⌘ *Author's Choice*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 35, pp. 29627–29635, August 24, 2012

Ca^{2+}/Calmodulin-dependent Protein Kinase IIα (αCaMKII) Controls the Activity of the Dopamine Transporter

IMPLICATIONS FOR ANGELMAN SYNDROME[*] [S]

Received for publication, March 30, 2012, and in revised form, June 13, 2012 Published, JBC Papers in Press, July 9, 2012, DOI 10.1074/jbc.M112.367219

Thomas Steinkellner[‡], Jae-Won Yang[‡], Therese R. Montgomery[‡§1], Wei-Qiang Chen[¶], Marie-Therese Winkler[‡], Sonja Sucic[‡], Gert Lubec[¶], Michael Freissmuth[‡], Ype Elgersma[‖], Harald H. Sitte[‡2], and Oliver Kudlacek[‡]

From the [‡]Institute of Pharmacology, Center for Physiology and Pharmacology, and [¶]Neuroproteomics Division, Department of Pediatrics, Medical University of Vienna, A-1090 Vienna, Austria, the [§]School of Biomolecular and Biomedical Science, University College Dublin, Dublin 4, Ireland, and the [‖]Department of Neuroscience, Erasmus University Medical Center, NL-3000 CA Rotterdam, The Netherlands

Background: αCaMKII modulates amphetamine-induced dopamine transporter-mediated substrate efflux.
Results: Mice with ablated or blunted αCaMKII function show decreased amphetamine-triggered efflux.
Conclusion: Dopamine transporter function is impaired in mice with targeted αCaMKII mutations and in a mouse model of the Angelman syndrome.
Significance: Such new insights into dopamine transporter function may further illuminate the complex pathophysiology of the Angelman syndrome.

The dopamine transporter (DAT) is a crucial regulator of dopaminergic neurotransmission, controlling the length and brevity of dopaminergic signaling. DAT is also the primary target of psychostimulant drugs such as cocaine and amphetamines. Conversely, methylphenidate and amphetamine are both used clinically in the treatment of attention-deficit hyperactivity disorder and narcolepsy. The action of amphetamines, which induce transport reversal, relies primarily on the ionic composition of the intra- and extracellular milieus. Recent findings suggest that DAT interacting proteins may also play a significant role in the modulation of reverse dopamine transport. The pharmacological inhibition of the serine/threonine kinase αCaMKII attenuates amphetamine-triggered DAT-mediated 1-methyl-4-phenylpyridinium (MPP^+) efflux. More importantly, αCaMKII has also been shown to bind DAT *in vitro* and is therefore believed to be an important player within the DAT interactome. Herein, we show that αCaMKII co-immunoprecipitates with DAT in mouse striatal synaptosomes. Mice, which lack αCaMKII or which express a permanently self-inhibited αCaMKII ($\alpha CaMKII^{T305D}$), exhibit significantly reduced amphetamine-triggered DAT-mediated MPP^+ efflux. Additionally, we investigated mice that mimic a neurogenetic disease known as Angelman syndrome. These mice possess reduced αCaMKII activity. Angelman syndrome mice demonstrated an impaired DAT efflux function, which was comparable with that of the αCaMKII mutant mice, indicating that DAT-mediated dopaminergic signaling is affected in Angelman syndrome.

The dopamine transporter (DAT)[3] is a member of the SLC6 gene family of neurotransmitter:sodium symporters. DAT mediates the reuptake of extracellular dopamine from the synaptic cleft into the presynaptic neuron (1–3), thus terminating dopaminergic neurotransmission (4, 5). DAT dysfunction has been associated with various neuropsychiatric diseases such as schizophrenia, Parkinson disease, attention deficit hyperactivity disorder, and drug addiction (6, 7).

DAT is also the primary target of a number of different illicit psychostimulant drugs such as cocaine and amphetamine. Most amphetamines, with the notable exception of methylphenidate, are DAT substrates and thereby induce a reversal of the normal transport process, ultimately resulting in the non-exocytotic transporter-mediated efflux of substrate (8, 9). An increase in the concentration of intracellular sodium is a known prerequisite of transporter reversal (10). However, the exact mechanistic details of reverse transport remain enigmatic. The role of DAT-interacting proteins in non-exocytotic transporter-mediated efflux has become more and more apparent recently, and they are now believed to be intricately involved in this process (2). For example, protein kinase Cβ (PKCβ) has previously been described as a regulator of DAT efflux (11, 12), and more recently, it was shown that the serine/threonine kinase Ca^{2+}/calmodulin-dependent protein kinase IIα (αCaMKII) binds to the DAT carboxyl terminus and phosphorylates its amino terminus (13). Furthermore, the pharmacological inhibition of αCaMKII by KN93 attenuates amphetamine-triggered DAT-mediated substrate

* This work was supported by the Austrian Science Foundation/FWF Grants SFB35 (to H. H. S. and M. F.) and DK W1232-B11 (to H. H. S.).
⌘ *Author's Choice*—Final version full access.
[S] This article contains supplemental Fig. 1.
[1] Recipient of a Health Research Board (HRB)-Marie Curie postdoctoral mobility fellowship.
[2] To whom correspondence should be addressed: Medical University Vienna, Center of Physiology and Pharmacology, Institute of Pharmacology, Waehringerstrasse 13a, A-1090 Vienna, Austria. Tel.: 43-1-4277-64123; Fax: 43-1-4277-9641; E-mail: harald.sitte@meduniwien.ac.at.

[3] The abbreviations used are: DAT, dopamine transporter; αCaMKII, calmodulin kinase IIα; AS, Angelman syndrome; AUC, area under the curve; ANOVA, analysis of variance; CFT, 2-carbo-methoxy-3-(4-fluorophenyl)-tropane.

efflux in human embryonic kidney cells (HEK293) and in rat striatal slices (13).

Angelman syndrome is a neurogenetic disease that arises from a defective maternally inherited allele in ubiquitin ligase Ube3A (14). The clinical phenotype of Angelman syndrome (AS) includes movement disorders and autism spectrum disorders, which collectively establish a connection to the dopaminergic system. Elucidating the underlying pathophysiology of Angelman syndrome is of vast therapeutic importance as there is, as yet, no effective pharmacotherapy available. Mice engineered to be maternally Ube3A-deficient ($Ube3A^{p+/m-}$) have previously been used as an animal model of human Angelman syndrome (15); $Ube3A^{p+/m-}$ causes the accumulation of autoinhibited αCaMKII in the brain (16). Because αCaMKII binds to the carboxyl terminus of the DAT and phosphorylates its amino terminus (13) to afford amphetamine-induced efflux (10), we reasoned that $Ube3A^{p+/m-}$ mice may phenocopy the effect of αCaMKII deficiency on DAT.

EXPERIMENTAL PROCEDURES

Reagents—D-Amphetamine-sulfate and methylphenidate were purchased from Sigma Aldrich; [^{3}H]2-carbo-methoxy-3-(4-fluorophenyl)tropane (CFT; 85.9 Ci/mmol) was obtained from PerkinElmer Life Sciences, and [^{3}H]MPP^+ (85 Ci/mmol) was provided by American Radiolabeled Chemicals. KN93 and autocamtide-2 related inhibitory peptide II were from Calbiochem. The cell culture media, supplements, and antibiotics were from Invitrogen. The rat anti-dopamine transporter antibody (MAB369) was provided by Millipore. The goat anti-dopamine transporter (C-20), mouse monoclonal anti-αCaMKII, and rabbit polyclonal anti-αCaMKII antibodies were all purchased from Santa Cruz Biotechnology. Mouse anti-α-tubulin (Sigma Aldrich), anti-rat Alexa Fluor 568, the anti-mouse Alexa Fluor 488 (Invitrogen), anti-rat horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technologies), and mouse and rabbit horseradish peroxidase-conjugated antibodies (GE Healthcare) were also used.

Animals—The generation of the Ube3A maternally deficient mice ($Ube3A^{p+/m-}$) and of the αCaMKII-knock-out (KO) and $\alpha CamKII^{T305D}$ mice has been described previously (15, 17).

The αCaMKII-KO and $\alpha CamKII^{T305D}$ mice were in the C57BL/6J background, and the $Ube3A^{p+/m-}$ mice were in the 129/Sv background. All experiments were performed using wild-type (WT) control mice of the appropriate genetic background. All experiments were performed *ex vivo*. The mice were bred in the Research Facility for Animal Experimentation (Forschungsanstalt für Versuchstierzucht; Himberg, Austria).

Preparation of Mouse Striatal Synaptosomes and Slices—Adult mice were sacrificed by cervical dislocation, and the brains were removed immediately. The striatum was dissected and either homogenized in ice-cold phosphate-buffered saline (PBS) containing 0.32 M sucrose and protease inhibitors (Roche complete) to prepare synaptosomes or else cut into slices (0.3 mm) using a McIllwain Tissue Chopper. For the preparation of synaptosomes, the suspension was centrifuged for 10 min at 1000 × *g*. The supernatant was again centrifuged for 15 min at 12,600 × *g*. The resulting pellet (P_2) was weighed and stored on ice until the start of the experiment. Slices were kept in Krebs-HEPES buffer (KHB, 25 mm HEPES; 120 mm NaCl; 5 mm KCl; 1.2 mm $CaCl_2$; and 1.2 mm $MgSO_4$ supplemented with 5 mm D-glucose) until the beginning of the experiment.

Preparation of Primary Dopaminergic Neurons—Midbrains from 1–3-day-old pups were dissected and cultured as described previously (18) with slight modifications. Briefly, ventral midbrains were digested in papain for 20 min at 37 °C and triturated afterward to dissociate the cells using increasingly smaller pipette tips. The cells were centrifuged for 4 min at 500 × *g* and resuspended in neuronal medium (Neurobasal A, 2% B27, 1% heat-inactivated calf serum, 0.4 mM glutamine, 50 μM kynurenic acid). The neurons were then seeded onto glial cells grown on coverslips. Glial-derived neurotrophic factor (Millipore) was added to each culture 2 h after seeding to stimulate sprouting and maturation of neurons. 5-Fluorodeoxyuridine was added to inhibit proliferation of glia. Cultures were used for experiments after at least 21 days *in vitro*.

Immunocytochemistry—Primary dopaminergic neurons were fixed using 4% paraformaldehyde in PBS for 20 min at room temperature. The neurons were washed 3× with PBS after fixation and blocked for 1 h using blocking/permeabilization buffer (5% goat serum in PBS, including 0.2% saponin). The neurons were then incubated overnight at 4 °C with a rat monoclonal dopamine transporter antibody and a mouse monoclonal αCaMKII antibody in blocking/permeabilization buffer. The next day, the neurons were washed 3× with PBS before being incubated with anti-rat Alexa Fluor 568 and anti-mouse Alexa Fluor 488 for 1 h at room temperature. The neurons were then washed 3× with PBS before being imaged using a Zeiss LSM510 laser scanning confocal microscope.

[^{3}H]CFT Binding—Binding was performed in striatal synaptosomes resuspended in assay buffer (20 mM Tris/HCl, 2 mM $MgCl_2$, 120 mM NaCl, 3 mM KCl; pH 7.4) to a final concentration of 0.3 mg of wet weight per 100 μl. The binding assays were conducted with increasing concentrations of [^{3}H]CFT, ranging from 1.25 to 40 nM. Nonspecific binding was determined in the presence of 3 μM methylphenidate. The final assay volume was 0.2 ml. Zn^{2+} (final concentration of 10 μM) was added to increase the affinity of [^{3}H]CFT for DAT (19). The reactions were performed at 22 °C for 10 min. After incubation, the assay tubes were rapidly filtered onto GF/B filters (Whatman) using an automatic cell harvester filtration device (Skatron Instruments AS). Binding was terminated following extensive washing with an ice-cold wash buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 120 mM NaCl, 10 μM $ZnCl_2$). The radioactivity on the filters was measured by liquid scintillation.

[^{3}H]MPP^+ Uptake—[^{3}H]MPP^+ has been used throughout the study because of its superior signal-to-noise ratio. Due to its fixed charge, MPP^+ does not permeate the membrane in the absence of transporter-mediated uptake. Accordingly, back-diffusion that may confound the analysis is obviated (20). [^{3}H]MPP^+ uptake (20 nM) was measured in striatal synaptosomes. P_2 pellet was resuspended in KHB to yield a final concentration of 0.3 mg of wet weight/50 μl in a final assay volume of 200 μl. The uptake assay was conducted in KHB for 5 min at 37 °C. Nonspecific uptake was determined in the presence of 10 μM mazindol. The assay was terminated by the addition of 2.5 ml of ice-cold KHB buffer and rapid filtration through GF/C

filters (Whatman) followed by two wash steps. Radioactivity on the filters was measured by liquid scintillation.

Superfusion—For superfusion assays, the P_2 pellets were dissolved in KHB to yield a final concentration of 1 mg of P_2 pellet in a total volume of 15 μl.

Synaptosomal fractions or slices for superfusions were incubated with 0.1 μM [^{3}H]MPP^+ (synaptosomes) or 0.4 μM [^{3}H]MPP^+ (slices) for 20 or 30 min, respectively, at 37 °C. After incubation, 15 μl of the P_2 suspension was loaded onto GF/B Whatman filters, and these filters were inserted into superfusion chambers. For slice superfusion, one slice was used per chamber. A washout period of 40 min at a flow of 0.7 ml/min (25 °C) was started, and it served to establish a basal efflux of radioactivity. Superfusate samples were collected at 2-min intervals. At min 6, [^{3}H]MPP^+-loaded synaptosomes were superfused by either KN93 or dimethyl sulfoxide (vehicle).

At min 12, synaptosomes were challenged by the addition of 6 μM D-amphetamine to induce DAT-mediated efflux. The experiment was stopped at min 20, synaptosomes containing filters or slices were recovered, lysed in 1% SDS, and mixed with scintillation mixture to determine the total amount of radioactivity present at the end of the experiment.

[^{3}H]MPP^+ efflux is expressed as percentage of total radioactivity present in each fraction or as area under the curve (AUC). AUC of amphetamine efflux was calculated by adding the total radioactivity present in each fraction upon amphetamine stimulation (*i.e.* min 12–20) and normalizing this sum to the baseline efflux (*i.e.* the mean of min 0–4). The AUC is proportional to substrate efflux.

Surface Biotinylation—Synaptosomes were resuspended in Krebs-Ringer bicarbonate (KRB) buffer (24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 146.2 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 10 mM glucose, 50 μM ascorbic acid) saturated with 95% O_2/5% CO_2 as described previously (12). 200 μg of synaptosomes were treated with sulfo-NHS-SS-biotin (1 mg/1 mg of protein) in KRB for 30min at 4 °C. This reaction was quenched using 100 mM glycine in KRB and incubated for 30 min at 4 °C. The synaptosomes were then lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS) supplemented with protease inhibitor mixture (Roche complete). The lysates were incubated with streptavidin beads (Thermo Fisher Scientific, Inc.) overnight at 4 °C. The next day, the beads were washed three times with lysis buffer and subsequently subjected to SDS-PAGE.

Co-immunoprecipitation of DAT—A crude membrane fraction was prepared from wild-type (C57Bl/6J background) and αCamKII knock-out or wild-type (129/Sv background) and Angelman syndrome striatal tissues. The crude membrane fraction (10 mg) was solubilized in lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 5 mM NaF, 5 mM sodium pyrophosphate, and a protease inhibitor mixture (Roche complete) on a tube rotator for 2 h at 4 °C. After centrifugation at 14,000 × *g* for 30 min at 4 °C, the supernatant was collected and incubated overnight with goat anti-DAT polyclonal antibody. After protein G bead (GE Healthcare) incubation and subsequent washing, bound proteins were eluted in SDS sample buffer at 95 °C for 3 min. Eluted proteins were size fractionated on SDS-PAGE gels and visualized by a Coomassie brilliant blue staining.

SDS-PAGE and Western Blot—Proteins were loaded onto an SDS-polyacrylamide gel and then transferred onto Protran™ nitrocellulose membranes (Whatman). Membranes were incubated overnight at 4 °C with primary antibodies: rat anti-DAT, goat anti-DAT, rabbit anti-αCaMKII, and mouse anti-α-tubulin.

The bands were visualized using the enhanced chemoluminiscence detection method (Thermo Scientific Pierce). Densitometric quantification of the bands was performed by NIH ImageJ software (Image Processing and Analysis in Java).

In-gel Digestion—The Coomassie Blue-stained bands were directly excised from SDS-PAGE gel, destained with 50% acetonitrile in 25 mM ammonium bicarbonate, and dried in a speed vacuum concentrator. After reduction and alkylation of cysteine residues, gel pieces were washed and dehydrated. Dried gel pieces were rehydrated with 25 mM ammonium bicarbonate (pH 8.0) containing 10 μg/ml trypsin (Promega, Madison, WI) and incubated for 18 h at 37 °C. Tryptic peptide mixtures were extracted with 50% acetonitrile in 5% formic acid and concentrated in a speed vacuum concentrator for LC-MS/MS.

LC-MS/MS—An ion trap mass spectrometer (HCT, Bruker Daltonics, Bremen, Germany) coupled with an Ultimate 3000 nano-HPLC system (Dionex, Sunnyvale, CA) was used for LC-MS/MS data acquisition. A PepMap100 C-18 trap column (300 μm × 5 mm) and PepMap100 C-18 analytic column (75 μm × 150 mm) were used for reverse phase chromatographic separation with a flow rate of 300 nl/min. The two buffers used for the reverse phase chromatography were 0.1% formic acid/water (buffer A) and 0.08% formic acid/acetonitrile (buffer B), with a 125 min gradient (4–30% B for 105 min, 80% B for 5 min, and 4% B for 15 min). Eluted peptides were then directly sprayed into the mass spectrometer to record peptide spectra over the mass range of m/z 350–1500 and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100–2800. Repeatedly, MS spectra were recorded followed by three data-dependent CID MS/MS spectra generated from four highest intensity precursor ions. The MS/MS spectra were interpreted with the Mascot search engine (Matrix Science, London, UK). Database searches through Mascot, were performed with a mass tolerance of 0.5 Da, a MS/MS tolerance of 0.5 Da, and three missing cleavage site. In addition, carbamidomethylation on cysteine, oxidation on methionine, deamidation on asparagine/glutamine, and phosphorylation on serine/threonine were allowed for database searches.

Statistics—All data were statistically evaluated using one-way ANOVA followed by a Tukey's post hoc test unless otherwise stated. Significance was established upon a p value < 0.05.

RESULTS

αCaMKII Interacts with Dopamine Transporter in Native Brain Preparations—A direct interaction of αCaMKII with the carboxyl terminus of human DAT was previously documented in a heterologous expression system *in vitro* (13). We examined this interaction *ex vivo* using corpora striata prepared from WT and αCaMKII-KO mice (17). DAT was immunopurified with an anti-DAT antibody from striatum. The immunoprecipitate

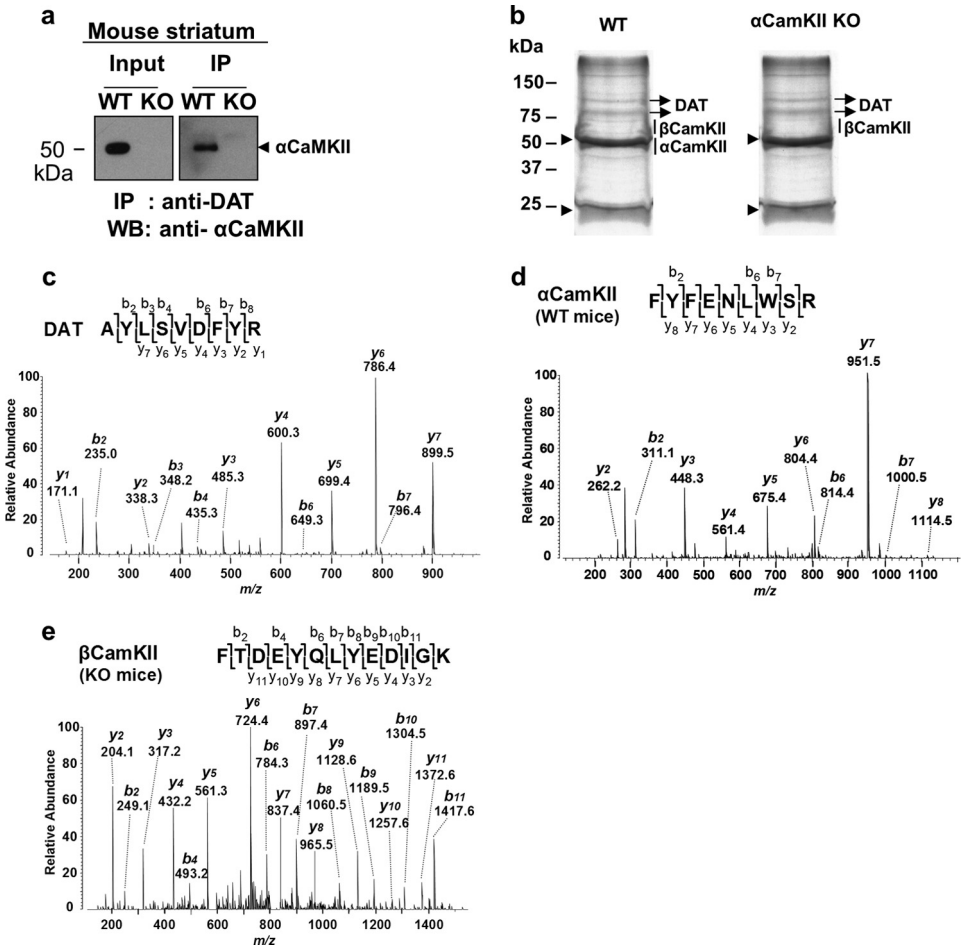

FIGURE 1. **αCaMKII co-precipitates with DAT from mouse striatum.** *a*, αCamKII co-immunoprecipitates with DAT from WT but not αCaMKII-KO striatal lysates. Synaptosomal fractions were solubilized (*input*) and immunoprecipitated (*IP*) by an affinity purified polyclonal anti-DAT antibody. Co-purified αCamKII was detected with a monoclonal anti-αCaMKII antibody. *b*, Coomassie Blue-stained denaturing gel of striatal tissues prepared from WT and αCaMKII-KO mice with protein complex immunopurified by an anti-DAT antibody. LC-MS/MS identified DAT and βCamKII in immunopurified protein complex from WT and αCaMKII-KO mice and αCamKII only from WT mice. *c*, the double-charged (2^+) MS/MS-spectrum obtained at *m/z* 567.33 was fragmented to produce a tandem mass spectrum with *y*- and *b*-ion series that identified the sequence AYLSVDFYR (amino acids 295–303) from mouse DAT. *d*, specific peptides of αCamKII and βCamKII (*e*) in immunoprecipitates from WT and αCaMKII-KO mice. The MS/MS spectrum at *m/z* 631.37 (2^+) showing FYFENLWSR of αCamKII was identified only in association with the DAT of WT mice. *e*, the MS/MS spectrum at *m/z* 810.94 (2^+) assigns the sequence FTDEYQLYEDIGK to βCamKII in immunoprecipitates from αCaMKII-KO mice.

prepared from WT striatal tissue contained immunoreactivity for αCaMKII, which was absent in immunoprecipitates from αCaMKII-deficient striata (Fig. 1*a*). This was confirmed by mass spectrometry (Fig. 1, *b*–*d*; supplemental Fig. 1).

We also investigated whether DAT and αCaMKII co-localized in primary dopaminergic neurons. For that purpose, we cultured ventral midbrain neurons from newborn pups and fixed and stained the neurons using specific antibodies against αCaMKII and DAT. Confocal imaging revealed co-staining for both αCaMKII and DAT, such as would be anticipated were these two proteins to interact (Fig. 2*a*).

Amphetamine-induced DAT-mediated Efflux Is Attenuated in αCaMKII Mutant Mice—Fog *et al.* (13) reported that the binding of αCaMKII to DAT and its subsequent phosphorylation of the DAT amino terminus supports the action of amphetamines. Accordingly, a consistent and statistically significant reduction in amphetamine-induced efflux of the DAT substrate 1-methyl [^{3}H]-4-phenylpyridinium ([^{3}H]MPP$^+$) was observed upon the comparison of either superfused striatal synaptosomes or slices prepared from αCaMKII-KO to those prepared from WT mice (Fig. 3, *a* and *b*).

We examined whether αCaMKII activity itself is required in addition to αCaMKII binding to the DAT (13) by using the αCaMKII^{T305D} mutant, which carries a threonine to aspartate mutation in the calcium/calmodulin binding domain. This amino acid substitution mimics inhibitory autophosphorylation at Thr-305 and renders (i) the αCaMKII protein inactive (17) and (ii) lowers the expression level in the striatum (Fig. 2, *b* and *c*) and hippocampus (17). We observed that the amphetamine-induced release in αCaMKII^{T305D} mice was significantly decreased and was therefore comparable to that seen in αCaMKII-KO mice (Fig. 3, *b* and *c*). However, there was no significant difference between the basal [^{3}H]MPP$^+$ efflux levels (wild-type, 5.2 ± 0.4 fmol/2 min; αCaMKII-KO, 4.3 ± 0.4 fmol/2 min; αCaMKII^{T305D}, 4.8 ± 0.2 fmol/2 min ($n = 5$) according to repeated measures ANOVA, followed by Tukey's post hoc tests). Hence, these results suggest that DAT activity requires not only αCaMKII binding but also its activation. To test whether the remaining DAT activity observed in our experiments was due to the activity of βCaMKII, which was also detected in DAT immunoprecipitates after MS/MS analysis (Fig. 1, *b*–*e*), we superfused striatal synaptosomes of WT and

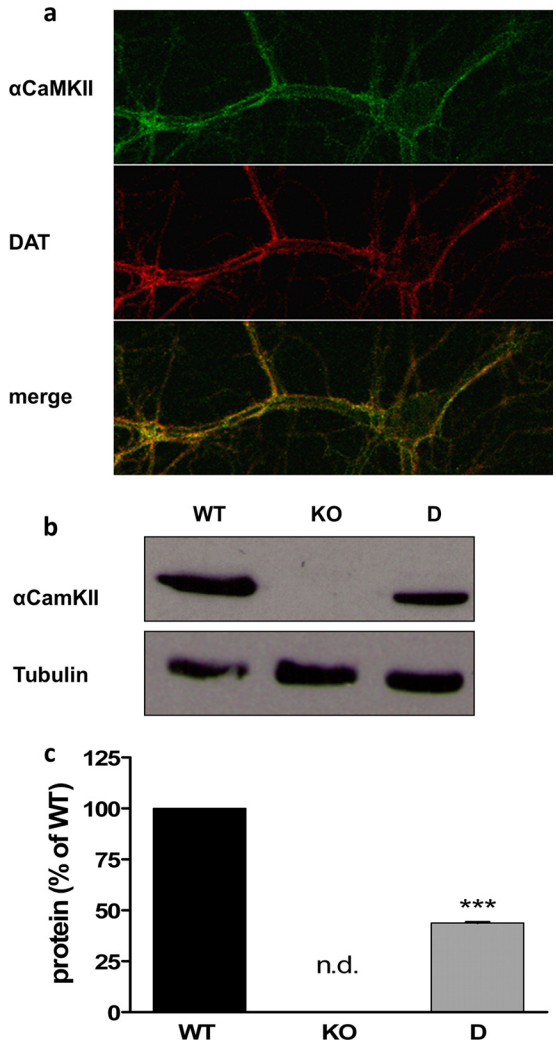

FIGURE 2. **Co-localization of αCaMKII and DAT in primary dopaminergic neurons and expression levels of αCaMKII in WT, αCaMKII-KO, and αCaMKIIT^{305D} mice.** *a*, αCamKII and DAT co-localize in primary dopaminergic neurons. Midbrain neurons were fixed with 4% PFA and stained with monoclonal antibodies against αCamKII and DAT, followed by fluorescently labeled secondary antibodies. Images were recorded using a Zeiss LSM510 microscope. *b*, representative immunoblot for αCamKII in WT, αCamKII-KO (KO), and αCamKII^{T305D} (*D*) striatal synaptosomes. Tubulin was used as a loading control. *c*, αCamKII bands were quantified by band densitometry and normalized to tubulin loading ($n = 5$ per genotype). *n.d.*, not detected. Statistically significant differences were assessed with a one-way ANOVA followed by Tukey's test. ***, $p < 0.001$ of αCaMKII^{T305D} against WT.

αCaMKII-KO mice in the presence of the general CaMKII inhibitor KN93. Amphetamine-induced release in synaptosomes obtained from WT mice was significantly reduced by KN93 (15 μM; $AUC_{WT,Co}$, 47 ± 1.3% ($n = 6$); $AUC_{WT,KN93}$, 31.6 ± 2.7% ($n = 13$, $p < 0.01$) but similar to that observed in synaptosomes of αCaMKII-KO mice ($AUC_{KO,Co}$, 31.8 ± 1.3%; $n = 23$). In addition, KN93 did not have any appreciable effect on synaptosomes of αCaMKII-KO ($AUC_{KO,KN93}$, 35.0 ± 1.8%; $n = 10$).

In an additional set of experiments, the more specific CaMKII inhibitor AIP (autocamtide-2-related inhibitory peptide II) was compared with KN93 (21). In accordance with the data described above, DAT-mediated efflux from striatal synaptosomes (WT mice) was reduced by AIP in a concentration-dependent manner and thus supported the findings with KN93 (Fig. 3*d*). The results thus demonstrate that amphetamine-induced efflux via the DAT is (i) contingent on a functional copy of αCaMKII, and (ii) βCamKII cannot substitute for αCaMKII and thereby sustain DAT efflux activity, although βCamKII also precipitates with DAT (Fig. 1, *d* and *e*; supplemental Fig. 1).

DAT Expression Remains Unchanged in αCaMKII Mutant Mice—Differences in release cannot be accounted for by variations in DAT levels in the striata of the mice because the DAT expression was unchanged as assessed by three independent approaches: (i) uptake of [^{3}H]MPP$^+$ into striatal synaptosomes (WT, 3.6 ± 0.3 fmol/mg/min; αCaMKII-KO, 3.3 ± 0.6 fmol/mg/min; αCaMKII^{T305D}, 3.1 ± 0.2 fmol/mg/min ($n = 4$ per genotype); Fig. 4*a*); (ii) high-affinity binding of the dopamine reuptake inhibitor [^{3}H]CFT to striatal membranes (K_d, 10.4 ± 4.7 nM (WT); 15.7 ± 4.8 nM (αCaMKII-KO); 9.4 ± 1.4 nM (αCaMKII^{T305D}); B_{max}, 0.087 ± 0.015 pmol/mg wet weight (WT); 0.115 ± 0.016 pmol/mg wet weight (αCaMKII-KO); 0.077 ± 0.004 pmol/mg wet weight (αCaMKII^{T305D}) ($n \geq 3$ per genotype; Fig. 4*b*), and (iii) the surface biotinylation of striatal DAT protein (Fig. 4, *c* and *d*). Thus, our data reveal that αCaMKII interacts with DAT in native brain preparations and that its activity plays a major role in supporting amphetamine-induced efflux.

Angelman Syndrome Mice Display Impaired Amphetamine-triggered DAT Efflux—Angelman syndrome is caused by the loss of functional E3 ubiquitin ligase Ube3A, which renders αCaMKII less active due to increased phosphorylation at the inactivating αCaMKII Thr-305/Thr-306 sites (22). Our experiments indicate that αCaMKII activity is required to regulate reverse transport by DAT, which may subsequently affect dopaminergic signaling (23); thus, we hypothesized that DAT function is impaired in Angelman syndrome. Accordingly, we examined Ube3A$^{p+/m-}$ mice (also known as Angelman syndrome mice), a mouse model of Angelman syndrome, (15) for a potential deficit in DAT function. Initially, we examined whether αCaMKII was still able to bind DAT in these mice by co-immunoprecipitation. αCaMKII was co-purified with DAT in both WT and Angelman syndrome mice. (Fig. 5, *a* and *b*).

Additionally, we assessed the total protein levels of αCaMKII in the striatum and found no significant difference (Fig. 5, *c* and *d*). We assessed amphetamine-induced efflux in Angelman syndrome mice as outlined above and compared striatal synaptosomes of Angelman syndrome mice to those of WT mice (Fig. 6*a*). Interestingly, basal [^{3}H]MPP$^+$ efflux levels were significantly higher in Angelman syndrome mice compared with WT mice (WT = 2.2 ± 0.1 fmol/2min, Angelman syndrome mice = 2.8 ± 0.02 fmol/2min; $n = 5$; $p < 0.01$, paired Student's t test, two-tailed); this finding was in contrast to the observations in αCaMKII mutant mice, which were slightly lower compared with WT mice (see above).

DAT expression in Angelman syndrome and WT mice was also investigated: surface biotinylation, uptake, and binding experiments (Fig. 6, *b*–*e*) verified that DAT was present at comparable levels and mediated substrate uptake at similar rate (uptake: WT, 2.3 ± 0.5 fmol/mg/min; Angelman syndrome, 2.5 ± 0.3 fmol/mg/min ($n = 3$ per genotype); binding: K_d WT, 10.3 ± 5.2 nM; Angelman syndrome, 8.6 ± 3.0 nM; B_{max} WT, 0.101 ± 0.02 pmol/mg wet weight; Angelman syndrome, 0.098 ± 0.012 pmol/mg wet weight ($n = 3$ per genotype); Fig. 6,

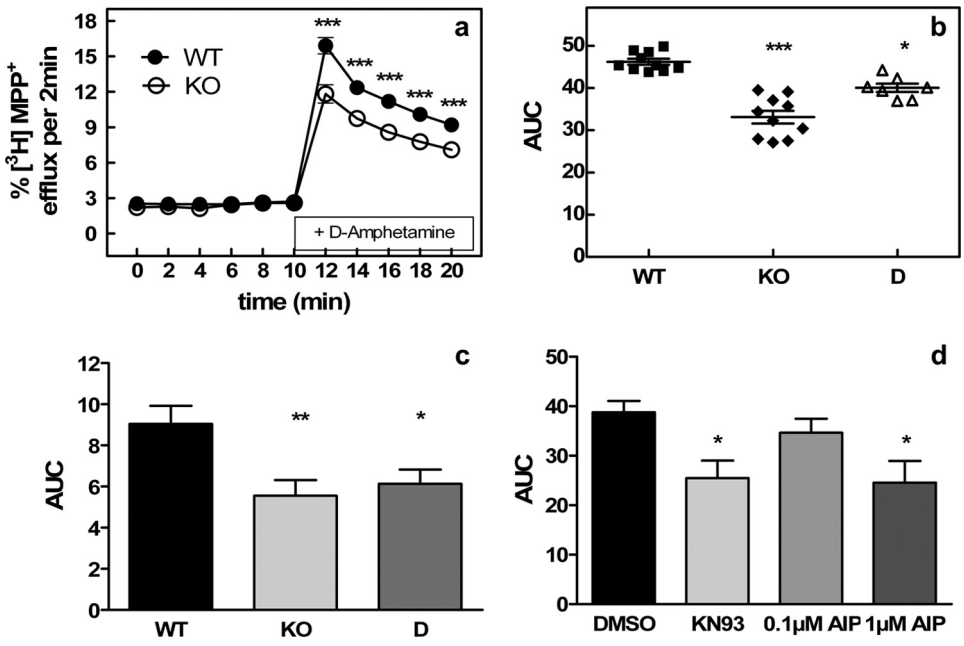

FIGURE 3. **Amphetamine-triggered substrate efflux in αCamKII mutant mice.** *a*, striatal synaptosomes prepared from wild-type (WT) and αCaMKII-KO (KO) mice were preloaded with [^{3}H]MPP$^+$ (0.1 μCi) for 20 min, loaded onto Whatman GF/B filters, and superfused. A stable base line was established by continuous superfusion for 40min. Thereafter, at time = 0 min, six fractions were collected. Basal efflux amounted to 2.47 ± 0.03% min^{-1}, *i.e.* 771 ± 19 dpm min^{-1} (n = 144). At time = 10 min, D-amphetamine (6 μM) was added to the buffer and at time = 20 min, filters were lysed, and remaining radioactivity was determined. Data are presented as fractional efflux, *i.e.* each fraction is expressed as the percentage of radioactivity present in the synaptosomes at the beginning of that fraction. Symbols represent means ± S.E. of five observations performed in triplicate. ANOVA with Bonferroni correction ***, $p < 0.001$. *b*, AUC values determined from superfusion experiments assessing synaptosomes prepared from different mice as indicated. Statistically significant differences were assessed with a one-way ANOVA followed by Tukey's test. ***, $p < 0.001$ of αCaMKII-KO against WT; *, $p < 0.05$ of αCaMKII^{T305D} (*D*) against WT. *c*, striatal slices from WT, αCaMKII-KO (KO), and αCaMKII^{T305D} (*D*) mice were prepared as described under "Experimental Procedures." AUC of D-amphetamine-induced efflux was calculated. Statistically significant differences were assessed with a one-way ANOVA followed by Tukey's test. **, $p < 0.01$ of αCaMKII-KO (KO) against WT, respectively. *, $p < 0.05$ of αCaMKII^{T305D} (*D*) against WT. *d*, AUC values determined from superfusion experiments assessing synaptosomes prepared from WT mice in the presence of KN93 (15 μM) or AIP (0.1 μM, 1 μM) as indicated. Statistically significant differences were assessed with a one-way ANOVA followed by Tukey's test. *, $p < 0.05$ of drug treatment against control (dimethyl sulfoxide; *DMSO*) (n = 3–5).

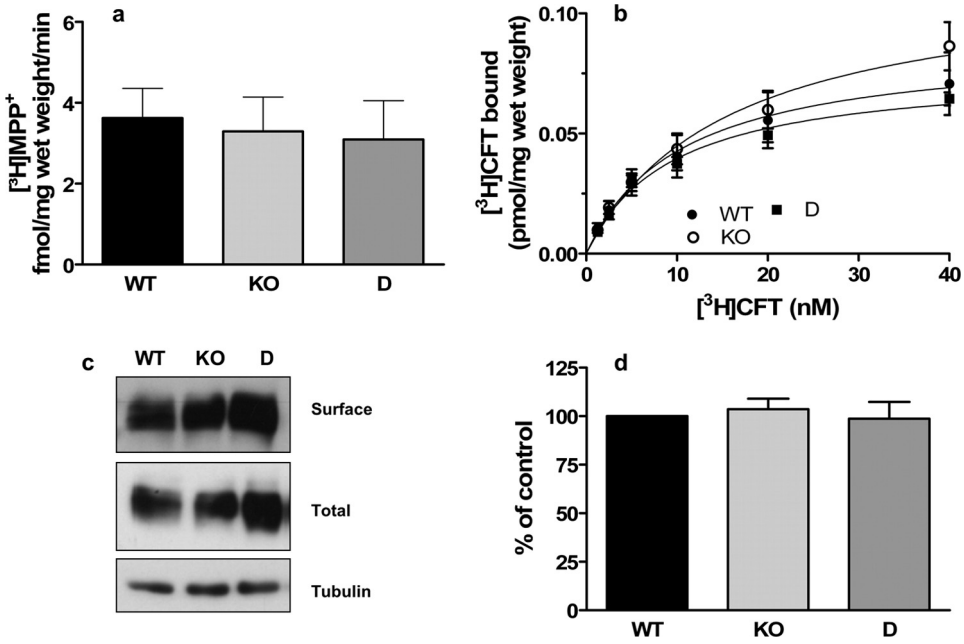

FIGURE 4. **DAT expression in αCamKII mutant mice.** *a*, uptake of [^{3}H]MPP$^+$ in mouse striatal synaptosomes prepared from the following mice: wild-type (WT), αCaMKII knock-out (KO), αCaMKII^{T305D} (*D*). Experiments were performed four times in triplicate. *b*, binding of [^{3}H]CFT to membranes prepared from corpora striata of wild-type, αCaMKII-KO (KO), αCaMKII^{T305D} (*D*). Membranes were incubated with [^{3}H]CFT (concentrations as indicated) in the presence of Zn^{2+} (10 μM) for 10 min (22 °C; V = 200 μl). Nonspecific binding was defined in the presence of 3 μM methylphenidate and was <5% at a concentration corresponding to K_D. *c*, surface biotinylation of DAT. Shown in the *upper panel* are surface-biotinylated samples of three different mouse strains: WT, αCaMKII-KO, αCaMKII^{T305D} (*D*). The *lower panel* shows DAT from total extracts. Densitometric evaluation of three experiments revealed comparable values relative to wild-type expression. *d*, bands were quantified by band densitometry and normalized to total DAT bands (n = 3 per genotype). No significant differences were assessed (one-way ANOVA followed by Tukey's test).

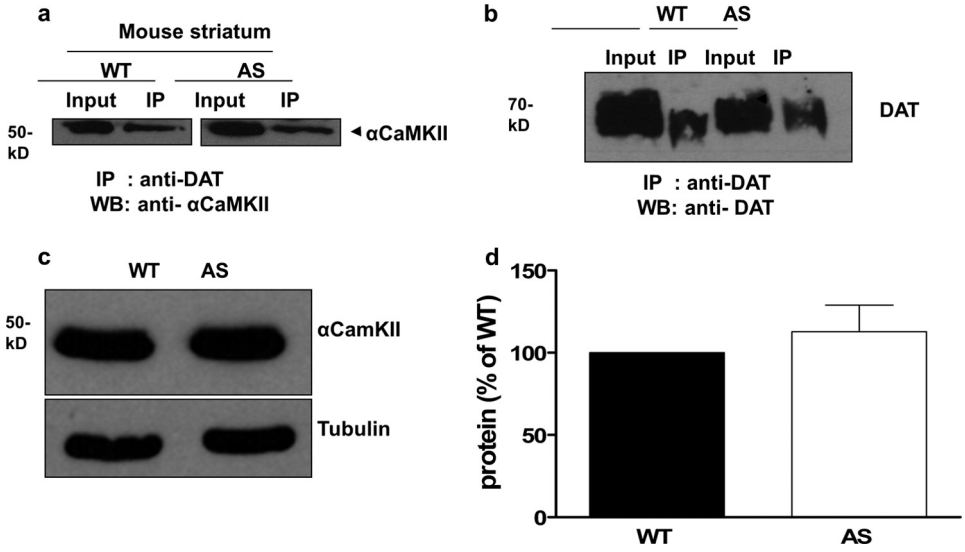

FIGURE 5. **DAT immunoprecipitation and αCamKII expression in Angelman syndrome mice.** *a*, αCamKII co-immunoprecipitates with DAT from wild-type (WT) and Angelman syndrome (AS) mouse striatal lysates. Synaptosomal fractions were solubilized (*input*) and immunoprecipitated (*IP*) by an affinity purified polyclonal anti-DAT antibody. Co-purified αCamKII was detected with a monoclonal anti-αCaMKII antibody. *b*, control for immunoprecipitation efficiency of samples from *a*. *c*, representative immunoblot for αCamKII in WT and AS striatal synaptosomes. Tubulin was used as a loading control. *d*, αCamKII bands were quantified by band densitometry and normalized to tubulin loading ($n = 4$ per genotype). No significant differences between WT and AS were assessed (Student's *t* test).

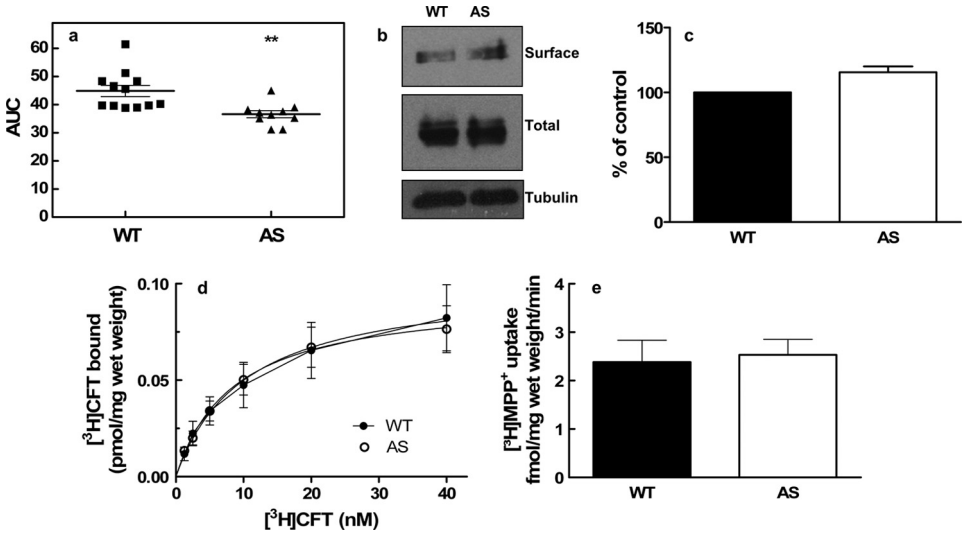

FIGURE 6. **Amphetamine-induced efflux and DAT expression in Angelman syndrome mice.** *a*, AUC values of amphetamine-induced [^{3}H]MPP$^+$ efflux determined from superfusion experiments of synaptosomes (WT, wild-type and AS, Angelman syndrome). Basal efflux amounted to 2.39 ± 0.03% min^{-1}, *i.e.* 629 ± 15 dpm min^{-1}; $n = 144$. Statistically significant differences were assessed by Student's *t* test. **, $p < 0.01$ of AS against WT. *b*, DAT cell surface biotinylation. Shown in the *upper panel* is surface-biotinylated DAT in wild-type and AS mice. The *lower panel* shows DAT from total extracts. *c*, bands were quantified by band densitometry and normalized to total DAT ($n = 3$ per genotype). No significant differences between WT and AS were assessed (Student's *t* test). *d*, binding of [^{3}H]CFT to membranes prepared from WT and AS striata. Membranes (0.15 mg wet weight) were incubated with [^{3}H]CFT (concentrations as indicated) in the presence of Zn^{2+} (10 μM) for 10min (22 °C; V = 200 μl). Nonspecific binding was defined in the presence of 3 μM methylphenidate and was <5% at the K_D concentration ($n = 3$). *e*, uptake of [^{3}H]MPP$^+$ in striatal synaptosomes prepared from the following mice: WT and AS. Experiments were performed three times in triplicate.

b–*e*). However, the amphetamine-induced efflux of [^{3}H]MPP$^+$ was significantly reduced in striatal synaptosomes from Angelman syndrome mice (Fig. 6*a*). This observation is consistent with the results obtained in the αCaMKII-KO and αCaMKII^{T305D} mice.

DISCUSSION

Unraveling the mechanisms of DAT function and regulation is vital to the understanding of a variety of neuropsychiatric disorders, ranging from Parkinson disease to attention deficit hyperactivity disorder and drug addiction. The recently identified interaction of DAT and αCaMKII prompted us to investigate whether or not the disturbance of this DAT-interacting protein has consequences on DAT function.

Angelman syndrome is caused by a loss of function mutation in Ube3A, a HECT (homologous to E6 carboxyl terminus) domain containing E3 ubiquitin ligase (14). Ube3A is normally expressed in a biallelic fashion in most tissues; however, in the brain, it is mainly expressed from the maternal allele (24). The maternal loss of Ube3A causes increased phosphorylation of αCaMKII at Thr-286 and Thr-305/Thr-306 by means that are

currently unknown, thereby rendering αCaMKII self-inhibited (16) (25). The resulting Angelman syndrome is characterized by severe developmental delay, including impaired motor coordination and language deficits (26). The identification of the molecular basis of Angelman syndrome pathophysiology is a subject of immense interest as there is currently no targeted drug therapy available to treat human Angelman syndrome patients (27). Recently, it has been unraveled that the unsilencing of the paternal Ube3a allele using topoisomerase inhibitors might be beneficial for the treatment of Angelman syndrome patients (28). Other possible treatment options attempt to target the dopaminergic system. Therefore, we investigated whether the previously reported interaction between αCaMKII and DAT (13) is of pathophysiological importance in Angelman syndrome. We used $Ube3A^{p+/m-}$ mice, a mouse model of Angelman syndrome, to assess whether the self-inhibition of αCaMKII observed in these mice affects the functionality of the DAT. We examined DAT-mediated uptake and amphetamine-induced substrate efflux in Angelman syndrome mice. As controls, we performed experiments in mice lacking αCaMKII (αCaMKII-KO mice) as well as mice in which αCaMKII function is inhibited by a point mutation at Thr-305 ($\alpha CaMKII^{T305D}$ mice). This mutant mimics the auto-phosphorylation of this residue, which is known to inactivate the kinase (17).

Our experiments show that amphetamine-induced DAT-mediated substrate efflux but not basal efflux is significantly attenuated in αCaMKII-KO and $\alpha CaMKII^{T305D}$ mutant mice. This decrease is a consequence of blunted αCaMKII function and not due to changes in DAT protein expression as substrate uptake, radioligand binding, and surface biotinylation did not reveal any significant changes in mutants compared with the wild-type upon acute exposure to amphetamine.

In contrast, a longer exposure to amphetamines regulates CaMKII activity and DAT function in a different manner: a 30-min amphetamine treatment of HEK293-hDAT cells stimulates CaMKII activity profoundly and initiates a decrease in Akt activity, which, in turn, results in a subsequent down-regulation of DAT surface expression (29). In addition, syntaxin 1A, a member of the family of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins has been shown to directly interact with monoamine transporters and regulate their function (30). CaMKII activity is required for an amphetamine-induced association between DAT and syntaxin 1A, which thereby increased amphetamine-induced substrate efflux (31).

Our experiments suggest that DAT is among the proteins affected by disturbed maternal Ube3A and this deficiency is most likely caused by inhibited αCaMKII. In fact, some clinical features of Angelman syndrome are plausibly explained by an impaired connection of αCaMKII and the defective Ube3A protein, which finally impacts on DAT function. The dopaminergic system plays a major role in both movement disorders (32) and autism (33), both of which are key manifestations of the Angelman syndrome phenotype in human patients (26). Based on our observations, it is tempting to speculate that an anomalous function of DAT may account for some of the manifestations of Angelman syndrome. Two possibilities for anomalous function of DAT should be taken into consideration: (i) in the case of Angelman syndrome, it is plausible that the disease arises from a defective interactome comprised of αCaMKII and the Ube3A protein; (ii) alternatively, anomalous function may also be ascribed to DAT itself, for *e.g.* the coding variant DAT^{A559V}, previously identified in a pedigree of male attention deficit hyperactivity disorder patients mediated exaggerated efflux (34). Whether or not anomalous dopamine efflux occurs via either one of these two possibilities, it certainly bears a marked pathophysiological relevance: efflux via DAT regulates dopaminergic signaling because released dopamine binds to dopamine D_2 receptors, which are in close association with DAT (35, 36). Stimulation of D_2 receptors increases DAT surface expression by an ERK1/2-dependent process (35), and increased DAT uptake has been implicated with states of hyperinsulinemia (37, 38). This, in turn, suggests that insulin signaling pathways may regulate dopamine transmission and thereby significantly influence the effects exerted by amphetamines.

In line with these observations, D_2 receptors are tonically activated in cells expressing the mutant DAT^{A559V} and thereby support the anomalous dopamine efflux via DAT^{A559V} (36). Furthermore, the hypothesis that dopaminergic signaling may be modulated by dopamine released from presynaptic sites via DAT has been fueled by observations made during electrophysiological and amperometric recordings in rat brain slices (23). Consistent with a role of DAT in Angelman syndrome, $Ube3A^{p+/m-}$ mice become hypodopaminergic and develop motor deficits that are equivalent to human Parkinson disease (39). In fact, frank (levodopa responsive) Parkinson disease has also been observed in young adults with Angelman syndrome (40). Hence, our data on DAT-mediated efflux in striata prepared from Angelman syndrome mice establishes an association between defective Ube3A and DAT and indicates that a defective αCaMKII is most likely the link between these two proteins.

Acknowledgment—We are indebted to Emilio Casanova for comments of during experimental work.

REFERENCES

1. Blakely, R. D., and Bauman, A. L. (2000) Biogenic amine transporters: Regulation in flux. *Curr. Opin. Neurobiol.* **10,** 328–336
2. Kristensen, A. S., Andersen, J., Jørgensen, T. N., Sørensen, L., Eriksen, J., Loland, C. J., Strømgaard, K., and Gether, U. (2011) SLC6 neurotransmitter transporters: Structure, function, and regulation. *Pharmacol. Rev.* **63,** 585–640
3. Ramamoorthy, S., Shippenberg, T. S., and Jayanthi, L. D. (2011) Regulation of monoamine transporters: Role of transporter phosphorylation. *Pharmacol. Ther.* **129,** 220–238
4. Iversen, L. L. (1971) Role of transmitter uptake mechanisms in synaptic neurotransmission. *Br J. Pharmacol.* **41,** 571–591
5. Amara, S. G., and Kuhar, M. J. (1993) Neurotransmitter transporters: Recent progress. *Annu. Rev. Neurosci.* **16,** 73–93
6. Steinkellner, T., Freissmuth, M., Sitte, H. H., and Montgomery, T. (2011) The ugly side of amphetamines: Short and long term toxicity of 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy"), methamphetamine, and D-amphetamine. *Biol. Chem.* **392,** 103–115
7. Fleckenstein, A. E., Volz, T. J., Riddle, E. L., Gibb, J. W., and Hanson, G. R. (2007) New insights into the mechanism of action of amphetamines. *Annu. Rev. Pharmacol. Toxicol.* **47,** 681–698

8. Seiden, L. S., Sabol, K. E., and Ricaurte, G. A. (1993) Amphetamine: Effects on catecholamine systems and behavior. *Annu. Rev. Pharmacol. Toxicol.* **33,** 639–677
9. Sulzer, D., Sonders, M. S., Poulsen, N. W., and Galli, A. (2005) Mechanisms of neurotransmitter release by amphetamines: A review. *Prog. Neurobiol.* **75,** 406–433
10. Sitte, H. H., and Freissmuth, M. (2010) The reverse operation of Na^+/Cl^--coupled neurotransmitter transporters: Why amphetamines take two to tango. *J. Neurochem.* **112,** 340–355
11. Johnson, L. A., Guptaroy, B., Lund, D., Shamban, S., and Gnegy, M. E. (2005) Regulation of amphetamine-stimulated dopamine efflux by protein kinase Cβ. *J. Biol. Chem.* **280,** 10914–10919
12. Chen, R., Furman, C. A., Zhang, M., Kim, M. N., Gereau, R. W., 4th, Leitges, M., and Gnegy, M. E. (2009) Protein kinase Cβ is a critical regulator of dopamine transporter trafficking and regulates the behavioral response to amphetamine in mice. *J. Pharmacol. Exp. Ther.* **328,** 912–920
13. Fog, J. U., Khoshbouei, H., Holy, M., Owens, W. A., Vaegter, C. B., Sen, N., Nikandrova, Y., Bowton, E., McMahon, D. G., Colbran, R. J., Daws, L. C., Sitte, H. H., Javitch, J. A., Galli, A., and Gether, U. (2006) Calmodulin kinase II interacts with the dopamine transporter C terminus to regulate amphetamine-induced reverse transport. *Neuron* **51,** 417–429
14. Mabb, A. M., Judson, M. C., Zylka, M. J., and Philpot, B. D. (2011) Angelman syndrome: Insights into genomic imprinting and neurodevelopmental phenotypes. *Trends Neurosci.* **34,** 293–303
15. Jiang, Y. H., Armstrong, D., Albrecht, U., Atkins, C. M., Noebels, J. L., Eichele, G., Sweatt, J. D., and Beaudet, A. L. (1998) Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long term potentiation. *Neuron* **21,** 799–811
16. Weeber, E. J., Jiang, Y. H., Elgersma, Y., Varga, A. W., Carrasquillo, Y., Brown, S. E., Christian, J. M., Mirnikjoo, B., Silva, A., Beaudet, A. L., and Sweatt, J. D. (2003) Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *J. Neurosci.* **23,** 2634–2644
17. Elgersma, Y., Fedorov, N. B., Ikonen, S., Choi, E. S., Elgersma, M., Carvalho, O. M., Giese, K. P., and Silva, A. J. (2002) Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. *Neuron* **36,** 493–505
18. Rayport, S., Sulzer, D., Shi, W. X., Sawasdikosol, S., Monaco, J., Batson, D., and Rajendran, G. (1992) Identified postnatal mesolimbic dopamine neurons in culture: Morphology and electrophysiology. *J. Neurosci.* **12,** 4264–4280
19. Sucic, S., Dallinger, S., Zdrazil, B., Weissensteiner, R., Jørgensen, T. N., Holy, M., Kudlacek, O., Seidel, S., Cha, J. H., Gether, U., Newman, A. H., Ecker, G. F., Freissmuth, M., and Sitte, H. H. (2010) The N terminus of monoamine transporters is a lever required for the action of amphetamines. *J. Biol. Chem.* **285,** 10924–10938
20. Scholze, P., Sitte, H. H., and Singer, E. A. (2001) Substantial loss of substrate by diffusion during uptake in HEK-293 cells expressing neurotransmitter transporters. *Neurosci. Lett.* **309,** 173–176
21. Bhatt, H. S., Conner, B. P., Prasanna, G., Yorio, T., and Easom, R. A. (2000) Dependence of insulin secretion from permeabilized pancreatic β-cells on the activation of Ca^{2+}/calmodulin-dependent protein kinase II. A re-evaluation of inhibitor studies. *Biochem. Pharmacol.* **60,** 1655–1663
22. van Woerden, G. M., Harris, K. D., Hojjati, M. R., Gustin, R. M., Qiu, S., de Avila Freire, R., Jiang, Y. H., Elgersma, Y., and Weeber, E. J. (2007) Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of αCaMKII inhibitory phosphorylation. *Nat. Neurosci.* **10,** 280–282
23. Falkenburger, B. H., Barstow, K. L., and Mintz, I. M. (2001) Dendrodendritic inhibition through reversal of dopamine transport. *Science* **293,** 2465–2470
24. Albrecht, U., Sutcliffe, J. S., Cattanach, B. M., Beechey, C. V., Armstrong, D., Eichele, G., and Beaudet, A. L. (1997) Imprinted expression of the murine Angelman syndrome gene, Ube3a, in hippocampal and Purkinje neurons. *Nat. Genet.* **17,** 75–78
25. Elgersma, Y., Sweatt, J. D., and Giese, K. P. (2004) Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. *J. Neurosci.* **24,** 8410–8415
26. Williams, C. A. (2010) The behavioral phenotype of the Angelman syndrome. *Am. J. Med. Genet. C Semin. Med. Genet.* **154C,** 432–437
27. Clayton-Smith, J., and Laan, L. (2003) Angelman syndrome: A review of the clinical and genetic aspects. *J. Med. Genet.* **40,** 87–95
28. Huang, H. S., Allen, J. A., Mabb, A. M., King, I. F., Miriyala, J., Taylor-Blake, B., Sciaky, N., Dutton, J. W., Jr., Lee, H. M., Chen, X., Jin, J., Bridges, A. S., Zylka, M. J., Roth, B. L., and Philpot, B. D. (2012) Topoisomerase inhibitors unsilence the dormant allele of Ube3a in neurons. *Nature* **481,** 185–189
29. Wei, Y., Williams, J. M., Dipace, C., Sung, U., Javitch, J. A., Galli, A., and Saunders, C. (2007) Dopamine transporter activity mediates amphetamine-induced inhibition of Akt through a Ca^{2+}/calmodulin-dependent kinase II-dependent mechanism. *Mol. Pharmacol.* **71,** 835–842
30. Quick, M. W. (2006) The role of SNARE proteins in trafficking and function of neurotransmitter transporters. *Handb. Exp. Pharmacol.* 181–196
31. Binda, F., Dipace, C., Bowton, E., Robertson, S. D., Lute, B. J., Fog, J. U., Zhang, M., Sen, N., Colbran, R. J., Gnegy, M. E., Gether, U., Javitch, J. A., Erreger, K., and Galli, A. (2008) Syntaxin 1A interaction with the dopamine transporter promotes amphetamine-induced dopamine efflux. *Mol. Pharmacol.* **74,** 1101–1108
32. Hornykiewicz, O. (2006) The discovery of dopamine deficiency in the parkinsonian brain. *J. Neural Transm. Suppl.* **70,** 9–15
33. Nakamura, K., Sekine, Y., Ouchi, Y., Tsujii, M., Yoshikawa, E., Futatsubashi, M., Tsuchiya, K. J., Sugihara, G., Iwata, Y., Suzuki, K., Matsuzaki, H., Suda, S., Sugiyama, T., Takei, N., and Mori, N. (2010) Brain serotonin and dopamine transporter bindings in adults with high-functioning autism. *Arch. Gen. Psychiatry* **67,** 59–68
34. Mazei-Robison, M. S., Bowton, E., Holy, M., Schmudermaier, M., Freissmuth, M., Sitte, H. H., Galli, A., and Blakely, R. D. (2008) Anomalous dopamine release associated with a human dopamine transporter coding variant. *J. Neurosci.* **28,** 7040–7046
35. Bolan, E. A., Kivell, B., Jaligam, V., Oz, M., Jayanthi, L. D., Han, Y., Sen, N., Urizar, E., Gomes, I., Devi, L. A., Ramamoorthy, S., Javitch, J. A., Zapata, A., and Shippenberg, T. S. (2007) D2 receptors regulate dopamine transporter function via an extracellular signal-regulated kinases 1 and 2-dependent and phosphoinositide 3-kinase-independent mechanism. *Mol. Pharmacol.* **71,** 1222–1232
36. Bowton, E., Saunders, C., Erreger, K., Sakrikar, D., Matthies, H. J., Sen, N., Jessen, T., Colbran, R. J., Caron, M. G., Javitch, J. A., Blakely, R. D., and Galli, A. (2010) Dysregulation of dopamine transporters via dopamine D2 autoreceptors triggers anomalous dopamine efflux associated with attention-deficit hyperactivity disorder. *J. Neurosci.* **30,** 6048–6057
37. Williams, J. M., Owens, W. A., Turner, G. H., Saunders, C., Dipace, C., Blakely, R. D., France, C. P., Gore, J. C., Daws, L. C., Avison, M. J., and Galli, A. (2007) Hypoinsulinemia regulates amphetamine-induced reverse transport of dopamine. *PLoS Biol.* **5,** e274
38. Owens, W. A., Williams, J. M., Saunders, C., Avison, M. J., Galli, A., and Daws, L. C. (2012) Rescue of dopamine transporter function in hypoinsulinemic rats by a D2 receptor-ERK-dependent mechanism. *J. Neurosci.* **32,** 2637–2647
39. Mulherkar, S. A., and Jana, N. R. (2010) Loss of dopaminergic neurons and resulting behavioural deficits in mouse model of Angelman syndrome. *Neurobiol. Dis.* **40,** 586–592
40. Harbord, M. (2001) Levodopa responsive Parkinsonism in adults with Angelman syndrome. *J. Clin. Neurosci.* **8,** 421–422