Amphetamine Action at the Cocaine- and Antidepressant-Sensitive Serotonin Transporter Is Modulated by α CaMKII

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Serotonergic neurotransmission is terminated by reuptake of extracellular serotonin (5-HT) by the high-affinity serotonin transporter (SERT). Selective 5-HT reuptake inhibitors (SSRIs) such as fluoxetine or escitalopram inhibit SERT and are currently the principal treatment for depression and anxiety disorders. In addition, SERT is a major molecular target for psychostimulants such as cocaine and amphetamines. Amphetamine-induced transport reversal at the closely related dopamine transporter (DAT) has been shown previously to be contingent upon modulation by calmodulin kinase II α (α CaMKII). Here, we show that not only DAT, but also SERT, is regulated by α CaMKII. Inhibition of α CaMKII activity markedly decreased amphetamine-triggered SERT-mediated substrate efflux in both cells coexpressing SERT and α CaMKII and brain tissue preparations. The interaction between SERT and α CaMKII was verified using biochemical assays and FRET analysis and colocalization of the two molecules was confirmed in primary serotonergic neurons in culture. Moreover, we found that genetic deletion of α CaMKII impaired the locomotor response of mice to 3,4-methylenedioxymethamphetamine (also known as “ecstasy”) and blunted D-fenfluramine-induced prolactin release, substantiating the importance of α CaMKII modulation for amphetamine action at SERT *in vivo* as well. SERT-mediated substrate uptake was neither affected by inhibition of nor genetic deficiency in α CaMKII. This finding supports the concept that uptake and efflux at monoamine transporters are asymmetric processes that can be targeted separately. Ultimately, this may provide a molecular mechanism for putative drug developments to treat amphetamine addiction.

Key words: addiction; amphetamines; monoamine transporters; reverse transport; serotonin

Introduction

Serotonergic signaling is terminated by the reuptake of extracellular serotonin (5-HT) through the serotonin transporter (SERT;

Murphy and Lesch, 2008). SERT is a member of the solute carrier 6 (SLC6) gene family, which also includes dopamine transporters (DATs) and norepinephrine transporters (NETs). These transporters use the sodium gradient to transport neurotransmitters against the concentration gradient (Kristensen et al., 2011). In the presynaptic specialization, 5-HT is retrieved into synaptic vesicles by the vesicular monoamine transporter (VMAT).

SERT is a major target of psychostimulants such as cocaine or amphetamines and is thus thought to be a key player in substance abuse and addiction (Müller et al., 2007; Kirby et al., 2011). Cocaine and amphetamines differ in two important ways: their mechanism of action and their selectivity. Cocaine is a nonselective inhibitor at monoamine transporters; it binds to all three transporters with comparable affinity (Kristensen et al., 2011). Therefore, cocaine elicits its stimulant action by raising synaptic monoamine levels. In contrast, different amphetamine derivatives are selective for the one or other monoamine transporter. For example, D-amphetamine (“speed”) has higher affinities for DAT and NET compared with SERT, whereas fenfluramine or 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) show a higher affinity for SERT (Green et al., 2003). Similarly, the mechanism of action of amphetamines is complex (Sitte and Freissmuth, 2015): amphetamines are recognized as substrates of

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both plasma membrane transporters and VMATs (Schuldiner et al., 1993). Accordingly, they accumulate within the synaptic vesicles, where they dissipate the proton gradient; therefore, 5-HT increases within the cytosol. In addition, amphetamines effectively trigger a sustained Na^+ inward current (Schicker et al., 2012). The cytosolic accumulation of both Na^+ and substrate allows for reverse transport. However, this simplified model does not take into account the asymmetrical nature of inward and outward transport: amphetamine-triggered reverse transport—but not substrate influx—for example, is contingent on the presence of phosphatidylinositol-4,5-bisphosphate (Buchmayer et al., 2013). Interestingly, calmodulin kinase II α (α CaMKII) was found to play a critical role in amphetamine-triggered reverse transport of dopamine by DAT via an interaction of the kinase with the C terminus of the transporter (Fog et al., 2006; Rickhag et al., 2013). This interaction facilitates N-terminal phosphorylation of DAT, which in turn promotes efflux through the transporter (Khoshbouei et al., 2004).

The hydrophobic core of SERT, NET, and DAT share extensive homology, but their intracellular segments, in particular, their N- and C termini are diverse. Therefore, it is conceivable that the interaction of DAT and α CaMKII is unique, as suggested originally (Fog et al., 2006). Here, we tested this hypothesis. Mass spectrometry identified α CaMKII in complex with SERT, which had been immunopurified from brain extracts, and an interaction was recapitulated *in vitro* and in cotransfected cells in which α CaMKII modulated amphetamine-triggered reverse transport through SERT. Finally, the behavioral and physiological action of MDMA and D-fenfluramine (D-FEN) were blunted in mice deficient in α CaMKII. Our findings demonstrate that α CaMKII is central to reverse transport by SERT and support the emerging role of the 5-HT system in psychostimulant abuse and addiction (Kirby et al., 2011).

Materials and Methods

Reagents. S(+)-MDMA, para-chloroamphetamine (pCA), D-FEN, D-amphetamine, pargyline, paroxetine, nisoxetine, and nomifensine were from Sigma-Aldrich; KN-93 and autocamtide-2 related inhibitory peptide II (AIP) were from Calbiochem. [^3H]S-citalopram (85 Ci/mmol; custom labeled) and [^3H]5-hydroxytryptamine-creatinesulfate (28.1 Ci/mmol) were obtained from PerkinElmer Life Sciences. [^3H]MPP $^+$ (85 Ci/mmol) was provided by American Radiolabeled Chemicals. Cell culture media, supplements, and antibiotics were from Invitrogen. The murine monoclonal anti-SERT antibody (H-45) has been described in Montgomery et al. (2014). The goat anti-SERT (sc-1458) and mouse monoclonal anti- α CaMKII antibodies were from Santa Cruz Biotechnology. The mouse anti- α -tubulin was from Sigma-Aldrich. Alexa Fluor 488 and 568 antibodies were from Invitrogen; the secondary horseradish peroxidase-conjugated antibodies directed against mouse, rabbit, or goat IgG were from GE Healthcare.

Animals. The generation of α CaMKII knock-out (KO) mice has been described previously (Elgersma et al., 2002). SERT-Cre mice were used to produce SERT KO mice and are described in detail in Zhuang et al. (2005) and Montgomery et al. (2014). All mice were bred on a C57BL/6J background and were housed under standard laboratory conditions (12 h light/12 h dark cycle). Food and water were provided *ad libitum*. For behavioral experiments, only male mice at 12–20 weeks of age were tested. Wild-type (WT) littermates were used as controls.

Female Sprague Dawley rats were obtained from the Research Facility for Animal Experimentation (Forschungsanstalt für Versuchstierzucht; Himberg, Austria). All animal experiments were conducted in accordance with protocols approved by the Animal Welfare Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (License BMWF • 66.009/0250-II/3b/2013).

Cell culture. A cell line stably expressing both, α CaMKII and an N-terminally YFP-tagged human SERT (YFP-SERT) was created by se-

quentially transfecting HEK-293 cells with plasmids encoding α CaMKII and YFP-SERT. The stable monoclonal α CaMKII/YhS cells were cultured in DMEM, 10% FCS, 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin, 5 $\mu\text{g}/\text{ml}$ blasticidin, 175 $\mu\text{g}/\text{ml}$ hygromycin, and 500 $\mu\text{g}/\text{ml}$ geneticin (G418). CAD cells were cultured in 50% DMEM/50% Ham's F12 medium supplemented with 10% FCS and 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin and transfected with TurboFect Transfection Reagent (Thermo Scientific) according to the manufacturer's instructions.

Primary serotonergic and hippocampal neurons. The procedure used for primary serotonergic and hippocampal neurons was described in detail in Montgomery et al. (2014). Briefly, dorsal raphe nuclei or hippocampi were isolated from 1- to 3-d-old rat pups. Tissue was digested in papain for 20 min at 37°C and subsequently triturated to dissociate the cells. Cells were centrifuged for 4 min at 500 $\times g$ and resuspended in neuronal medium (Neurobasal A, 2% B27, 1% heat-inactivated calf serum, 0.4 mM glutamine, and 50 μM kynurenic acid). Raphe neurons were then seeded onto a confluent monolayer of rat glial cells grown on 15 mm glass coverslips precoated with poly-D-lysine. Hippocampal neurons were seeded onto poly-D-lysine-coated Ibidi μ -slide chambered coverslips. Glial-derived neurotrophic factor (Millipore) was added to raphe cultures 2 h after plating to stimulate the sprouting and maturation of neurons. 5-Fluorodeoxyuridine was added to inhibit the proliferation of glia in both raphe and hippocampal cultures.

FRET. FRET (Schmid and Sitt, 2003) was measured with a Carl Zeiss Axiovert 200 epifluorescence microscope. We used either HEK-293 cells or rat hippocampal neurons transiently transfected with plasmid cDNA (1.7 μg) by means of the calcium phosphate coprecipitation method, as described previously (Egaña et al., 2009; Sucic et al., 2010). Cells were transfected directly in Ibidi μ -slide chambered coverslips with 8 wells. Directly before each FRET microscopy experiment, every well was washed with 300 μl of Krebs–HEPES buffer (KHB) containing the following (in mM): 10 HEPES, 130 NaCl, 1.3 KH_2PO_4 , 1.5 CaCl_2 , and 0.5 MgSO_4 , pH 7.4 adjusted with NaOH, and incubated in 150 μl of KHB. The “three-filter method” was performed as described previously (Bartholomäus et al., 2008). Images were acquired using a 63 $\times$ oil-immersion objective under continuous usage of a gray filter (20% density). LUDL filter wheels allowed for a rapid excitation and emission filter exchange. The LUDL filter wheels were configured as follows: CFP (I_{Donor} ; excitation: 436 nm, emission: 480 nm, and dichroic mirror: 455 nm), YFP (I_{Acceptor} ; excitation: 500 nm, emission: 535 nm, and dichroic mirror: 515 nm), and FRET (I_{FRET} ; excitation: 436 nm, emission: 535 nm, and dichroic mirror: 455 nm). Images were acquired with a CCD camera (Coolsnap fx; Roper Scientific) using the MetaMorph of Meta-Series software package (release 4.6; Universal Imaging). Pixelshift was corrected whenever necessary using the ImageJ plugins TurboReg and StackReg (Thevenaz et al., 1998). Background fluorescence was subtracted from all images. We analyzed the images pixel by pixel using ImageJ version 1.43b and the ImageJ plugin PixFRET (Pixel by Pixel analysis of FRET with ImageJ, version 1.6.0_10; Feige et al., 2005) spectral bleed-through parameters were determined for the donor bleed-through (BT) and the acceptor BT. Next, FRET efficiency (E) was computed: the mean FRET efficiency was measured at the region of interest using the computed FRET efficiency image. The regions of interest were selected in the CFP (donor) or YFP (acceptor) image (to avoid bleaching-associated bias) and transmitted to the FRET image (equivalent to the Youvan-image, FRETc; Youvan et al., 1997) using the ImageJ Multi Measure Tool. All experiments were conducted for three to five individual transfections; seven to 10 wide-field images were captured during each experiment and one to seven transfected cells per image were included in the study.

Preparation of synaptosomes and slices. Adult mice were killed by cervical dislocation and brains were removed immediately. Hippocampus and cortex were dissected and homogenized in ice-cold PBS containing 0.32 M sucrose and protease inhibitors (Roche Complete) to prepare synaptosomes. The suspension was centrifuged for 10 min at 1000 $\times g$. The supernatant was again centrifuged for 15 min at 12,600 $\times g$. The resulting pellet (P_2) was weighed and stored on ice until the start of the experiment. Alternatively, the tissue was cut into slices (0.3 mm) using a McIlwain tissue chopper. Slices were kept in KRB containing the follow-

ing (in mM): 24.9 NaHCO₃, 1.2 KH₂PO₄, 146.2 NaCl, 2.7 KCl, 1.0 MgCl₂, and 10 glucose, along with 50 μ M ascorbate and 50 μ M pargyline, until the beginning of the experiment. Nisoxetine (10 nM) and nomifensine (100 nM) were added to KRB throughout the whole experiment to block NET and DAT, respectively.

Efflux. Superfusion experiments on cells and synaptosomes are described in detail in Susic et al. (2010) and Steinkellner et al. (2012), respectively. Briefly, P₂ pellets were resuspended in KRB to yield a final concentration of 1 mg (wet weight) of P₂ pellet in a total volume of 15 μ l. Synaptosomal fractions or slices were incubated with 0.4 μ M [³H]5-HT for 30 min at 37°C; cells were incubated with 0.1 μ M [³H]MPP⁺ in KHB for 20 at 37°C. Subsequently, cells, slices, or P₂ fractions were placed in superfusion chambers that were perfused for 40 min at a flow rate of 0.7 ml/min (25°C) to establish a stable basal efflux of radioactivity. The end of this washout period corresponded to the zero time point. The superfusate was collected in intervals of 2 min, resulting in fractions of 1.4 ml. After 6 min, KN-93 (final concentration 1 to 10 μ M), AIP (0.1 to 1 μ M), or the corresponding amount of DMSO (vehicle) was added to the superfusate. After an additional interval of 6 min, the cells, slices, or P₂ fractions were challenged by the addition of amphetamines (in concentrations indicated in the pertinent figures) to induce efflux. The superfusion with amphetamine lasted for 10 min, resulting in the collection of 5 fractions; thereafter, cells, slices, or P₂ pellets 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. Efflux of radioactive substrate is expressed as percentage of total radioactivity (i.e., the sum of the total remaining radioactivity and the released radioactivity) present at each time point. Alternatively, the released amount was expressed as area under the curve (AUC). AUC of amphetamine-induced efflux was calculated from the sum of the released radioactivity in the five fractions collected in the presence of amphetamine and normalizing this sum to the baseline efflux (i.e., the mean of the first three fractions).

Uptake. Uptake of [³H]5-HT by cortical synaptosomes was measured as follows. The P₂ pellet was resuspended in KRB (20 to 30 μ g of protein/assay). The final assay volume was 0.2 ml of KRB containing 10 nM nisoxetine and 100 nM nomifensine to block DAT and NET, respectively, and 20 nM [³H]5-HT (final concentration). Nonspecific uptake was determined in the presence of 1 μ M paroxetine. After 5 min at 37°C, the assay was terminated by the addition of 2.5 ml of ice-cold KRB and rapid filtration through GF/B filters (Whatman), followed by 2 more wash steps. Radioactivity on the filters was measured by liquid scintillation. SERT-dependent cellular uptake of [³H]5-HT was determined as in Susic et al. (2010).

Radioligand binding. Cortical synaptosomes (P₂; 20–30 μ g of protein/reaction) were resuspended in buffer containing the following (in mM): 20 Tris-HCl, pH = 7.4, 2 MgCl₂, 120 NaCl, and 3 KCl, along with 0.3–30 nM [³H]S-citalopram. Nonspecific binding was determined in the presence of 10 μ M paroxetine. After 20 min at 22°C, the binding reaction was stopped by rapid filtration over GF/B filters presoaked in 2% polyethylenimine using an automated cell harvester filtration device (Skatron Instruments). The radioactivity bound to the filters was measured by liquid scintillation counting.

Immunoprecipitation. Immunoprecipitations (IPs) were performed on detergent extracts prepared from HEK-293 cells or from synaptosomal membranes (P₂ pellet) obtained from rat or mouse brain. Cells were washed 3 times with ice-cold PBS and lysed in buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 supplemented with protease inhibitors (Roche Complete). The suspension was incubated for 30 min at 4°C on a rotator. For extracts from synaptosomes, P₂ pellets were resuspended in buffer of similar composition except that the concentration of Triton X-100 was 0.7%. Detergent extracts were obtained by centrifugation for 10 min at 13,000 $\times$ g. The protein concentration in the supernatant was measured using the BCA protein assay (Thermo Scientific). The detergent extracts were incubated overnight at 4°C on a rotator with the H-45 murine monoclonal antibody, which specifically recognizes SERT but not DAT or NET (Montgomery et al., 2014). On the next day, preequilibrated protein G Sepharose was added and samples were incubated for 3–4 h on a rotator at 4°C. Thereafter, beads were washed at least 3 times with 1 ml of ice-cold

buffer. Bound proteins were released by denaturation in 50 μ l of electrophoresis sample buffer for 3 min at 95°C. Subsequently, the immunoprecipitated proteins were resolved by denaturing gel electrophoresis, transferred to nitrocellulose, and visualized by immunoblotting with the pertinent antibodies.

In-gel digestion. Detergent extracts were prepared from rat whole brains and SERT was immunoprecipitated as described above. After electrophoretic separation of the immunoprecipitated proteins, Coomassie-blue-stained bands were excised from the polyacrylamide gel. Gel pieces were destained with 50% acetonitrile in 50 mM ammonium bicarbonate and dried in a speed vacuum concentrator. After reduction and alkylation of cysteine residues, gel pieces were washed and again dehydrated. Dried gel pieces were rehydrated with 25 mM ammonium bicarbonate, pH 8.0, containing 10 ng/ μ l trypsin (Promega) and incubated for 18 h at 37°C. The 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. For LC-MS/MS, samples were injected into an Ultimate 3000 nano-HPLC system (Dionex) equipped with PepMap100 C-18 trap column (300 μ m $\times$ 5 mm) and PepMap100 C-18 analytic column (75 μ m $\times$ 150 mm) and connected in-line to an ion trap mass spectrometer (HCT; Bruker Daltonics). Peptides were separated by reversed phase chromatography in 0.1% formic acid in water (buffer A) and 0.08% formic acid in 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 sprayed directly 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 collision-induced dissociation MS/MS spectra generated from four highest intensity precursor ions. The MS/MS spectra were interpreted with the Mascot search engine (Matrix Science). Database searches through Mascot were performed with a precursor ion mass tolerance of 0.2 Da and a MS/MS tolerance of 0.5 Da; in addition, search parameters allowed for up to three missing cleavage sites, carbamidomethylation of cysteine, oxidation of methionine, and deamidation of asparagine/glutamine (Kang et al., 2009).

Glutathione-S-transferase pull-down. Fusion proteins comprising glutathione-S-transferase (GST) from *Schistosoma japonicum* and the N and C terminus of SERT or DAT were generated by amplifying the sequences encoding the N and C terminus by PCR using the cDNA of human SERT or a synthetic human DAT (Saunders et al., 2000), respectively, as the template. GST and GST fusion proteins were expressed in *E. coli* BL21(DE3) and purified from bacterial lysates as outlined in Farhan et al. (2007).

In pull-down assays, purified GST fusion proteins or GST (30 μ g) and detergent extract prepared from HEK-293 cells expressing α CaMKII (100 μ g) were incubated in a total volume of 0.5 ml of buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100 for 1 h on ice. Glutathione Sepharose 4B (50 μ l of pre-equilibrated 1:2 slurry) was added and the incubation continued on a rotator for 16 h at 4°C. Thereafter, beads were washed 3 times with 1 ml of buffer; bound proteins were released by denaturation for 5 min at 85°C in 50 μ l of reducing sample buffer.

Proteins were resolved by denaturing electrophoresis and transferred to nitrocellulose membranes; GST and GST fusion proteins were visualized by Ponceau S staining of nitrocellulose membranes or by Coomassie blue staining of the gel. Membranes were then sequentially incubated with the murine monoclonal anti- α CaMKII antibody (Santa Cruz Biotechnology) and the secondary horseradish peroxidase-conjugated antibody directed against mouse IgG. The immunoreactive material was visualized by enhanced chemoluminescence.

Immunocytochemistry. Cells were washed 3 times with PBS and fixed with acetone/methanol (1:1) for 2 min at room temperature. After fixation, cells were permeabilized and blocked in PBS containing 5% normal goat serum and 0.2% saponin for 1 h at room temperature. Primary antibodies were added to blocking/permeabilization buffer and incubated overnight at 4°C on a shaker. The next day, cells were washed 3 times with PBS and incubated with fluorescently labeled secondary anti-

bodies (Alexa Fluor 488 or 568; 1:3000) for 1 h at room temperature, followed by 3 washing steps in PBS. Immunofluorescence was visualized using a Zeiss confocal laser scanning microscope (LSM 780).

D-FEN-induced prolactin release. WT and α CaMKII KO mice were injected intraperitoneally with D-FEN (10 mg/kg) or saline and killed 30 min after injection. Blood was collected into EDTA microtubes (1.3 ml, K3 EDTA; Sarstedt). Serum was obtained via centrifugation ($3000 \times g$ at 4°C for 10 min) and examined using a Prolactin ELISA kit (RayBioR Mouse Prolactin ELISA Kit). The Prolactin ELISA was performed according to the manufacturer's protocol. In brief, serum samples were diluted in assay diluent A (including $1 \times$ proteinase inhibitor; Roche) 5-fold and 50-fold, respectively. Then, 100 μl of standard solution or diluted serum samples was added to each well and incubated overnight at 4°C . A standard curve was prepared according to the manufacturer's instructions. Next, 100 μl of biotin antibody solution was added to each well and incubated for 1 h at room temperature. After the addition of 100 μl of streptavidin solution, the mixture was incubated for 45 min at room temperature. Finally, 100 μl of 3,3',5,5'-tetramethylbenzidine One-Step Substrate Reagent was added to each well and the plate was subsequently incubated for 30 min at room temperature under light protection. After the addition of 50 μl of stop solution to each well, the absorption was immediately determined at 450 nm.

Behavioral pharmacology. Horizontal locomotion (total distance traveled) was measured in open-field square boxes ($36 \times 36 \times 45$ cm) using a video camera mounted above the box and analyzed using Anymaze version 4.7 software from Stoelting. Distances traveled were recorded for 60 min. Acute drug effects were assessed as follows: mice were administered an intraperitoneal injection of saline on day zero (d0); their locomotion were measured for 60 min to establish a baseline. On the next day (d1), mice were administered MDMA (10 mg/kg) by intraperitoneal injection. Distances traveled were again recorded for 60 min. Acute drug effects were normalized to the distances traveled upon injection of saline and are expressed as the fold increase in locomotion.

Statistics. The statistical significance of differences was evaluated using one-way ANOVA followed by Tukey's *post hoc* test or Student's *t* test where appropriate. Data are shown as mean $\pm$ SEM.

Results

SERT and α CaMKII are found within the same protein complex and interact physically *in vitro*

DAT seems to be a substrate of α CaMKII: *in vitro*, it has been shown that a DAT N-terminal peptide was phosphorylated by α CaMKII, so it was hypothesized that the kinase is first recruited via its association domain to the C terminus of DAT and subsequently phosphorylates N-terminal residues, which finally supports amphetamine-induced reverse transport (Fog et al., 2006). In this previous study, there was no evidence for an interaction between SERT and α CaMKII. This is surprising, because SERT and DAT are closely related and mass spectrometry indicates that SERT is a phosphoprotein containing multiple phosphorylated serine and threonine residues (J.-W.Y. and H.H.S., unpublished observation; Ramamoorthy et al., 2011). Because the original approach relied on heterologous coexpression of SERT and α CaMKII (Fog et al., 2006), we generated detergent extracts from brain membranes and immunopurified SERT with a specific monoclonal SERT antibody (H-45) (Montgomery et al., 2014; Fig. 1A). The immunopurified fraction was subsequently subjected to mass spectrometry. The analysis revealed that α CaMKII was coimmunopurified with SERT from rat brain lysates (Fig. 1B,C). Similar to DAT, we also detected an interaction between the β isoform of CaMKII and SERT (Fig. 1D). It is worth noting that the association was observed in the absence of prior activation of α CaMKII. This most probably accounts, at least in part, for the fact that the levels of α CaMKII (and of β CaMKII) were substoichiometric—that is, substantially lower than that of SERT

(cf. Fig. 1A). We used two approaches to verify that the coimmunopurification of α CaMKII was specific: detergent extracts were prepared from hippocampal membranes of SERT-deficient and α CaMKII-deficient mice. As anticipated, immunoprecipitation of SERT retrieved α CaMKII from hippocampal extracts of WT mice but not of SERT KO or of α CaMKII KO animals (Fig. 1E). These observations suggest that SERT and α CaMKII can form a complex in the brain. We therefore investigated whether this interaction might occur via direct interaction by fusing the first 87 aa of the SERT N terminus and the last 35 aa of the SERT C terminus to GST. Although GST pull-downs do not formally prove direct protein–protein interactions, they at least give a strong indication that this might be the case. The purified GST fusion proteins were incubated with a lysate containing overexpressed α CaMKII. We used GST fusion proteins comprising the N terminus (i.e., the first 66 aa) or the C terminus (i.e., the last 43 residues) of DAT and GST as positive and negative controls, respectively. From Figure 2A, it is evident that the C terminus, but not the N terminus, of SERT retrieved α CaMKII (Fig. 2A). As expected (Fog et al., 2006), there was robust binding of α CaMKII to the C terminus of DAT and a weak interaction with its N terminus (Fig. 2A). We quantified the level of recovered α CaMKII by pull-down and related this to the amount of GST fusion protein to gauge the mutual affinity of α CaMKII and the GST fusion protein. Based on this estimate, the C terminus of SERT pulled down α CaMKII less efficiently than the C terminus of DAT, but more efficiently than its N terminus (Fig. 2B). We performed a statistical analysis to test whether there is a difference in binding of α CaMKII to DAT- or SERT-GST fusion proteins (or a complex thereof involving additional unknown proteins). *Post hoc* analysis (one-way ANOVA) indicated that there is a significant difference between the binding of α CaMKII to the DAT-C terminus and the SERT-C terminus ($n = 3$; $F = 9.619$, $df = 7$; $p < 0.05$), suggesting that the binding affinities of α CaMKII differ between DAT and SERT N termini, with binding of α CaMKII to DAT being stronger compared with SERT, at least under the *in vitro* conditions used in our assays. GST alone did not pull down any α CaMKII (Fig. 2A).

FRET reveals interaction of SERT and α CaMKII in live HEK-293 cells and hippocampal neurons

We used FRET microscopy to visualize the interaction of α CaMKII and SERT in live cells. For that purpose, HEK-293 cells were transfected with various combinations of either N-terminally or C-terminally CFP-tagged SERT constructs (C-SERT) and N-terminally CFP or YFP-tagged α CaMKII (C-CaMKII, Y-CaMKII). First, however, we gauged our system by measuring cells expressing CFP and YFP, which interact only spuriously and therefore serve as a negative control (FRET efficiency: 3.8 ± 0.4); a fusion protein between the two results in strong resonance energy transfer (FRET efficiency: 36.6 ± 2.2 ; Schmid et al., 2001) or the SERT with both fluorophores attached to both intracellular termini (CFP-SERT-YFP, FRET efficiency: 35.5 ± 0.8 ; Just et al., 2004).

Subsequent FRET analysis indicated that C-CaMKII and Y-CaMKII had a high mean FRET efficiency of $31.6 \pm 1.4\%$ (Fig. 3A,B). This is expected because CaMKII is a dodecameric complex. The examination of C-SERT and Y-CaMKII yielded a mean FRET efficiency of $25.7 \pm 1.2\%$ (Fig. 3A,B), which indicated that the two proteins were present in close proximity to allow for resonance energy transfer to occur. This observation provides an independent confirmation of our biochemical results reported

Figure 1. Mass spectrometry of SERT-containing complexes immunoprecipitated from native brain. **A**, Coomassie-blue-stained SDS-PAGE gel with SERT protein complex immunopurified by an anti-SERT antibody. LC-MS/MS identified SERT, CaMKII α , and CaMKII β subunits in immunopurified protein complex as indicated on the left. The vertical lines on the right indicate the cut-out gel areas used for LC-MS/MS analysis which contained the indicated CaMKII subunits. **B**, Double-charged (2^+) MS/MS-spectrum obtained at m/z 764.93 was fragmented to produce a tandem mass spectrum with y - and b -ion series that identified the sequence EMLGSPGWFWR (aa 527–538 with methionine oxidation) from rat SERT. **C**, MS/MS spectrum at m/z 631.39 (2^+) showing FYFENLWSR (aa 397–405) of α CaMKII was identified from the immunopurified SERT protein complex. **D**, MS/MS spectrum obtained at m/z 810.91 (2^+) assigns the sequence FTDEYQLYEDIGK (aa 10–22) to β CaMKII in the SERT coimmunoprecipitates. **E**, Immunoprecipitation of α CaMKII with SERT from mouse hippocampus: WT, α CaMKII KO, or SERT KO hippocampal lysates were incubated with a specific anti-SERT antibody (H-45) and probed for the presence of α CaMKII by Western blotting. Shown is a representative immunoblot ($n = 3$).

above. Importantly, the coexpression of a SERT construct with C-terminal attachment of CFP and Y-CaMKII resulted in a low FRET efficiency (3.2 ± 0.8 ; Fig. 3A,B), a value that is comparable to the FRET efficiency obtained with our negative control CFP and YFP. Therefore, these results suggest that the CFP tag at the C terminus of SERT precludes its interaction with α CaMKII. This is consistent with our GST pull-down data, in which only a SERT C-terminal GST construct was able to coprecipitate CaMKII. Therefore, from our pull-down and FRET data, we surmise that the interaction of SERT and CaMKII is most likely mediated by the C terminus of SERT, although we cannot formally exclude an

indirect interaction through additional, yet undiscovered intermediary proteins.

In addition, we used hippocampal neurons transfected with C-SERT and Y-CaMKII to verify that SERT and CaMKII also interacted in neuronal primary cells. FRET analysis revealed a FRET efficiency of 24.8 ± 2.1 , indicating that this interaction also occurred in native neurons (Fig. 3A).

α CaMKII colocalizes with SERT in serotonergic neurons

If α CaMKII modulated reverse transport by SERT through a direct interaction *in vivo*, the two proteins ought to be present in the

Figure 2. GST pull-down: **A**, Top, Representative GST pull-down experiment ($n = 3$) of α CaMKII from lysates of HEK-293 cells stably transfected with α CaMKII using GST alone or GST fused to either the DAT N termini, DAT C termini, SERT N termini, or SERT C termini. Bound α CaMKII was detected by Western blotting using a specific anti- α CaMKII antibody. Bottom, Ponceau S staining of GST fusion proteins on nitrocellulose membrane after electrotransfer as a loading control. **B**, α CaMKII band densities after GST pull-downs were quantified by densitometry using ImageJ and normalized to GST input ($n = 3$); n.d., Not detected.

same cellular compartment. Accordingly, we first looked at possible colocalization using HEK-293 cells transfected with both YFP-tagged SERT and untagged mouse α CaMKII. Figure 4A shows confocal microscopy images of SERT α CaMKII colocalizing closely at the plasma membrane. In addition, we prepared primary serotonergic neuronal cultures from the dorsal raphe nuclei of newborn rats. These cultures were previously extensively characterized and shown to support axonal delivery of SERT (Montgomery et al., 2014). After 14 d *in vitro*, the cells were fixed and stained with antibodies against SERT and α CaMKII. Figure 4B shows that both α CaMKII and SERT were coexpressed within the same neuron. Merging the pictures revealed considerable colocalization (Fig. 4B1,B2, right). We also stained the cultures for tryptophan hydroxylase 2 (TpH2), the rate-limiting enzyme in the 5-HT synthesis and a specific marker for 5-HT neurons, to verify the serotonergic nature of the neurons. Immunoreactivity for TpH2 was visualized in the same cells as SERT (Fig. 4C).

Inhibition of α CaMKII function impairs amphetamine-induced efflux but does not affect substrate uptake at SERT in cells

Fog et al. (2006) demonstrated that the small-molecule CaMKII-inhibitor KN-93 decreased the ability of amphetamine to induce reverse transport by DAT. SERT and DAT share a high degree of homology and both are targets for amphetamines. Therefore, we investigated whether α CaMKII also modulated the action of amphetamines on SERT. For that purpose, we measured

amphetamine-induced efflux in HEK-293 cells, which stably expressed YFP-tagged human SERT and α CaMKII, using a superfusion system. Cells were preloaded with [3 H]MPP $^+$, a radiolabeled substrate of SERT, and subsequently superfused with buffer. The addition of pCA (3 μ M) induced robust SERT-mediated [3 H]MPP $^+$ efflux (Fig. 5A). This efflux was decreased by the addition of KN-93 to the superfusion buffer in a concentration-dependent manner (Fig. 5A,B). We also used the neuronal-like cell line CAD, which endogenously expresses CaMKII (Dipace et al., 2007). CAD cells were transfected with hSERT and efflux was monitored in the presence or absence of KN-93. A concentration of 10 μ M KN-93 also significantly reduced SERT-mediated efflux in CAD cells in the presence of physiological CaMKII levels (Fig. 5C). KN-93 may also affect kinases other than α CaMKII. Accordingly, we tested whether the α CaMKII-specific inhibitor autocamtide 2-related inhibitory peptide II (AIP) reduced pCA-triggered efflux. As may be seen from Figure 5D, AIP also decreased SERT-mediated reverse transport in YFP-hSERT/ α CaMKII HEK-293 cells in a concentration-dependent manner. We verified that inhibition of α CaMKII did not affect substrate influx by measuring substrate uptake in HEK-293 and CAD cells in the presence of CaMKII inhibitors (Fig. 5E,F). These observations

are consistent with the concept that uptake and efflux via monoamine transporters are asymmetrical processes (Sitte et al., 1998).

Inhibition or genetic ablation of α CaMKII attenuate amphetamine-induced SERT-mediated efflux in mice

We verified next that α CaMKII modulated the action of amphetamines on SERT in native brain preparations by measuring efflux using cortical synaptosomes that had been preloaded with [3 H]5-HT. Inhibition of α CaMKII by KN-93 (10 μ M) or AIP (100 nM) significantly attenuated pCA-induced SERT-mediated [3 H]5-HT efflux in cortical synaptosomes of WT mice (Fig. 6A). These findings predicted that synaptosomes of α CaMKII-deficient mice would exhibit impaired amphetamine-induced efflux. This prediction was verified: pCA-induced [3 H]5-HT efflux was reduced by $\sim$ 50% (Fig. 6A). Finally, synaptosomes from α CaMKII-deficient mice also allowed for examining the specificity of KN-93: when synaptosomes prepared from α CaMKII KO animals were superfused with 10 μ M KN-93, the compound did not decrease pCA-triggered efflux (AUC in the presence of 10 μ M KN-93 = $100.5 \pm 21.5\%$ of AUC in the presence of DMSO; $n = 3$; means $\pm$ SEM). This observation formally proves that the decrease in SERT-dependent efflux elicited by KN-93 is accounted for by a specific action on α CaMKII rather than an off-target effect.

We ruled out that WT and α CaMKII KO animals differed in SERT levels using three different approaches: (1) SERT-mediated, paroxetine-sensitive [3 H]5-HT uptake was comparable in synap-

Figure 3. FRET microscopy of SERT and α CaMKII in HEK-293 cells and hippocampal neurons. **A**, HEK-293 cells were transfected with plasmids encoding CFP-CaMKII and YFP-CaMKII, CFP-SERT and YFP-CaMKII or SERT-CFP and YFP-CaMKII, respectively. The last two lines show representative FRET images of hippocampal neurons transfected with plasmids encoding C-SERT and Y-CaMKII. The columns show CFP and YFP images as indicated; the third column shows a false color rendering of the BT-corrected FRET image. All images are representative of 3 experimental days and corrected for background. Scale bar, 10 μ m. **B**, FRET efficiency was determined as described previously (Fenollar-Ferrer et al., 2014); cells expressed the indicated constructs: CFP-CaMKII + YFP-CaMKII ($n = 55$ cells), CFP-SERT + YFP-CaMKII ($n = 27$), SERT-CFP and YFP-CaMKII ($n = 26$); differences were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test ($F = 109.3$, $df = 107$, $p < 0.0001$).

Figure 4. Confocal microscopy showing colocalization of SERT and α CaMKII in HEK-293 cells and in cultured serotonergic neurons. **A**, HEK-293 cells were transfected with plasmids encoding for YFP-tagged SERT (YFP-SERT) and untagged mouse α CaMKII; cells were fixed 48 h after transfection and stained with a monoclonal antibody against α CaMKII followed by an Alexa Fluor 568 secondary antibody. **B**, **C**, Primary serotonergic neurons were isolated from the dorsal raphe nuclei of rats and cultured *in vitro* for 14 d before they were fixed with acetone/methanol (1:1) and stained with antibodies against SERT (+ Alexa Fluor 488) and α CaMKII (+ Alexa Fluor 568) (**B1**, **B2**) or TpH2 (+ Alexa Fluor 568) (**C**). Images taken using an LSM710 confocal microscope at an overall magnification of $63\times$.

some prepared from WT and α CaMKII-deficient mice (Fig. 6B); (2) saturation hyperbolas for binding of [3 H]S-citalopram overlapped resulting in comparable affinity (K_D) and maximal binding (B_{max}) of the radioligand (Fig. 6C); and (3) immunoblotting for SERT did not reveal any significant differences between α CaMKII-deficient mice and WT controls (Fig. 6D). Therefore, the decrease observed in *p*CA-induced efflux is a functional consequence of blunted or absent α CaMKII activity rather than a change in SERT expression levels.

Behavioral and physiological effects of MDMA and D-FEN in mice lacking α CaMKII

We surmised that the *in vitro* and *ex vivo* findings summarized in Figures 5 and 6 ought to translate into a behavioral and physiological phenotype in α CaMKII-deficient mice. Accordingly, we investigated the effect of two SERT-specific amphetamine-derivatives, MDMA and D-FEN, *in vivo*.

First, we challenged WT and α CaMKII-deficient mice with the SERT-preferring amphetamine derivative MDMA and measured the locomotor response of mice. On the first day, mice received an intraperitoneal injection of saline and were placed into open-field chambers. Distances were recorded for 60 min to define locomotion under baseline conditions. On the next day, mice received an injection of MDMA (10 mg/kg, i.p.) and horizontal locomotion was again recorded for 60 min. Mice lacking α CaMKII were expected to respond with less locomotor activation than WT controls because less 5-HT was released in response to MDMA. This was indeed the case: locomotion of α CaMKII KO animals increased by only ~ 2 -fold compared with WT animals, which traveled an approximately 3-fold longer distance than upon saline injection (Fig. 7A). We also performed experiments in which we investigated the acute locomotor responses of WT and α CaMKII KO animals to cocaine (20 mg/kg). The psychostimulant action of cocaine is predicted to be independent of the modulation of DAT or SERT by α CaMKII because cocaine merely blocks these transporters in a competitive fashion. As expected and in contrast to MDMA and D-amphetamine (Steinkellner et al., 2014), WT and α CaMKII KO mice displayed comparable responses in horizontal locomotion when challenged with cocaine (Fig. 7B). We ruled out that the difference observed upon drug challenges might have been confounded by changes in habituation to the open-field chamber by assessing

Figure 5. Inhibition of α CaMKII reduces pCA-induced $[^3\text{H}]\text{MPP}^+$ efflux in HEK-293 cells stably transfected with hSERT and α CaMKII and in CAD cells transfected with hSERT. **A**, Cells were superfused with KHB and either incubated with vehicle (0.1% DMSO) or KN-93 (1 μM , 3 μM , 10 μM). pCA (3 μM) was added at minute 10. Superfusates containing $[^3\text{H}]\text{MPP}^+$ were collected and analyzed by liquid scintillation counting. **B**, Bar graph on the right represents the AUCs of pCA-induced $[^3\text{H}]\text{MPP}^+$ efflux ($n = 3$; one-way ANOVA followed by Tukey's multiple-comparisons test, $F = 25.67$; $df = 32$; $*p < 0.05$, $***p < 0.001$). **C**, AUCs of $[^3\text{H}]\text{MPP}^+$ efflux in CAD cells transfected with SERT; 3 μM pCA were added to induce efflux in the presence or absence of 10 μM KN-93. **D**, AUCs of pCA-induced $[^3\text{H}]\text{MPP}^+$ efflux in HEK-293 cells superfused in the presence of AIP (100 nM, 1 μM) or vehicle (0.1% DMSO). Data represent means $\pm$ SEM from 3 independent experiments; statistically significant differences were determined by one-way ANOVA followed by Dunnett's multiple-comparisons test ($F = 3.068$, $df = 25$; $*p < 0.05$). **E**, HEK-293 cells were preincubated for 15 min with vehicle (0.1% DMSO), KN-93 (1, 3, and 10 μM), or AIP (0.1 and 1 μM). Uptake of 100 nM $[^3\text{H}]\text{5-HT}$ was measured in the presence of vehicle or inhibitors for 1 min at room temperature. Nonspecific uptake was determined in the presence of 10 μM paroxetine ($n \geq 3$, measured in triplicate). Data were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test ($F = 2.593$; $df = 65$; $p > 0.05$). **F**, Saturation uptake of $[^3\text{H}]\text{5-HT}$ in CAD cells in the presence (10 μM) or absence (DMSO) of KN-93.

the extent of habituation to the open field. Both WT and α CaMKII KO mice habituated to the open-field on the second day of exposure compared with the first day, measured as a reduction in the horizontal locomotor activity (WT mice: day1: distance = 81.29 ± 29.87 m; day 2: distance = 48.97 ± 31.13 m; paired t test, $p < 0.001$; $n = 15$; KO mice: day 1: distance = 168.60 ± 66.80 m; day 2: distance = 145.60 ± 50.28 m; paired t test, $p < 0.05$; $n = 14$).

Second, we injected mice with D-FEN (10 mg/kg) to measure 5-HT-induced prolactin release in plasma of WT and α CaMKII KO mice as a marker for SERT-mediated efflux. Mice were killed 60 min after D-FEN injection and plasma prolactin levels were measured using an ELISA. Consistent with our hypothesis that α CaMKII modulates amphetamine action at SERT, we found that α CaMKII KO animals displayed significantly less prolactin release in response to D-FEN compared with WT controls, indi-

Figure 6. AUCs of $p\text{CA}$ -induced $[^3\text{H}]5\text{HT}$ efflux in cortical synaptosomes of WT and αCaMKII KO mice in the presence or absence of CaMKII inhibition: **A**, Quantification of $[^3\text{H}]5\text{HT}$ efflux triggered by $p\text{CA}$ ($3\ \mu\text{M}$). The graph shows the AUCs calculated from superfusion experiments performed as outlined in Figure 4 for synaptosomes prepared from WT mice in the absence and presence of $15\ \mu\text{M}$ KN-93, $100\ \text{nM}$ AIP, and from αCaMKII -deficient animals (KO). Data are from at least three independent experiments. Error bars represent SEM. Statistically significant differences were determined by one-way ANOVA followed by Tukey's multiple-comparisons test ($F = 6.944$, $df = 81$; $*p < 0.05$, $**p < 0.01$). **B**, $[^3\text{H}]5\text{HT}$ uptake ($20\ \text{nM}$ final concentration) was measured in cortical synaptosomes for $5\ \text{min}$ at 37°C . Nonspecific uptake was determined in the presence of $1\ \mu\text{M}$ paroxetine ($n = 5$, experiments were performed in duplicates; Student's t test: $t = 0.9823$, $df = 21$, $p = 0.3371$). **C**, $[^3\text{H}]S$ -citalopram saturation binding to cortical synaptosomes. Nonspecific uptake was determined in the presence of $1\ \mu\text{M}$ paroxetine. Data are means $\pm$ SEM from three independent experiments done in duplicate. The statistical comparison of S -citalopram binding parameters (K_d , B_{max}) was done by Student's t test (K_d : $t = 0.506$, $df = 4$, $p = 0.6395$; B_{max} : $t = 0.3251$, $df = 4$, $p = 0.7614$). **D**, SERT protein expression is not altered in the cortex of αCaMKII -deficient mice: SERT immunoreactivity was assessed in cortical lysates by immunoblotting using the H-45 mouse monoclonal SERT antibody. Blots were stripped afterward and checked for anti- α -tubulin (loading control). Band intensities of SERT were analyzed by ImageJ and normalized to α -tubulin ($n = 3$). Student's t test was used to compare the differences between SERT levels in WT and αCaMKII -deficient (KO) mice ($t = 0.6296$, $df = 4$, $p = 0.5361$).

cating that D-FEN induces less 5-HT efflux in mice lacking αCaMKII (Fig. 7B). Baseline prolactin levels after injection of vehicle (PBS) did not differ between genotypes (αCaMKII KO + PBS: $201.5 \pm 42.77\ \text{pg/ml}$; WT + PBS: $248.8 \pm 32.61\ \text{pg/ml}$; $n = 4$; Student's t test: $t = 0.8785$, $df = 6$, $p = 0.4134$).

Because $p\text{CA}$ was used to induce $[^3\text{H}]5\text{-HT}$ efflux from synaptosomes in the experiments described above (cf. Fig. 6), we also measured efflux elicited by MDMA to document that the decrease in locomotion seen with MDMA was also reflected by a reduced 5-HT release. In addition, these experiments were done in hippocampal slices of αCaMKII -deficient and WT mice rather than synaptosomes to recapitulate the findings in a less disrupted preparation: the serotonergic axon terminals, in which SERT and VMAT2 operate in a relay, are preserved in slices. When challenged with $10\ \mu\text{M}$ MDMA, hippocampal slices of αCaMKII KO mice released significantly less $[^3\text{H}]5\text{-HT}$ than those of WT animals (Fig. 7C). In addition, we performed the same experiment using D-FEN ($10\ \mu\text{M}$) as used for the prolactin release assay. There was again a significant decrease in $[^3\text{H}]5\text{-HT}$ efflux from slices of αCaMKII -deficient mice (Fig. 7D).

Discussion

Currently, amphetamines are the second most commonly abused drugs in Europe (European Monitoring Center for Drugs and Drug Addiction, 2013). The prevalence of their abuse is even higher in the United States and Australia (Johnson et al., 2013). Recently, synthetic derivatives of cathinone and piperazine-substituted compounds have become increasingly popular as amphetamine replacements and some may be purchased legally as "bath salts" or under other names on the internet (Baumann et al., 2013). Therefore, abuse of and addiction to amphetamines and modified derivatives thereof—referred to as "designer drugs"—represent a current major health burden to society because their repeated intake can be detrimental both mentally and physically and may even result in fatalities (Karila and Reynaud, 2011). Insights into the molecular underpinnings of amphetamine action are therefore crucial for developing new strategies to treat and prevent amphetamine abuse. Here, we reexamined the role of αCaMKII in regulating substrate flux through SERT.

Figure 7. MDMA- and cocaine-induced horizontal locomotion, *D*-FEN-induced prolactin levels, and [3 H]5-HT efflux in hippocampal slices of α CaMKII KO and WT mice. **A, B**, Normalized acute locomotor responses to intraperitoneal injection of MDMA or cocaine in WT and α CaMKII KO mice. Mice were habituated to the open field for 60 min 1 d before they received an injection of MDMA (10 mg/kg) or cocaine (20 mg/kg) and subsequent recording of distances for another 60 min. Total distances traveled after MDMA or cocaine were normalized to baseline locomotion and are expressed as the fold increase in locomotion (total distances covered in meters). **A**, MDMA: WT saline: 87.7 ± 9.6 m, WT MDMA: 256.4 ± 13.4 m; α CaMKII KO saline: 161.3 ± 16.2 m, α CaMKII KO MDMA: 327.2 ± 38.5 m; $n \geq 8$; **B**, cocaine: WT saline: 73.9 ± 4.0 m, WT cocaine: 224.3 ± 30.3 m; $n = 12$; α CaMKII KO saline: 152.2 ± 13.2 m, α CaMKII KO cocaine: 354.9 ± 35.8 m; $n = 16$. Student's *t* test was used for statistical analysis: **A**, MDMA: $n \geq 8$, $t = 2.939$, $df = 17$, $p = 0.0092$; **B**, cocaine: $n = 12$ – 16 , $t = 0.3072$, $df = 24$, $p = 0.7614$. **C**, *D*-FEN-induced prolactin levels in serum of α CaMKII KO or WT animals (α CaMKII KO + *D*-FEN: 216.3 ± 45.51 pg/ml; WT + *D*-FEN: 389.4 ± 17.47 pg/ml; $n = 4$; data were analyzed by Student's *t* test: $t = 3.551$; $df = 6$, $*p = 0.0121$; **D, E**, MDMA- (**D**) and *D*-FEN- (**E**) induced [3 H]5HT efflux in hippocampal slices of WT and α CaMKII KO mice: Hippocampal slices obtained from WT and α CaMKII KO mice were preloaded with [3 H]5-HT for 30 min at 37°C before superfusion. S(+)-MDMA (**D**) or *D*-FEN (**E**) were added to a final concentration of 10 μ M at minute 10 into the superfusion buffer to induce [3 H]5HT efflux. Graph shows the AUC of MDMA/*D*-FEN-induced efflux ($n = 3$ – 4 , Student's *t* test: MDMA: $t = 2.085$, $df = 31$, $p = 0.0454$; *D*-FEN: $t = 2.293$, $df = 21$, $p = 0.0323$).

Previous pull-down experiments documented a direct interaction between the C terminus of DAT and α CaMKII, but failed to detect any binding of α CaMKII to the C terminus of SERT (Fog et al., 2006). However, these experiments relied on a fusion protein that only contained the last 24 aa of SERT and it is the proximal portion of the C terminus where DAT and SERT share homologous sequences. Accordingly, in the current experiments, the GST fusion protein used covered the entire C terminus of SERT. We found that α CaMKII was pulled down by this GST fusion protein *in vitro*, albeit to a significantly lower extent than by a GST fusion protein comprising the C terminus of DAT. There are five additional lines of argument that substantiate our conclusions that SERT is a target for α CaMKII. First, α CaMKII was coimmunoprecipitated with SERT from native brain preparations and unequivocally identified by mass spectrometry. Second, α CaMKII colocalized with SERT in transfected HEK-293 cells and endogenous SERT in primary cultures of rat dorsal raphe serotonergic neurons. Third, α CaMKII and SERT resulted in high FRET efficiency values in HEK-293 cells and this was confined to the availability of a binding competent C terminus only because its tagging with the fluorophore precluded the interaction; furthermore, comparable FRET efficiencies have been obtained from transfected primary hippocampal neurons. Fourth, reverse transport elicited by SERT-preferring amphetamines was modulated by α CaMKII in synaptosomal and slice preparations and this regulation was recapitulated in cotransfected HEK-293 cells. Fifth, and most importantly, our experiments are consistent with the notion that the interaction between α CaMKII and SERT is relevant to the behavioral action of MDMA, as well as to the physiological effect of D-FEN. In mice deficient in α CaMKII, the locomotor response elicited by the amphetamine derivative MDMA, but not the monoamine transporter blocker cocaine, was blunted and the D-FEN-induced prolactin release was decreased. Interestingly, a recent study also reported that α CaMKII directly phosphorylated SERT *in vitro* (Sørensen et al., 2014), which further substantiates the assertion that α CaMKII interacts with SERT.

MDMA has a higher affinity for SERT than for DAT (Rothman et al., 2001; Green et al., 2003). Accordingly, MDMA is more efficacious in releasing 5-HT than in triggering dopamine efflux (Gudelsky and Nash, 1996; Verrico et al., 2007; Baumann et al., 2008). In addition, MDMA-induced 5-HT release is required for its locomotor-stimulating effects in rodents (Callaway et al., 1990; Bengel et al., 1998; Baumann et al., 2008). For example, inhibition of SERT by 5-HT reuptake inhibitors (SSRIs) prevented MDMA-induced hyperlocomotion (Callaway et al., 1990) and mice lacking SERT did not respond to MDMA with an increase in locomotion (Bengel et al., 1998). Therefore, MDMA-induced locomotion is a sensitive and specific paradigm to monitor the impact of reverse transport through SERT *in vivo*. The locomotor response to MDMA was blunted in mice lacking α CaMKII to an extent that was consistent with the reduction in MDMA-induced 5-HT efflux, which was observed *ex vivo* in slices and synaptosomes. The rewarding properties of amphetamines do not rely exclusively on their action on DAT and on the ensuing increase in dopamine release; efflux of 5-HT through SERT and serotonergic transmission also contribute to gratification. For example, mice lacking DAT still develop conditioned place preference for amphetamine, which is abolished upon inhibition of 5HT_{1A} receptors (Budygin et al., 2004). Similarly, mice lacking SERT do not self-administer MDMA (Trigo et al., 2007). Finally, repeated administration of amphetamines induces

long-term sensitization, which is thought to rely on enhanced signaling via 5-HT_{2A} (and α_{1b} -adrenergic) receptors, and this contributes to the addictive properties of amphetamines (Tassin, 2008). Most importantly, 5-HT and glutamate corelease from dorsal neurons has recently been demonstrated to signal reward directly in animal models (Liu et al., 2014) and thus further substantiates the emerging evidence for a role of the serotonergic system in substance abuse, reward, and addiction (Müller et al., 2007; Kirby et al., 2011).

Because we cannot fully exclude dopaminergic components in the results obtained in our MDMA *in vivo* experiments, we included another physiological assay in which we looked at D-FEN-induced prolactin release in the plasma of WT and α CaMKII KO mice. 5-HT is an indirect but potent modulator of prolactin release and probably acts via activation of 5HT_{2A/C} receptors on neurons in the paraventricular nucleus of the hypothalamus (Quattrone et al., 1983; Emiliano and Fudge, 2004). Increased prolactin levels are well known side effects of SSRIs, tricyclic antidepressants, and monoamine oxidase inhibitors (Torre and Falorni, 2007). Therefore, D-FEN-induced 5-HT-mediated prolactin release is an indirect outcome parameter with which to measure reverse transport efficacy of SERT (Jacobsen et al., 2012). This and the fact that D-FEN is highly specific for SERT (Baumann et al., 2014) render it an ideal tool with which to approach SERT reverse transport *in vivo* in an alternative manner. Consistent with our hypothesis, we found that mice lacking α CaMKII displayed less D-FEN-induced prolactin serum levels compared with controls, further strengthening the idea that α CaMKII is a critical modulator of amphetamine action at SERT in a physiological context as well.

A previous report suggested a role for CaMKII in SERT-mediated uptake of 5-HT: in that study, inhibition of CaMKII decreased 5-HT uptake in the human chorioncarcinoma cell line JAR (Jayanthi et al., 1994). We also investigated whether 5-HT uptake was affected by CaMKII activity in our model systems. For that purpose, we determined uptake of [³H]5-HT in HEK-293 cells engineered to coexpress α CaMKII and YFP-hSERT and in hSERT-transfected CAD cells, which endogenously expressed CaMKII, but we did not observe any effect on uptake when α CaMKII was inhibited. We also measured [³H]5-HT uptake in synaptosomes prepared from α CaMKII-deficient mice and, again, [³H]5-HT uptake was not different from WT controls. Our finding that α CaMKII does not affect SERT uptake is consistent with a report by Cinquanta et al. (1997), who also reported no appreciable effect on 5-HT uptake in rat synaptosomes upon inhibition of CaMKII. The discrepancy between our uptake results and the findings by Jayanthi et al. (1994) is difficult to explain. It may be due to different levels of CaMKII expression or to differences in subunit composition. For example, we failed to detect α CaMKII in JAR cells (data not shown). In addition, small-molecule inhibitors of CaMKII elicit off-target site effects at higher concentrations (Ledoux et al., 1999; Gao et al., 2006). Our experimental approach allowed us to verify that these did not confound our analysis. At the concentrations used, CaMKII inhibitors did not blunt inward transport of substrate through SERT regardless of whether α CaMKII was present or absent. More importantly, these observations and related findings with DAT (Steinkellner et al., 2012, 2014) stress the apparent asymmetry of substrate uptake by and efflux through monoamine transporters (Sitte and Freissmuth, 2015). A comparable asymmetry in uptake and efflux has also been found for the interaction of SERT and DAT with phosphoinositides: depletion of phosphatidylinositol-4,5-bisphosphate impaired amphetamine-induced

efflux through SERT and DAT, but had no effect on substrate uptake (Buchmayer et al., 2013; Hamilton et al., 2014). These studies imply that substrate uptake and efflux are, at least in part, subject to different regulatory input. This can be rationalized by considering the fact that the affinity of monoamine transporters for their substrate is low in the inward-facing conformation. In fact, the K_M for reverse outward transport of 5-HT by SERT is some 500-fold lower than that for inward transport (Sitte et al., 2001). The access pathway of substrate in the outward and inward conformation is defined by the pseudosymmetry of the transporter, which creates an outer and inner vestibule during the transport cycle. Conceptually, these can also be targeted independently, for example, by manipulations that address the interaction interface of α CaMKII and the transporters or affect the membrane microenvironment in the inner leaflet. We propose that strategies relying on these or related approaches be explored because they may prove to be suitable in the treatment of amphetamine addiction without interfering with monoamine uptake.

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