The Alkylamine Stimulant 1,3-Dimethylamylamine Exhibits Substrate-Like Regulation of Dopamine Transporter Function and Localization

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Abstract

The alkylamine stimulant 1,3-dimethylamylamine (DMAA) is used non-medically as an appetite suppressant and exercise performance enhancer despite adverse cardiovascular effects that have limited its legal status. There is scant research describing the mechanism of action of DMAA, making it difficult to gauge risks or therapeutic potential. An important molecular target of structurally related phenethylamines, such as amphetamine, for regulating mood, cognition, movement, and the development of substance use disorder is the dopamine transporter, which limits the range and magnitude of dopamine signaling via re-uptake from the extracellular space. The present studies were therefore initiated to characterize the effects of DMAA on dopamine transporter function. Specifically, we tested the hypothesis that DMAA exhibits substrate-like effects on dopamine transporter function and trafficking. In transport assays in human embryonic kidney cells, DMAA inhibited dopamine uptake by the human dopamine transporter in a competitive manner. Docking analysis and molecular dynamics simulations supported these findings, revealing that DMAA binds to the S1 substrate binding site and induces a conformational change from outward-facing open to outward-facing closed states, similar to the known substrates. Further supporting substrate-like effects of DMAA, the drug stimulated dopamine transporter endocytosis in a heterologous expression system via cocaine- and protein kinase A-sensitive mechanisms, mirroring findings with amphetamine. Together, these data indicate that DMAA elicits neurological effects by binding to and regulating function of the dopamine transporter. Furthermore, pharmacologic distinctions from amphetamine reveal structural determinants for regulating transporter conformation and add mechanistic insight for the regulation of dopamine transporter endocytosis.
Significance Statement

The alkylamine stimulant 1,3-dimethylamylamine (DMAA) is used as an appetite suppressant and athletic performance enhancer and is structurally similar to amphetamine, but there is scant research describing its mechanism of action. Characterizing the effects of DMAA on dopamine transporter function supports evaluation of potential risks and therapeutic potential, while also revealing mechanistic details of dynamic transporter-substrate interactions.
Introduction

Derivatives of phenethylamine include stimulants (e.g. amphetamine, methylphenidate), neurotoxins (e.g. MPP), sympathomimetics (e.g. phenylephrine), antidepressants (e.g. bupropion), and psychedelics (e.g. mescaline). From this broad class of compounds, pharmacodynamic evidence of interactions with monoamine neurotransmitter systems has been used to predict or understand physiological and behavioral effects, and to gauge potential therapeutic benefits or risks. An understanding of the regulation of dopamine (DA) signaling is of particular interest, due to the role of DA in controlling aspects of mood, cognition, and movement and its involvement in a variety of neuropsychiatric disorders, such as substance use disorder, Attention Deficit Hyperactivity Disorder, Schizophrenia, and Parkinson’s Disease (Lalonde and Botez-Marquard, n.d.; Gainetdinov and Caron, 2003; Spiga et al., 2008; Wise, 2008). Although the mechanisms differ, most addictive drugs increase DA concentrations in the nucleus accumbens, a projection area of DA neurons in the ventral tegmental area (Deadwyler, 2010; Willuhn et al., 2010).

A primary regulator of dopaminergic neurotransmission is the DA transporter (DAT), which is responsible for re-uptake of DA back into the pre-synaptic neuron (Giros et al., 1991; Jaber et al., 1997; Kristensen et al., 2011). Since DAT functions at the surface of neurons, its expression levels in the plasma membrane are critical for DA-regulated behaviors. Consequently, drugs and endogenous molecules can modulate DAT function through direct interactions and by altering DAT trafficking. For example, the DAT ligand cocaine inhibits DA transport, but has also been observed to increase the plasma membrane expression of the transporter (Little, 2002;
Mash et al., 2004). Additionally, the DAT substrate amphetamine induces DAT reverse transport (Fog et al., 2006; Steinkellner et al., 2014; Sitte and Freissmuth, 2015), but also stimulates endocytic pathways to decrease transporter expression at the cell surface (Zahniser and Sorkin, 2004; Kristensen et al., 2011). The molecular events underlying amphetamine-induced DAT endocytosis have been the subject of numerous studies, and there is on-going debate regarding the trafficking itineraries and molecular mechanisms involved, especially with regard to the role of signaling (Eriksen et al., 2010; Cremona et al., 2011; Rao et al., 2012; Gabriel et al., 2013; Wu et al., 2015; Melikian and Buckley, 2018; Sorkina et al., 2018).

In addition to the phenethylamines, there is strong evidence that structurally similar alkylamines influence catecholamine physiology. 1,3-dimethylamylamine (DMAA, methylhexanamine), which resembles amphetamine closely, was first introduced as a decongestant. It has since been marketed as an exercise stimulant and appetite suppressant, as have its structural analogs 1,4-DMAA and 1,3-dimethylbutylamine, being included in over 200 products (Cohen, 2012; Cohen et al., 2015, 2018; Rychert and Wilkins, 2016; Pawar and Grundel, 2017). A number of adverse cardiovascular events led to safety evaluation and eventually, banning by the US FDA (Gee et al., 2010, 2012; Eliason et al., 2013; Forrester, 2013; Smith et al., 2014; Karnatovskaia et al., 2015; Pawar and Grundel, 2017; Ronis et al., 2018). Although it is difficult to ascertain the current popularity, the substance, remains available for purchase and has also been detected in commercial products as an unlisted ingredient (van Hout and Hearne, 2015; Cohen et al., 2018). For example, analysis of doping violations from 2003 to 2020 in Norway revealed 9% from DMAA, compared to 26% from cannabinoids (Lauritzen, 2022), suggesting that DMAA has been and remains a drug of concern for populations seeking positive affective, cognitive, and energy effects of stimulants, especially
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athletes. Of notable concern, DMAA has been used recreationally in powder form and mixed with other ingredients in “party pills” (Gee et al., 2010, 2012; van Hout and Hearne, 2015). Furthermore, DMAA was found to induce cocaine- and methamphetamine-like discriminative effects and conditioned place preference in rats (Dolan and Gatch, 2015). Recently, DMAA was found to inhibit the function of the DAT and, to a greater extent, the norepinephrine transporter in a heterologous expression system (Rickli et al., 2019).

Given the sociocultural usage patterns, structural similarity to amphetamine, and emerging pharmacodynamic evidence, we sought to better characterize the effects of DMAA on DAT function and expression. Using a combination of computer modeling and cell culture-based assays of transporter function and endocytosis, we tested the hypothesis that the DAT regulation by 1,3-DMAA occurs in an amphetamine-like manner.

Materials and Methods

Materials

\(^3\)H-DA was from Perkin Elmer. 1,3-dimethylamylamine, d-amphetamine, cocaine hydrochloride and other chemicals were from Sigma. Antibodies were purchased from the following source: mouse monoclonal antibodies against the HA epitope HA11 (16B12) were from BioLegend (cat. # 901502); Secondary goat anti-mouse antibodies conjugated with Cy5 or Cy3 were from Jackson Immuno Research (West Grove, PA). Phorbol 12-myristate, 13-acetate (PMA), forskolin, and H89 were from LC laboratories. 3-Isobutyl-1-methylxanthine (IBMX) and KN-93 were from Tocris. Bisindolylmaleimide-I was from EMD Millipore. All chemical inhibitors were prepared as 1,000X to 10,000X stock solutions in DMSO and stored in aliquots at -80°C.
Other supplies and reagents were from ThermoFisher or Midwest Scientific.

**Cells**

HEK-293A cells were purchased from Invitrogen and stable clones were generated by transfection with the specified plasmid using Lipofectamine 2000 according to manufacturer’s protocol (Invitrogen). Cells were cultured in DMEM supplemented with 5% fetal bovine serum according to standard procedures.

**DA uptake assays**

HEK-293A cells with stable expression of human DAT (pcDNA3.1-hDAT, Addgene plasmid #32810) were grown to confluence in poly-d-lysine-coated 24-well dishes and treatments and assays were conducted as described previously (Cheng *et al.*, 2015) in room-temperature (21°C) phosphate-buffered saline (PBS) supplemented with 0.1 mM CaCl₂, 1 mM MgCl₂, and 10 mM glucose (PBS-CMG). Following ten-minute uptake of ³H-DA (Perkin Elmer NET673), cells were washed twice with ice-cold PBS-CMG prior to lysis in water with 1% SDS and 0.1 N NaOH and scintillation counting. Background uptake was established by inclusion of 0.1 mM cocaine. Each replicate experiment used both DMAA and amphetamine so the fold difference between the two could be measured directly within the same experiment. For kinetic assays, DMAA was added 10 minutes prior to and during uptake.

**Structural model of hDAT and ligand binding simulations**

Homology model for hDAT in the outward-facing (OF) *open* (OFo) state was taken from the previous work (Cheng and Bahar, 2015; Cheng *et al.*, 2015). The 3D molecular structure of
DMAA (CID_7753) was downloaded from the PubChem database (Kim et al., 2021). Molecular structures of DA and AMPH were adopted from our previous studies (Cheng and Bahar, 2015; Cheng et al., 2015). The ligand binding sites and binding poses on the OFo hDAT were assessed using docking simulation software AutoDock Vina (Trott and Olson, 2009), following the previous approach (Romanazzi et al., 2021). In brief, AutoDock Vina simulations were carried out using a grid with dimensions set to cover the entire transporter. The exhaustiveness of the simulation was set to 50 and the algorithm returned 20 binding modes of interest for each conformer.

**Molecular Dynamics simulations of DMAA binding to hDAT**

All Molecular Dynamics (MD) simulations were performed using the NAMD2 software (version 2.13) (Phillips et al., 2005), adopting previous simulation protocol (Cheng and Bahar, 2015; Cheng et al., 2015, 2018). MD simulation systems were set up using VMD (Humphrey et al., 1996). The control system without DMAA binding was taken from the previous study (Cheng and Bahar, 2015; Cheng et al., 2015), in which two sodium ions and one chloride resolved in the crystal structure of dDAT (Penmatsa et al., 2013) were included and the transmembrane domain of hDAT OFo was inserted into the center of pre-equilibrated and pre-solvated POPC (1-palmitoyl-2-oleoylphosphatidyl choline) lipid bilayer. The DMAA-bound system was constructed from the control, where the DMAA ligand was docked to the substrate-binding site S1 (see Figure 2A). Fully equilibrated TIP3P waters were added to form a box of $104.6 \times 104.6 \times 150 \, \text{Å}^3$. Na$^+$ and Cl$^-$ ions corresponding to a 0.15 M solution were added to neutralize the system.
CHARMM36 force field with CMAP corrections was used for hDAT, water and lipid molecules (Huang et al., 2017). DMAA was protonated using Open Babel (O’Boyle et al., 2011) at the amine moiety, as assumed for DA and AMPH in the previous studies (Cheng and Bahar, 2015; Cheng et al., 2015, 2018). Force field parameters for DMAA were obtained from the CHARMM General Force Field (CGenFF) for drug-like molecules (Vancouver et al., 2010), using the web server ParamChem. Two independent MD runs of 100 ns were performed following the previous protocol (Cheng and Bahar, 2015; Cheng et al., 2015). In brief, prior to productive runs, each system was energy-minimized for 50,000 step, followed by 0.5 ns constant volume and temperature (T = 310K) (NVT) simulations and a subsequent 4 ns Nosé-Hoover (Nosé, 1984; Hoover, 1985) constant pressure and temperature (1 bar, 310 K) (NPT) simulation, during which the protein was fixed and constraints on the POPC head groups were gradually released. Subsequently, the constraints on the protein backbone were reduced from 10 kcal/mol to zero within 3 ns. Finally, the unconstrained protein was subjected to NPT simulations for 100 ns.

DAT endocytosis “HA antibody feeding” assay and image analysis

HEK-293A cells with stable expression of plasmid encoding DAT with an HA epitope inserted in the second extracellular loop (HA-DAT) (Sorkina et al., 2006) were cultured on glass coverslips. Cells were incubated with 1 mg/ml mouse anti-HA antibodies in room-temperature growth medium for one hour. Antibodies were removed, cells washed three times with growth medium, and then incubated with 37℃ growth medium containing the specified concentration of 1,3-DMAA, amphetamine, or equivalent volume of water (vehicle). For experiments requiring use of signaling inducer or inhibitor (Figure 4), the chemical was added 20 minutes prior to
adding DMAA or amphetamine and remained present throughout. After 30-60 minutes, cells were fixed by addition of paraformaldehyde to a final concentration of 4%. After washing in PBS, Cy3-conjugated goat anti-mouse antibodies (1 μg/ml) were added for one hour to label cell surface HA-DAT. Following ten-minute fixation with 4% paraformaldehyde and five-minute permeabilization with 0.1% Triton-x-100, Cy5-conjugated goat anti-mouse antibodies (0.5 μg/ml) were added to label endocytosed and remaining unlabeled surface HA-DAT. Coverslips were mounted on slides using MOWIOL mounting medium. Z-stacks of x-y confocal images were acquired using a spinning disc confocal imaging system based on a Zeiss Axio Observer Z1 inverted fluorescence microscope (with 63x Plan Apo PH NA 1.4) equipped with a computer-controlled Spherical Aberration Correction unit, Yokogawa CSU-X1, Photometrics Evolve 16-bit EMCCD camera, environmental chamber and piezo stage controller and lasers (405, 445, 488, 515, 515, 561, and 640 nm), all controlled by SLIDEBOOK6 software (Intelligent Imaging Innovations, Inc). Image acquisition settings were identical in all experiments. For quantitation, 3-D images of at least four random fields from each condition were acquired through 561 nm (Cy3) and 640 nm (Cy5) channels. Quantitation of the amount of Cy3 (surface HA-DAT) and Cy5 (surface plus internalized HA-DAT) fluorescence was performed using the statistics module of SLIDEBOOK6. The background-subtracted 3D images were segmented using a minimal intensity of Cy3 or Cy5 fluorescence as a low threshold to obtain segment Masks #1 and #2. Mask #1 was subtracted from Mask #2 to obtain Mask #3 corresponding to voxels containing only intracellular Cy5 fluorescence (internalized HA11 complexes with HA-DAT). The ratios of integrated intensities of Mask#3 to Mask#1 were calculated to determine the apparent extent of DAT endocytosis.
Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA). For comparisons of two groups, unpaired Student’s t-test was used after verification of equal variance using F-test. Welch’s correction was performed when the variance across groups was assumed to be unequal. For multiple comparison analyses, a one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was used. All experiments were performed at least three times. Differences were considered significant when the p-value was <0.05.

Results

DMAA is a competitive inhibitor of the DAT

To test the hypothesis that 1,3-DMAA inhibits DAT function, we measured uptake of $^3$H-DA by HEK-293A cells that express the human DAT. 1,3-DMAA inhibited uptake of $^3$H-DA with a mean IC$_{50}$ of 29.4 µM (+/- 14.8 SD, range 12.5 to 66.2), while d-amphetamine inhibited uptake with an IC$_{50}$ of 0.66 µM (+/- 0.21 SD, range 0.4 to 0.9), similar to reported values (Han and Gu, 2006). Representative results from one of four replicate experiments are shown in Figure 1A. Overall, DMAA exhibited a roughly 60-fold decrease in potency compared to d-amphetamine across the four replicate experiments. To explore the mechanism of transport inhibition further, we examined the effect of 1,3-DMAA on DAT substrate affinity and capacity. In the presence of 100 µM 1,3-DMAA, there was a nearly ten-fold decreased affinity of DA for the transporter, with little change in transport capacity (Figure 1B). These results suggest that DMAA inhibits DA transport by competitive as opposed to allosteric inhibition of DA binding.
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Binding of DMAA triggers the reconfiguration of hDAT from the OFo to OFc*

To investigate the mechanism of 1,3-DMAA binding to human DAT, we performed ligand docking simulations using AutoDock Vina. Preliminary docking analysis indicated that the 1S, 3S enantiomer of DMAA bound to DAT similarly to d-amphetamine, so that was used for subsequent studies. The most favorable binding site of DMAA bound to the hDAT OFo was predicted to be substrate binding site S1, which is approximately halfway across the membrane within the EC vestibule (Figure 2A). Figures 2A-C compare binding poses and binding-coordinating residues of DMAA, DA, and d-amphetamine to the hDAT OFo conformer. The binding poses of those three ligands exhibit high similarities: their amine groups are involved into attractive electrostatic interactions with D79 via hydrogen bonding or salt bridging, and their hydrophobic moieties interact with hydrophobic residues F76, V152, and F326. Differently, the aromatic rings from DA and AMPH were wedged more tightly between the TM3 and TM10 via aromatic interaction with Y156, while DMAA displayed high flexibility for the binding of its aliphatic moiety. The binding affinities of DMAA, d-amphetamine and DA to the S1 site were calculated to be -4.3±0.5, -5.5±0.3 and -5.8±0.3 kcal/mol, respectively, by AutoDock Vina.

To further explore whether the binding of DMAA triggers the reconfiguration of hDAT, we performed MD simulations of the hDAT OFo conformer in the presence of DMAA bound to the S1 site. Interestingly, within 100 ns of MD simulation courses, we consistently observed that binding of DMAA triggered significant conformational changes in the EC vestibule, which led to the OFc* (outward-facing closed) state (see Figure 2D-E). The OFo and OFc* states were distinguished by three major criteria (Cheng and Bahar, 2015, 2019; Cheng et al., 2015): opening/closure of the outer and inner EC gates, inter-helix packing, and hydration pattern of
both the EC and IC vestibules. First, the F320 side chain underwent a rotational isomerization, enabling its association with Y156 to form the inner EC gate within tens of nanoseconds. The salt-bridge of R85-D476 that serves as the outer EC gate spontaneously formed, which was further stabilized after the formation of the inner EC gate (Figure 2E-G). The closure of these two EC-gates (R85-D476 and Y156-F320) triggered by DMAA binding exhibited striking similarity to the sequence of events observed in our previous MD simulations with the substrates DA or d-amphetamine binding to hDAT (Cheng and Bahar, 2015; Cheng et al., 2015), which is predisposed to substrate translocation and release as confirmed in our earlier work on LeuT (Cheng and Bahar, 2013, 2014). Secondly, the binding of DMAA prompted an inward tilting of TM1b (10~15°) and TM6a (2~10°) segments towards the center of the EC vestibule. The inter-helical packing geometry of the EC vestibule reached values typical of OFc* state as we observed in the DA or amphetamine-bound hDAT (Cheng and Bahar, 2015; Cheng et al., 2015). Thirdly, in contrast to the OFo state where there is a continuous water occupancy in the EC vestibule, in the OFc* state, the site S1 is minimally hydrated.

**DMAA stimulates DAT endocytosis**

DA uptake, docking analysis, and MD simulations suggested that DMAA inhibits DAT function and induces conformational changes by binding in a manner similar to amphetamine, albeit with lower affinity. Amphetamine, DA, and other DAT substrates stimulate DAT endocytosis (Saunders et al., 2000; Vaughan and Foster, 2013; Wheeler et al., 2015). To determine whether DMAA also exhibits this property of DAT substrates, we measured DAT endocytosis using confocal microscopy and a two fluorophore “antibody feeding” protocol. HEK-293A cells stably expressing a mutant DAT containing an HA epitope inserted in the second extracellular
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loop (HA-DAT) carry bound HA antibodies from the plasma membrane to endosomes, allowing efficient visualization and quantitation of endocytosis (see (Sorkina et al., 2006) and Methods for details). HA-DAT accumulated in intracellular compartments following 30-minute treatment with 100 µM 1,3-DMAA or d-amphetamine (Figure 3A). DMAA stimulated a dose-dependent increase in DAT endocytosis with a slightly decreased potency compared to amphetamine (Figure 3B-C).

Next, we sought to determine whether DMAA induces DAT endocytosis by employing similar cellular mechanisms as amphetamine. DMAA- and amphetamine-induced endocytosis were inhibited by the presence of cocaine (Figure 4A), suggesting that direct interaction with DAT or transport to an intracellular target is necessary for endocytosis induced by both drugs. The role of signaling on DAT endocytosis has been the subject of extensive study (Zahniser and Sorkin, 2004; Kristensen et al., 2011; Vaughan and Foster, 2013). There is evidence for regulation by PKC (Melikian and Buckley, 1999; Holton et al., 2005; Sorkina et al., 2006), CaMKII (Cervinski et al., 2005; Wei et al., 2007), PI3K (Lin et al., 2003; Garcia et al., 2005), MAPK (Morón et al., 2003), PKA (Pristupa et al., 1998; Page et al., 2004; Xie and Miller, 2007; Gorentla et al., 2009; Wheeler et al., 2015; Batchelor and Schenk, 2018), and RhoA (Wheeler et al., 2015), with CaMKII, PKA, and RhoA being implicated directly in amphetamine-induced endocytosis. Initially, we screened the effects of signaling inhibitors on amphetamine- and DMAA-induced HA-DAT endocytosis (Figure 4B). Inhibitors of PKC (Bisindolylmaleimide-I), CaMKII (KN93), the RhoA effector ROCK (Y27632), and PKA (among other kinases (Davies et al., 2000; Xie and Miller, 2009), H89) were examined. Generally, chemical inhibitors should be assumed to be non-specific and are best used to rule out the involvement of signaling pathways (Davies et al., 2000; Bain et al., 2007). None of these treatments significantly inhibited DAT
endocytosis by DMAA or amphetamine (Figure 4B). To test the hypothesis that PKA negatively regulates DAT endocytosis, which has been observed previously in cultured DA neurons (Wheeler et al., 2015), we examined the effects of enhancing PKA activity on amphetamine- and DMAA-induced endocytosis with the adenylyl cyclase activator forskolin and the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). As shown in Figures 4C and D, this method of increasing cAMP significantly inhibited drug-induced endocytosis. All together, these results suggest that DMAA induces DAT endocytosis in the same manner as amphetamine, that is, through a mechanism dependent on transporter interaction that is sensitive to PKA.

Discussion

Pharmacologic profiles of many natural and synthetic amphetamine-like compounds are relatively uncharacterized despite their recreational use or inclusion in diet and exercise supplements (Pawar and Grundel, 2017). Improved definitions of interactions with the DAT and effects on DA physiology can aid identification of public health risks, such as abuse potential due to increased DA transmission. On the other hand, some drugs may carry untapped therapeutic potential, as phenethylamines are used to treat ADHD as well as substance use disorder (e.g. bupropion for cigarette smoking cessation and prevention of cocaine relapse) (Grabowski et al., 2001; Shearer and Gowing, 2004; Rothman, 2005; Shearer et al., 2009; Lee et al., 2018; Buchholz and Saxon, 2019). Here, we took a key step toward understanding potential risks and benefits of 1,3-DMAA by testing the hypothesis that it influences DAT function and endocytosis in a manner similar to amphetamine. Specifically, we have acquired evidence that
DMAA is a competitive DAT inhibitor with an affinity roughly 20-fold lower than d-amphetamine; DMAA binds the DAT and induces conformational changes in manner similar to amphetamine; and DMAA induces DAT endocytosis by a cocaine- and cAMP-sensitive mechanism.

The mean IC50 value for inhibition of transport by DAT obtained here (29 µM) was lower than that reported in a recent study characterizing interactions of stimulants with monoamine transporters and receptors (106 µM) (Rickli et al., 2019), although the relative decreases in affinity compared to amphetamine were similar, 60-fold compared to 80-fold (Rickli et al., 2019). In rats, 10 mg/kg of DMAA produced discriminative effects similar to 1 mg/kg methamphetamine (Dolan and Gatch, 2015). Reported doses of 1,3-DMAA for performance enhancement or recreation in humans are roughly 10-20 times higher than doses of amphetamine used for ADHD. Dosage of DMAA can be difficult to estimate due to unregulated and illicit sources, as well as mixture with other substances, but seems to range from 25 to 300 mg for oral consumption (Gee et al., 2012; Cohen et al., 2018). Pharmacokinetic analysis indicates that doses in this range result in plasma DMAA concentrations of 0.6 to 20 µM (Gee et al., 2012; Schilling et al., 2013). Studies in rats (Sood et al., 2009; Folgering et al., 2019) and non-human primates (Folgering et al., 2019) comparing pharmacokinetic distribution of d-amphetamine in plasma and brain extracellular fluid indicated similar concentrations in both compartments. Since DMAA has similar chemical properties as amphetamine for crossing the blood-brain barrier (e.g., LogP), these results suggest that consumption of higher doses of DMAA can result in acute brain exposure to effective concentrations examined in the present studies. If there is chiral bias for DMAA binding to the DAT, as there is for amphetamine, then the 1S, 3S enantiomer of DMAA that was used in the present modeling studies may require
lower dose than the racemic mixture used in the functional assays to achieve DAT-related
effects.

Physiologic and behavioral effects of DMAA from interactions with other targets should
considered. Importantly, DMAA inhibits the norepinephrine transporter (NET), another known
amphetamine target, with an IC50 of 0.41 µM (Rickli et al., 2019). Amphetamine can stimulate
NET endocytosis (Underhill et al., 2020), so it is likely that DMAA mediates biological effects
through interactions with the NET as well. As DMAA has a greater preference for NET:DAT
than d-amphetamine, it may carry greater risks from pressor actions but lower risks from positive
reinforcement, limiting abuse potential.

In addition to expanding knowledge of DMAA pharmacodynamics, the present studies
reveal novel drug-dependent structural changes in DAT conformation. Our molecular modeling
and simulations (Figure 2) suggest that DMAA can bind to the substrate binding site of the
hDAT, with less binding affinity compared to DA or amphetamine, in agreement with uptake
data (Figure 1). Hydrogen bonding or salt bridging formed between DMAA and D79 facilitates
its binding to the S1 site, which is further strengthened via favorable hydrophobic interactions
with F76, V152, and F326. Multiple MD simulations consistently showed that binding of
DMAA to the S1 site was able to trigger hDAT conformational changes from OFo to OFc*,
which may be predisposed to substrate translocation and release as observed for DA binding to
hDAT (Cheng and Bahar, 2015, 2019). In the OFo state, hydrogen bonding is formed between
D79 and Y156 (Figure 2D). The binding of DMAA inclined to break this hydrogen bonding via
forming salt bridge or hydrogen bond with D79, thus releasing Y156 to form aromatic
interaction with F320 (Figure 2D). We suggest that the amine group in DMAA is important to
trigger the reconfiguration of hDAT, and furthermore, that a complete phenyl ring is not
necessary for substrate translocation. These findings suggest that other aliphatic amine stimulants, such as 1,4-DMAA, 1,3-dimethylbutylamine, and tuaminoheptane also exert biological effects through DAT interactions. Supporting this theory, tuaminoheptane interacted with the bacterial DAT orthologue LeuT \textit{in silico} and inhibited the NET \textit{in vitro} (Schlessinger \textit{et al.}, 2011).

Investigating DMAA-induced DAT endocytosis may reveal mechanistic details of DAT endocytic trafficking and regulation by amphetamine. \textbf{Figures 3 and 4} indicate that DMAA-induced endocytosis is inhibited by cocaine, as has been reported previously for amphetamine. This suggests that interaction with the transporter or access to the cytosol, assuming DMAA is a substrate, is necessary for stimulating endocytosis. Conclusive evidence for DMAA being a DAT substrate would include cocaine-sensitive intracellular accumulation of radiolabeled DMAA. If DMAA were an inhibitor as opposed to a substrate, it would be the first described to induce DAT endocytosis and would suggest that transporter interactions alone are sufficient to stimulate endocytosis. Alternatively, a proposed intracellular target for amphetamine is the Trace Amine Associated Receptor 1 (TAAR1), a GPCR that is expressed on intracellular membranes in DA neurons (Miller, 2012). Phenethylamine stimulants have been proposed to activate TAAR1, leading to increased cAMP generation and RhoA activation, with subsequent enhancement of DAT reverse transport and endocytosis (Xie and Miller, 2007, 2008, 2009; Underhill \textit{et al.}, 2021). Methamphetamine-induced DAT endocytosis was found to be dependent on TAAR1 expression and PKA activity, as suggested by use of the kinase inhibitor H89 (Xie and Miller, 2009). However, evidence indicating amphetamine-induced endocytosis is independent of TAAR1 includes 1) HEK-293 cells do not express TAAR1 (Reese \textit{et al.}, 2007; Xie and Miller, 2007) but do exhibit amphetamine-induced DAT endocytosis (present studies...
and (Saunders et al., 2014; Cheng et al., 2015; Wheeler et al., 2015)), 2) cAMP and PKA activation, which are stimulated by TAAR1, antagonized amphetamine-induced DAT endocytosis in heterologous cells and DA neurons (present studies and (Wheeler et al., 2015)), and 3) in cell lines and rodent striatal synaptosomes, PKA activation increased DAT Vmax, consistent with increased plasma membrane expression (Pristupa et al., 1998; Page et al., 2004; Batchelor and Schenk, 2018). Additionally, DMAA induced DAT endocytosis (Figures 3 and 4) despite exhibiting no activity at human TAAR1 in receptor binding studies (Rickli et al., 2019). Therefore, while some evidence does support a role of TAAR1 in modulating amphetamine-induced DAT endocytosis, the present studies suggest that DMAA and amphetamine promote DAT endocytosis through a TAAR1-independent mechanism. If DMAA and amphetamine regulate distinct cytoplasmic targets, their ability to induce similar DAT structural transitions supports a role for DAT conformation in regulating trafficking events, which has been suggested previously using conformationally biased DAT mutations and ligands (Sorkina et al., 2009; Ma et al., 2017).

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Author Contributions

Participated in research design: Small, Cheng, Sorkin, Block

Conducted experiments: Small, Cheng, Belay, Bulloch, Zimmerman, Block

Contributed new reagents or analytic tools: Sorkin
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Performed data analysis: Small, Cheng, Belay, Bulloch, Zimmerman, Sorkin, Block

Wrote or contributed to the writing of the manuscript: Cheng, Sorkin, Block

References


Cohen PA, Travis JC, Keizers PHJ, Deuster P, and Venhuis BJ (2018) Four experimental stimulants found in sports and weight loss supplements: 2-amino-6-methylheptane (octodrine), 1,4-dimethylamylamine (1,4-DMAA), 1,3-dimethylamylamine (1,3-DMAA) and 1,3-dimethylbutylamine (1,3-DMBA). *Clin Toxicol* **56**:421–426, Informa UK Limited, trading as Taylor & Francis Group.


Forrester MB (2013) Exposures to 1,3-dimethylamylamine-containing products reported to Texas poison centers. *Hum Exp Toxicol* **32**:18–23.


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**Footnotes**

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Figure Legends

Figure 1: DMAA inhibits DA uptake in a competitive manner

(A) Uptake of 20 nM \(^3\)H-DA by HEK-293A cells expressing human DAT was measured in the presence of the indicated concentration of d-amphetamine (AMPH) or 1,3-DMAA. Means of three replicates +/- S.E.M. were plotted. Drug concentrations for 50% inhibition of DA uptake (IC\(_{50}\)) were calculated. (B) Uptake of the indicated concentrations of \(^3\)H-DA by HEK cells expressing human DAT was measured in the presence of no drug (vehicle) or 100 \(\mu\)M DMAA. Mean +/- S.E.M. of three replicates were plotted and Michaelis-Menten non-linear regression analysis was performed, providing transporter dopamine affinity (Km) and capacity (Vmax) for each condition.
Figure 2: DMAA binding to the substrate-binding (S1) site of outward facing open (Ofo) DAT leads to the closure of EC gates

(A) Most energetically favorable binding pose of DMAA to the Ofo hDAT (top two panels), in comparison with the predicted binding poses of d-amphetamine (AMPH) (B) and (C) DA to the S1 site. DMAA, AMPH and DA are shown in stick format with meshes. Cyan, red, blue and white represent the carbon, oxygen, nitrogen and hydrogen atoms, respectively. Interacting residues making atom-atom contacts closer than 4 Å with those ligands are shown in the right panel. All three small molecules form hydrogen bonding with D79. MD simulations revealed a reconfiguration of hDAT from the (D) Ofo to © outward-facing closed (Ofc*) state after DMAA binding. Time evolution of the distances between R85 and D476 (F) and between Y156 and F320 (G) in two independent runs. Distances were calculated based on the center of mass.

Figure 3: DMAA stimulates DAT endocytosis

HEK-293A cells expressing HA-DAT were treated for 30 minutes with water (Vehicle), or the indicated concentrations of amphetamine (Amph) or DMAA and were subjected to the “HA antibody feeding” assay as described in Methods prior to imaging by confocal fluorescence microscopy. Surface transporters appear red and green (yellow in merged images) while internalized transporters appear in green only. Single channel and merged image sets are shown in (A) for 100 μM conditions; merged images are shown in (B) for 1 and 10 μM conditions. Representative images from three replicate coverslips are shown. (C) The fold change compared to vehicle (represented by dashed line) in the ratio of internalized:surface transporter was determined by quantitative analysis as described in Methods. Values are means of six fields of view and error bars are S.E.M. Significant difference from vehicle (denoted by *) indicates
Regulation of DAT by DMAA

*p<0.05* as determined by one-way ANOVA followed by the Tukey post-test for multiple comparisons. Results were similar in three replicate experiments.

**Figure 4: Similar mechanisms regulate DMAA- and amphetamine-induced DAT endocytosis**

HEK cells expressing HA-DAT were subjected to the “HA antibody feeding” assay and quantitative image analysis as described in Methods. (A) Cells were treated for 15 minutes with 1 mM cocaine as indicated prior to and during exposure to 100 µM DMAA. (B) Image sets from five separate “HA antibody feeding” experiments were quantitated and collected in a single chart. Each experiment consisted of duplicate coverslips exposed to 10 µM of indicated inhibitor or vehicle (“no inhibitor,” water or a final concentration of 0.1% DMSO) for 15 minutes prior to and during application of 10 µM amphetamine or 100 µM DMAA. The ratio of internalized:surface HA-DAT in the amphetamine or DMAA condition was set to 1.0 within each experiment (amphetamine and DMAA did not necessarily yield identical levels of endocytosis in all experiments). Values are means of six determinations and error bars are S.E.M. Statistical analysis was performed within individual experiments (as opposed to the normalized and compiled data) on all treatment conditions, including the untreated cells. Significant difference from the “no inhibitor” condition (denoted by *) indicates *p<0.05* as determined by one-way ANOVA followed by the Tukey post-test for multiple comparisons. (C) Cells were treated for 15 minutes with 10 µM forskolin and 50 µM IBMX as indicated prior to and during exposure to 10 µM amphetamine or 100 µM DMAA. (D) Quantitative analysis of images from experiments in C, which also included treatment with 10 µM of the PKA activator dibutyryl-cyclic-AMP (db-cAMP). Values are means of six determinations and error bars are
S.E.M. Significant difference from vehicle (denoted by *) indicates p<0.05 as determined by one-way ANOVA followed by the Tukey post-test for multiple comparisons. Results were similar in at least three replicate experiments.
Figure 1

A

B

<table>
<thead>
<tr>
<th></th>
<th>AMPH</th>
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<tr>
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</table>
Figure 3

A

Surface

Vehicle 100 μM Amph 100 μM DMAA

Surface & endocytosed

13000 13000 13000

1000 1000 1000

merge

B

Amph DMAA

1 μM

10 μM

C

Endocytosed/surface (fold change vehicle)

1 μM 10 μM 100 μM

AMPH DMAA