Body temperature was maintained by a water-circulated heating pad. To locally deliver test compounds (see below) close to the recording site, a glass multibarrel micropipette (FHC) was positioned adjacent to the microelectrode using sticky wax (Moyco). The center-to-center distance between the microelectrode and the micropipette ejector was ~200 μm. The micropipette was filled with AMPH (800 μM, Sigma), the peptide of interest (20 μM) TAT-C24WT, TAT-C24Scr, TAT-C24AAA, or vehicle (aCSF). Artificial CSF and AMPH solutions were prepared fresh each day. The peptides were initially dissolved in DMSO to make 20 mM stock solutions and then frozen. On the day of the experiment, peptide stocks were diluted to 20 μM (0.1% DMSO) in fresh aCSF. The electrode/micropipette assembly was lowered into the striatum at the following coordinates (in mm from bregma, Ref. 50): anterior/posterior, +1.1; medial/lateral, ±1.4; dorsal/ventral, –2.25.

The application of drug solutions was accomplished using a Picospriiter II (General Valve Corp.) in an ejection volume of 125 nl for AMPH to deliver 100 pmol, and 50 or 100 nl for peptides to deliver 1 or 2 pmol (5–25 p.s.i. for 0.25–3 s). After ejection of test agents, there is an estimated 10–200-fold dilution caused by diffusion through the extracellular matrix to reach a concentration of 4–80 μM (AMPH) or 0.1–2 μM (peptide of interest) at the recording electrode (30). It is worth noting that the estimated concentration of AMPH reaching the recording electrode approximates those reported in striatum (2–10 mM), as measured by microdialysis and HPLC, after a single, behaviorally effective systemic injection (31). To record the AMPH-evoked efflux of DA at the active electrode, oxidation potentials consisting of 100-ms pulses of 550 mV, each separated by a 1-s interval during which the resting potential was maintained at 0 mV, were applied with respect to a Ag/AgCl reference electrode implanted into the contralateral superficial cortex. Oxidation and reduction currents were digitally integrated during the last 80 ms of each 100-ms voltage pulse. For each recording session, DA was identified by its reduction/oxidation current ratio of 0.55–0.80.

AMPH and peptides were ejected according to the following sequence. AMPH was pressure-ejected intrastriatally to evoke the release of DA. As soon as DA was cleared from extracellular fluid, typically 5–30 min after AMPH application, the peptide of interest or equivalent volume of aCSF was locally applied to striatum, and 45 min and 90 min later the same amount of AMPH was again pressure-ejected.

At the termination of each experiment, an electrolytic lesion was made to mark the placement of the recording electrode tip. Mice were then decapitated while still anesthetized, and their brains were removed, frozen on dry ice, and stored at ~80 °C until sectioned (20 μm) for histological verification of electrode location within the striatum. Data were analyzed using one-way ANOVA with Dunnett’s multiple comparison test (GraphPad Prism) using the maximal signal amplitude of the released DA (in micromolars) to define AMPH-evoked DA release.

**Results**

**Intracellular Localization of Cell-permeable DAT C-terminal Peptides in HEK293 Cells.—**We have previously shown evidence for a direct interaction between CaMKIIα and the distal 24 residues of the DAT C terminus and that this interaction might be critical for AMPH-induced DA efflux in cells (17). To further assess the significance of the DAT C terminus for AMPH-induced DA efflux, we decided to investigate whether a C-terminal peptide corresponding to the CaMKIIα binding domain would be able to act in a dominant-negative fashion and blunt the response to AMPH by impairing CaMKIIα binding. The peptide was made membrane-permeable by fusing to the N terminus of the cell membrane transduction domain of the human immunodeficiency virus type 1 (HIV-1) Tat protein (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) (Fig. 1A) (32, 33). Previous reports have successfully used TAT-conjugated dominant-negative peptides to efficiently perturb particular protein-protein interactions (32–36). In addition to a TAT-conjugated WT peptide (TAT-C24WT), we generated an equivalent scrambled peptide (TAT-C24Scr) (Fig. 1A). Moreover, we generated biotinylated variants to assess cell membrane permeability. Importantly, both peptides accumulated efficiently in HEK293 cells as determined by visualizing the peptides with fluorescently tagged streptavidin (fluorescein streptavidin) and confocal microscopy (Fig. 1B).

**TAT-C24WT Peptide Blocks CaMKIIα Interaction with DAT—**CaMKIIα associates with the DAT C terminus as shown in a pulldown assay using C-terminal glutathione S-transferase fusion proteins containing the last 24 residues (C24) of DAT (17). The interaction has been further supported by co-immunoprecipitation in both heterologous cells and in synaptosomal preparations (17, 26). Here, we show that pCaMKIIα is observed in immunoprecipitates from T-Rex 293 CaMKIIα YFP-hDAT cells obtained with rat anti-DAT antibody, thus substantiating an association of phosphorylated CaMKIIα with the DAT C terminus (Fig. 2). Pretreatment of...
the cells with TAT-C24WT strongly inhibited the amount of co-immunoprecipitated pCaMKIIα, whereas TAT-C24Scr showed significantly less efficacy (*, p < 0.05, one-sample t-test, Fig. 2). In agreement with our prediction, these data support that TAT-C24WT, but not the scrambled TAT-C24WT peptide, is capable of blocking the CaMKIIα-DAT interaction in living cells.

AMPH-induced MPP⁺ Efflux in Heterologous Cells Co-expressing DAT and CaMKIIα Is Attenuated by TAT Peptides Mimicking the DAT C Terminus—We then assessed the effect of the TAT-C24 peptides on AMPH-induced [³H]MPP⁺ efflux in T-Rex 293 CaMKIIα FLAG-hDAT cells induced with tetracycline (Fig. 3, A and B). We have previously shown by immunoblotting that CaMKIIα is robustly induced in these cells following tetracycline treatment without influencing DA uptake (17). Cells were preloaded with [³H]MPP⁺, a hydrophilic DAT substrate resistant to enzymatic degradation and with minimal nonspecific diffusion (27). Cells were then superfused, and the experiment was initiated with collection of 2-min fractions; AMPH (3 µM) was added after 10 min. Cells were treated with TAT-C24WT (50 nM), TAT-C24Scr (50 nM), or vehicle during preloading sequence. AMPH-evoked [³H]MPP⁺ efflux was significantly attenuated by preincubation with the TAT-C24 WT peptide (*, p < 0.05, Fig. 3A). Importantly, TAT-C24Scr had no inhibitory effect on efflux, validating the efficacy of the WT peptide (Fig. 3A). These results were compared with experiments employing the CaMKII inhibitor, KN-93 (15 µM), which markedly decreased AMPH-induced efflux (***, p < 0.0001, Fig. 3B). KN-93 served as a control to show that AMPH-induced efflux of [³H]MPP⁺ could be dramatically decreased as shown previously (17). Furthermore, preincubation with both TAT-C24WT and KN-93 did not produce an additive inhibitory effect compared with KN-93 alone, indicating that their effects likely are exerted via the same pathway (Fig. 3B).

We also tested the effect of the peptides on [³H]DA uptake in the T-Rex 293 CaMKIIα YFP-hDAT cells. These experiments showed that both DA uptake and the apparent affinity of DA for DAT are not affected by application of cell-permeable peptides mimicking the DAT C terminus; TAT-C24WT and TAT-C24Scr peptides were applied 30 min prior to DA uptake, and there was no significant effect on uptake capacity (V_max) and the K_m value for DA uptake (Fig. 3, C and D). This excludes
Acute effects of the peptides on DAT catalytic activity and implies that TAT-C24WT selectively impaired AMPH-induced [3H]MPP\(^+\) efflux in DAT-expressing cells consistent with a dominant-negative effect on the DAT-CaMKII interaction. To exclude any effect of the peptides on AMPH interaction with the transporter, we also performed competition of [3H]DA uptake experiments with AMPH. These experiments suggested unaltered AMPH affinity for DAT upon preincubation with TAT peptides (\(K_i\) for AMPH after TAT-C24WT pretreatment, 1.8 \(\mu\)M; \(K_i\) for AMPH after TAT-C24Scr pretreatment, 0.8 \(\mu\)M; \(K_i\) control, 1.6 \(\mu\)M, \(n = 2\)).

Finally, we wanted to exclude that TAT-C24WT or TAT-C24Scr directly affected trafficking and surface expression of the transporter. Cells were treated for 1 h with TAT-C24WT, TAT-C24Scr, or vehicle before assessing DAT surface expression using a surface biotinylation protocol. As shown in Fig. 3E,
**DAT C-terminal Interactions and AMPH-evoked DA Release**

**FIGURE 4.** AMPH-evoked DA efflux in striatum following administration of TAT-C24 DAT peptides mimicking DAT C terminus. Representative oxidation currents (converted to micromolar values) produced by pressure ejection of AMPH into the striatum. A, oxidation currents before and 90 min following application of TAT-C24Scr show no effect on DA release. B, oxidation currents before and 90 min following application of TAT-C24WT demonstrate substantial decrease in DA efflux. C, oxidation currents before and 90 min following co-application of TAT-C24WT and KN-93. D, fractional AMPH-induced DA release (A) for local application of vehicle (acSF) (n = 10), TAT-C24Scr (n = 11), TAT-C24WT (n = 14), KN-93 (n = 9), and C24WT+KN-93 (n = 3); *p < 0.05, one-way ANOVA with Dunnett’s multiple comparison test.

Immunoblots for surface-expressed DAT and total DAT levels revealed no effect of the TAT peptides.

**TAT Peptides Mimicking the DAT C Terminus Inhibit DA Efflux in the Striatum**—We utilized high speed chronoamperometric recordings in striatum to assess kinetics of DA release in vivo following administration of TAT peptides mimicking the DAT C terminus. AMPH was pressure-ejected intrastriatally to evoke the release of DA. As soon as DA was cleared from the extracellular fluid, typically 5–30 min after AMPH application, the peptide of interest or equivalent volume of acSF (vehicle) was locally applied to the striatum, and 45 and 90 min later, the same amount of AMPH was again pressure-ejected. Representative traces of DA efflux in mouse striatum upon AMPH stimulation are shown in Fig. 4, A–C. Application of TAT-C24Scr did not influence DA release while TAT-C24WT inhibited release (Fig. 4, A and B). In agreement with our previous findings (17), administration of the CaMKII inhibitor KN-93 reduced DA release (Fig. 4D). The inhibition was similar to that seen in response to TAT-C24WT and, importantly, combined administration of TAT-C24WT and KN-93 resulted in a similar attenuation of DA efflux compared with KN-93 alone suggesting no additive effect (Fig. 4, C and D). Data are presented as fractional AMPH-induced DA release to illustrate a significant decrease in DA efflux upon WT peptide (TAT-C24WT) and KN-93 administration, whereas vehicle (acSF) and scrambled peptide (TAT-C24Scr) did not have any effect (Fig. 4D). This is consistent with data from the heterologous cells and shows that disrupting interactions with DAT C terminus by cell-permeant peptides significantly decreases DA efflux in mouse striatum upon AMPH challenge.

Because the DAT C terminus contains a prototypic PDZ-binding sequence known to interact with the PDZ domain protein PICK1 (16, 18), the TAT-C24WT peptide might also compete for binding of PDZ domain proteins to DAT in vivo. To address this issue, we generated an additional peptide, TAT-C24AAA, where the C-terminal PDZ target sequence (LLV) was substituted for three alanines (-AAA) resulting in abolished PDZ-mediated protein-protein interactions (18). The triple alanine substitution does not, however, affect CaMKII binding (17). The peptide should therefore only associate with CaMKIIα and not PICK1 since the PDZ motif has been disrupted. First, we investigated the effect of the peptide in T-Rex 293 CaMKIIα FLAG-hDAT cells. The cells were preloaded with [3H]MPP⁺ before superfusion and initiation of the experiment with a collection of 2-min fractions. During the preloading sequence, cells were treated with either TAT-C24AAA (50 nM), TAT-C24Scr (50 nM), or vehicle, and AMPH was added after 10 min. Intriguingly, AMPH-induced [3H]MPP⁺ efflux was significantly reduced following preincubation with the TAT-C24AAA peptide compared with vehicle and TAT-C24Scr (control peptide) (***, p < 0.001, Fig. 5A).

Chronoamperometric recordings were then performed to assess the effect of TAT-C24AAA on DA release in response to AMPH challenge. Representative traces for DA release in striatum before (base line) and 90 min following TAT-C24AAA peptide administration demonstrate a decrease in DA efflux (Fig. 5B). Fractional AMPH-induced DA release showed a more pronounced attenuation 90 min following TAT-C24AAA administration compared with 45 min, whereas no effect was seen for vehicle (*, p < 0.05, Fig. 5C). Hence, preadministration of the TAT-C24AAA peptide markedly reduces AMPH-evoked DA release in striatum as determined by in vivo high speed chronoamperometry.

**TAT-C24WT Peptide Attenuates AMPH-induced Hyperactivity**—Several previous reports have shown efficient delivery of TAT-conjugated peptides across the blood-brain barrier and into the brain tissue upon systemic administration (32, 34, 35). We therefore wished to investigate whether in vivo administration of TAT-C24WT would be able to impair AMPH-induced locomotor hyperactivity in mice. To investigate the effect of TAT-C24WT and TAT-C24Scr peptides on basal locomotion per se, mice were administered 100 μg of TAT-C24WT or TAT-C24Scr in the tail vein (intravenously). Saline (SA) solution or AMPH (2 mg/kg) was administered intraperitoneally. To allow sufficient time for peptide accumulation in brain tissue, 45 min was permitted to elapse before beginning a 60-min period of locomotor activity recordings. This time window has been shown to be sufficient to allow peptide accumulation in brain tissue (35). Our data showed that the membrane-permeable peptides did not affect locomotor activity per se (C24WT/SA, C24Scr/SA, Fig. 6). However, mice pretreated with TAT-C24Scr and subsequently challenged with AMPH showed a robust increase in locomotor activity that peaked 30 min after...
AMPH injection. Administration of the TAT-C24WT peptide significantly attenuated the AMPH-induced locomotor hyperactivity compared with treatment with TAT-C24Scr. Data are means ± S.E.; *, p < 0.05; **, p < 0.01 TAT-C24Scr/AMPH versus TAT-C24WT/AMPH; *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus own saline group; Bonferroni post hoc tests are after repeated measures two-way ANOVA (n_24WT/AMPH = 16 and n_24Scr/AMPH = 10). Note that administration of TAT peptides prior to saline administration did not affect locomotor activity per se (n_24WT/SA = 11 and n_24Scr/SA = 11).

**DISCUSSION**

DAT-mediated reverse transport of DA is critical for the addictive and rewarding properties of AMPH. AMPH promotes DA efflux by inhibiting loading of cytoplasmic DA into vesicles causing an accumulation of cytosolic DA and subsequent reverse transport of DA via DAT into the synaptic cleft (9). We have previously shown evidence that CaMKIIα plays a role in AMPH-induced DA efflux (17). CaMKIIα was found to bind the DAT C terminus, which in turn was suggested to facilitate phosphorylation of the DAT N terminus and mediate the AMPH-induced DA efflux (17). Recently, it was observed that AMPH-induced efflux is impaired in synaptosomes from mice with ablated CaMKIIα activity, including a mouse model of the neurogenetic disease known as Angelman syndrome (26). In addition, studies in *Drosophila* have supported a role of DAT N-terminal phosphorylation in AMPH-induced DA efflux *in vivo* (37). To further characterize the role of the DAT C terminus and CaMKIIα in AMPH-evoked DA efflux, we employ here dominant-negative peptides to disrupt DAT C-terminal protein-protein interactions. In agreement with our hypothesis, we find that the cell-permeant peptides mimicking the DAT C terminus can reduce AMPH-evoked MPP⁺ and DA release both *in vitro* and *in vivo*. By use of co-immunoprecipitation experiments, we were also able to validate that indeed the WT, but not a scrambled peptide, was able to block the association between CaMKIIα and DAT.

In heterologous cells, preloaded with [³H]MPP⁺ and expressing both DAT and CaMKIIα, we found that the TAT-C24WT peptide, but not the control peptide (TAT-C24Scr), efficiently attenuated AMPH-induced efflux. This is consistent with a dominant-negative action of the peptide preventing binding of CaMKIIα to the endogenous DAT C terminus. The inability of TAT-C24WT to further inhibit [³H]MPP⁺ efflux upon KN-93-mediated blockade of CaMKIIα activity further supports this conclusion, *i.e.* no additive effect was seen. The peptides had no effect on DA uptake and DAT surface levels, according to uptake and surface biotinylation experiments, respectively, consistent with no effect of the peptides on transporter turnover rate and transporter trafficking. A recent study analyzed the effect of a corresponding DAT peptide, and this peptide also showed no effect on DA uptake in the WT transporter (38).

To assess the effects of the peptides on DA release *in vivo*, we utilized high speed chronamperometry providing a high time resolution on release kinetics compared with microdialysis (39). AMPH-evoked DA efflux in striatum was significantly reduced following application of TAT-C24WT, whereas efflux was not altered upon administration of vehicle or TAT-C24Scr. Equivalently, AMPH-induced locomotor hyperactivity in mice was substantially lower upon *in vivo* administration of TAT-

**FIGURE 5. Role of DAT PDZ domain interactions for AMPH-evoked DA efflux.** A, T-Rex 293/CaMKIIα FLAG hDAT cells were preloaded with [³H]MPP⁺ and superfused, and the experiment was initiated with collection of 2-min fractions; during the preloading sequence, cells were treated with either TAT-C24Scr (50 nM), TAT-C24AAA (50 nM), or vehicle. AMPH (3 μM) was added after 10 min. Data are presented as areas-under-curve (AUC, arbitrary units) and show significant reduction in efflux upon TAT-C24AAA treatment (means ± S.E. of 12 observations from 3 experimental days); ***, p < 0.001 one-way ANOVA with Bonferroni’s multiple comparison test. B, representative oxidation currents (converted to micromolar values) produced by intrastriatal pressure ejection of AMPH before and 90 min following application of TAT-C24AAA. C, data are presented as fractional AMPH-induced DA efflux after local application of vehicle (aCSF) (n = 10), and TAT-C24AAA (n = 8); *, p < 0.05, one-way ANOVA with Dunnett’s multiple comparison test.

**FIGURE 6. Effect of TAT-C24 DAT peptides mimicking DAT C terminus on AMPH-induced locomotor hyperactivity.** Intravenous administration of TAT-C24WT peptide prior to AMPH administration significantly attenuates the AMPH-induced hyperactivity compared with treatment with TAT-C24Scr. Data are means ± S.E.; #, p < 0.05; ##, p < 0.01 TAT-C24Scr/AMPH versus TAT-C24WT/AMPH; *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus own saline group; Bonferroni post hoc tests are after repeated measures two-way ANOVA (n_24WT/AMPH = 16 and n_24Scr/AMPH = 10). Note that administration of TAT peptides prior to saline administration did not affect locomotor activity per se (n_24WT/SA = 11 and n_24Scr/SA = 11).
C24WT as compared with TAT-C24Scr. Altogether, the data are consistent with a critical role of C-terminal protein-protein interactions for AMPH-induced efflux and AMPH-induced locomotor hyperactivity.

Interestingly, the PDZ domain of PICK1 can bind both the DAT C terminus and the C terminus of protein kinase Cα (PKCα), and thus PICK1 could potentially act as a scaffold to bring PKCα in close proximity of the DAT N terminus. Notably, such a function of PICK1 has been proposed in relation to its binding to ASIC1/2 (acid-sensing ion channels 1/2) and the GluR2 subunit of the AMPA receptor (40, 41). It has also previously been suggested that PKC isoforms, including PKCα and PKCβ, might play a role in AMPH-mediated DA efflux (17, 23, 42–44). Furthermore, the dominant-negative TAT-C24WT peptide would be predicted to abolish not only the association of CaMKIIα to endogenous DAT but also the association of the PDZ domain protein PICK1 (17). It is therefore important that we assess the effect of a TAT peptide containing a disrupted PDZ-binding motif (TAT-C24AAA), and we found that this attenuated [3H]MPP+ release to a similar extent as TAT-C24WT. This finding in heterologous cells was further supported by chronoamperometric recordings in striatum preinjected with dominant-negative TAT-C24AAA peptide, i.e., the chronoamperometric recordings showed markedly impaired DA release upon AMPH challenge. Although a role in efflux of interactions with PDZ domain proteins like PICK1 still cannot be excluded, the data do suggest that PDZ domain interactions are less important for DA efflux compared with the association of CaMKIIα to the DAT C terminus.

Protein-protein interactions involving the DAT N terminus have also been shown to play an important role in AMPH-evoked DA efflux. A recent study demonstrated that AMPH stimulates binding of syntaxin1A to the DAT N terminus in a CaMKII-dependent manner in both heterologous cells and synaptosomal preparations. Inhibition of CaMKIIα activity using a membrane-permeable CaMKIIα inhibitor decreased the interaction between DAT and syntaxin1A. Importantly, similar effects were obtained by inhibiting CaMKIIα with KN-93, and it was hypothesized that AMPH-mediated activation of CaMKIIα increases DAT-syntaxin1A interactions resulting in reversed operation of DAT (45). This is in accordance with our data showing attenuated DA release upon TAT peptides abolishing DAT C-terminal interactions, and it emphasizes the importance of CaMKIIα for AMPH-induced DA efflux. However, the mechanisms by which N-terminal phosphorylation and syntaxin1A binding mechanistically can alter the function of the transporter and cause reverse transport is still not understood. It was suggested that N-terminal phosphorylations shift the transporter from a reluctant to a willing state for AMPH-induced DA efflux (22). It has been speculated that this involves a conformational change in the transporter that might favor substrate efflux by increasing the intracellular affinity of DA and/or sodium for DAT (46). Alternatively, the phosphorylations may promote a channel-like mode of the transporter leading to rapid bursts of DA efflux (47). The conformational flexibility of the N terminus for AMPH-induced efflux might also be important; hence, tethering the N terminus of SERT to the single transmembrane domain Tac (α-subunit of the interleukin-2 receptor) was found to strongly impair AMPH-induced efflux (48).

Intriguingly, the present data suggest that the effects of psychostimulants can be modulated by targeting DAT protein-protein interactions. Our data also corroborate the use of the TAT sequence as a simple way of solving the challenging problem of targeting peptides to the intracellular compartment. The TAT sequence is derived from the HIV-1 Tat protein and is capable of promoting intracellular accumulation of even larger proteins such as the β-galactosidase protein (32). There are now several examples substantiating the usefulness of the TAT sequence in relation to both studies of protein-protein interactions and in brain disease. Of specific interest for this study, a TAT peptide containing an internal sequence from the C terminus of the GluR2 subunit of the AMPA-type ionotropic glutamate receptor was shown to partially blunt behavioral sensitization to AMPH. The synthetic GluR2-derived peptide blocked regulated AMPA receptor endocytosis (35). Also, TAT fusion peptides with sequences from the C terminus of the N-methyl-D-aspartate (NMDA)-type ionotropic glutamate receptor were shown to perturb NMDA receptor-PSD-95 interaction and thereby attenuate ischemic brain damage (33). Targeting the PDZ domain(s) of PSD-95 represents a pharmacological strategy for treatment of acute stroke (33, 36).

Several DAT-interacting proteins have been identified implying that DAT is associated with a multiprotein network within the presynaptic terminal that controls critical aspects of DAT function (4, 14). A recent investigation showed that the Ras-like GTPase Rin interacts with the DAT and is required for PKC-mediated DAT internalization (49). The authors speculate that Rin may be involved in CaMKII activation, thereby representing a putative link between DAT, CaMKII, and MAPK signaling. Altogether, it is becoming increasingly clear that DAT is subject to complex and delicate regulatory mechanisms that govern trafficking and catalytic activity of the protein via complex protein-protein interactions and post-translational modifications. Some interactions and pathways might even serve dual roles such as CaMKIIα and protein kinase C, for example, that have both been suggested to be involved in controlling trafficking and DA efflux (13, 17, 23, 42–44). Further studies are required to dissect this complicated interplay. In this study, we have focused on one particular protein-protein interaction and have shown how its modulation is likely to change reverse transport and therefore might represent a strategy for therapeutic intervention in relation to abuse of AMPHs. It is interesting to consider how future detailed characterization of other DAT protein-protein interactions might identify new peptide targets and thereby new putative strategies for treating diseases characterized by dopaminergic dysfunction.

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DAT C-terminal Interactions and AMPH-evoked DA Release

304–312

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