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Amphetamine activates calcium channels through dopamine transporter-mediated depolarization

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ABSTRACT

Amphetamine (AMPH) and its more potent enantiomer S(+)AMPH are psychostimulants used therapeutically to treat attention deficit hyperactivity disorder and have significant abuse liability. AMPH is a dopamine transporter (DAT) substrate that inhibits dopamine (DA) uptake and is implicated in DA release. Furthermore, AMPH activates ionic currents through DAT that modify cell excitability presumably by modulating voltage-gated channel activity. Indeed, several studies suggest that monoamine transporter-induced depolarization opens voltage-gated Ca^{2+} channels (Ca_V), which would constitute an additional AMPH mechanism of action. In this study we co-express human DAT (hDAT) with Ca^{2+} channels that have decreasing sensitivity to membrane depolarization ($Ca_V1.3$, $Ca_V1.2$ or $Ca_V2.2$). Although S(+)AMPH is more potent than DA in transport-competition assays and inward-current generation, at saturating concentrations both substrates indirectly activate voltage-gated L-type Ca^{2+} channels ($Ca_V1.3$ and $Ca_V1.2$) but not the N-type Ca^{2+} channel ($Ca_V2.2$). Furthermore, the potency to achieve hDAT-Ca_V electrical coupling is dominated by the substrate affinity on hDAT, with negligible influence of L-type channel voltage sensitivity. In contrast, the maximal *coupling-strength* (defined as Ca^{2+} signal change per unit hDAT current) is influenced by Ca_V voltage sensitivity, which is greater in $Ca_V1.3$- than in $Ca_V1.2$-expressing cells. Moreover, relative to DA, S(+)AMPH showed greater *coupling-strength* at concentrations that induced relatively small hDAT-mediated currents. Therefore S(+)AMPH is not only more potent than DA at inducing hDAT-mediated L-type Ca^{2+} channel currents but is a better depolarizing agent since it produces tighter electrical coupling between hDAT-mediated depolarization and L-type Ca^{2+} channel activation.

1. Introduction

The dopamine transporter (DAT) is a Na^+/Cl^--dependent symporter expressed in dopaminergic neurons; its principal function is to limit dopamine receptor signaling by restricting the extracellular concentration of dopamine (DA) [1,2]. Amphetamine (AMPH) is a DAT substrate and its more potent enantiomer, S(+)AMPH, is used therapeutically to treat attention deficit hyperactivity disorder and narcolepsy [2,3]. AMPH competes with and diminishes DA uptake. In addition, intracellular AMPH disrupts DA's internal stores and induces the reverse transport of DA through DAT, increasing extracellular DA concentration [4,5]. Accordingly, the activation of dopaminergic pathways in the brain accounts for both the therapeutic properties and addictive liability of AMPH and its active derivatives [2,6,7].

An additional level of complexity for AMPH's action in cells is the generation of DAT-mediated, AMPH-induced inward currents [8–11]. Although substrate-induced currents through monoamine transporters are widely accepted [12–15] and they have been implicated in neurotransmitter depletion in the brain [16], the physiological significance of such currents are still under debate [17,18]. Recently, we showed that depolarization induced by serotonin (5HT) or S(+)3,4-methylenedioxymethamphetamine (MDMA, ecstasy) in skeletal muscle cells engineered to express the human serotonin transporter (hSERT) activates the L-type Ca^{2+} channel $Ca_V1.1$ [19]. Similarly, hSERT-mediated depolarization activates the L-type Ca^{2+} channel $Ca_V1.3$ in HEK cells, whereas hSERT activation is unable to open the N-type Ca^{2+} channel $Ca_V2.2$ under identical experimental conditions [19]. The L-type Ca^{2+} channels are important modulators of signal transduction and excitability in excitable cells. In particular, $Ca_V1.3$ and $Ca_V1.2$ have been extensively studied upstream of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and cAMP response element-binding

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protein (CREB) signaling pathways in neurons [20–22]. Furthermore, the lower-threshold L-type $Ca_V1.3$ channel is implicated in pace-making in dopaminergic neurons, and in neuroendocrine cells, such as adrenal chromaffin cells [23,24]. Since L-type channels $Ca_V1.2$ and $Ca_V1.3$ are expressed with monoamine transporters in several excitable cells [23–29], determining a functional interaction between these two classes of proteins could constitute an additional molecular mechanism of AMPH action.

In the present study we co-expressed the human DAT (hDAT) with $Ca_V1.2$, $Ca_V1.3$ or $Ca_V2.2$ in Flp-In™ T-REx™ 293 cells, and measured the effect of S(+)AMPH- or DA-induced DAT currents on Ca_V activation. These experiments were designed to study the interplay between two variables: (1) the affinity of S(+)AMPH and DA on hDAT, and (2) the voltage sensitivity of the Ca^{2+} channels studied, in achieving effective hDAT-Ca_V coupling. The results show that, regardless of the compound affinity on hDAT, DA and S(+)AMPH can couple indirectly to both L-type channels ($Ca_V1.2$ and $Ca_V1.3$) but not to the N-type channel ($Ca_V2.2$) under identical conditions. In addition, whereas the potency to achieve hDAT-Ca_V electrical coupling is dominated by substrate-hDAT affinity, the *coupling-strength*, defined as the Ca^{2+} signal change per unit hDAT current, is influenced by the sensitivity of Ca^{2+} channels to voltage. Moreover, S(+)AMPH showed larger *coupling-strength* compared to DA at concentrations that induced relatively small hDAT-mediated currents. These results suggest that S(+)AMPH- and DA-induced currents through hDAT are qualitatively different, because the S(+)AMPH-induced current is pharmacologically and electrically stronger at activating L-type channels.

2. Materials and methods

2.1. Generation of Flp-In™ T-REx™ cells expressing the human dopamine transporter (Flp-hDAT cells) and Ca_V channel transfection

The generation of the hDAT stable inducible cell line (Flp-hDAT) was done using the Flp-In™ T-REx™ 293 system (Life Technologies). The hDAT cDNA (accession number: NM_001044) was subcloned into the pcDNA5/FRT/TO plasmid and the targeted single site recombination and cell selection were performed as described previously [19]. The Ca^{2+} channels used in this study were $Ca_V2.2$ ($\alpha1_B$, Addgene #26570), $Ca_V1.3$ ($\alpha1_D$, Addgene #26576), $Ca_V1.2$ ($\alpha1_C$ accession number: NM_001136522), β_3 (Addgene #26574) and $\alpha2\delta1$ (Addgene #26575). All these plasmids were kindly provided by Dr. Diane Lipscombe (Department of Neuroscience, Brown University, Providence, Rhode Island, USA) except $Ca_V1.2$, which was kindly provided by Dr. Manfred Grabner (Department of Medical Genetics, Molecular, and Clinical Pharmacology, Innsbruck Medical University, Innsbruck, Austria). The $Ca_V1.2$ cDNA was subcloned into the pcDNA6 expression plasmid thus all $\alpha1$ subunits are expressed under the same background vector. EGFP expression plasmid was used as a transfection marker. The cells were co-transfected with the DNA ratio $\alpha1{:}\beta3{:}\alpha2\delta1{:}$EGFP = 1:1:1:0.2 using Fugene 6 (Promega) as the transfection reagent.

2.2. Immunofluorescence

Sample fixation and labeling was performed as described earlier [30]. The primary antibody used was a rat monoclonal-anti DAT (Santa Cruz Biotechnology, Cat# sc-32258) and the secondary antibody used was Alexa Fluor 555 goat anti-rat IgG (Invitrogen, Cat# A21434). The nuclei were stained with DAPI. The specimens were visualized in a Zeiss 710 confocal microscope.

2.3. [^{3}H]DA uptake

Flp-DAT cells were counted and 1×10^6 cells were exposed to different concentrations of DA where 1% of the total concentration consisted of [^{3}H]DA. The uptake reaction was performed for 10 min at 37 °C in an external solution containing (in mM): 130 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH adjusted to 7.4. Non-specific uptake was determined adding 10 μM methylenedioxypyrovalerone (MDPV, a potent hDAT blocker) [31,32]. After the incubation period, cells were centrifuged, washed once with PBS, centrifuged again and the cell pellets were resuspended in Ecoscint H (National Diagnostics, Atlanta, GA, USA); radioactivity was measured in a liquid scintillation counter.

Dose response-experiments were fit to the following expression:

$$Y(x) = \frac{Y_{max}}{1 + 10\exp[\{\log EC_{50} - \log x\} * n]} \quad (1)$$

where x is the concentration of the tested compound, $Y(x)$ is the response measured, Y_{max} is the maximal response, EC_{50} is the concentration that yields half-maximal response, and n is the Hill slope parameter. Competition assays were carried out adding a variable concentration of cold DA or cold S(+)AMPH to a constant 10 μM DA solution containing 1% [^{3}H]DA. The inhibition constant (K_i) was estimated using the Cheng–Prusoff equation.

2.4. Electrophysiology

2.4.1. Determination of hDAT substrate-induced currents

Patch pipettes made from borosilicate glass capillary tubing and coated with Sylgard were filled with the following internal solution (in mM): 133 K Gluconate, 5.9 NaCl, 1 $CaCl_2$, 0.7 $MgCl_2$, 10 EGTA, 10 HEPES, pH adjusted to 7.2 with KOH. In this condition the pipettes showed a tip resistance of ~4 MΩ. The external solution used was (in mM): 130 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH adjusted to 7.4. Patch-clamp recordings were performed under constant perfusion at 35 °C (AutoMate Scientific) and currents were acquired using an Axopatch 200 A amplifier, Digidata 1322 A acquisition system and Clampex 8.2 software (Molecular Devices); current traces were acquired at 1 kHz at −60 mV holding potential. Drugs were applied at various concentrations following a 30 μM dopamine pre-pulse. Holding currents for all traces were subtracted and divided by the DA pre-pulse peak current for cell to cell comparison.

2.4.2. Determination of Ca^{2+} currents

The Ca^{2+} currents were determined in HEK293T cells transfected with $Ca_V1.2$, $Ca_V1.3$ or $Ca_V2.2$ plus β3, $\alpha2\delta1$, and EGFP as described previously [19]. The external solution used was (in mM): 155 tetraethylammonium (TEA)-Cl, 5 $CaCl_2$, 10 Hepes, pH 7.4 with TEA-OH. The internal solution composition was (in mM): 130 CsCl, 10 Cs-EGTA, 1 $CaCl_2$, 4 MgATP and 10 HEPES, pH adjusted to 7.3 with CsOH. The effective serial resistance was corrected to 80% using the built-in circuit of the Axopatch 200B amplifier (remaining voltage error < 1.2 mV). The leak current was subtracted using a –P/6 protocol. The microelectrodes were made from 8520 glass capillary (Warner Instruments, #64-0817), fire polished, and Sylgard coated. The electrodes tip resistance was ~2.5 MΩ when filled with the internal solution. The whole-cell patch-clamp parameters of the recordings were: cell capacitance = 22.7 ± 2.3 pF, access resistance = 5.3 ± 0.4 MΩ, and time constant (τ) = 121 ± 16.4 μs (n = 23). The current was set to zero using the "pipette offset" command of the amplifier when the pipette was immersed in the external solution and no additional correction to the liquid-junction potential was performed. The recorded signals were acquired at 10 kHz and filtered at 5 kHz.