3,4-Methylenedioxypyrovalerone: Neuropharmacological Impact of a Designer Stimulant of Abuse on Monoamine Transporters

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MDPV	3,4-Methylenedioxypyrovalerone
METH	Methamphetamine
SCH23390	(*R*(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride)

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Abstract

Methylenedioxypyrovalerone (MDPV) is an abused synthetic cathinone, commonly referred to as a “bath salt.” As the dopamine (DA) transporter (DAT) and vesicular monoamine transporter-2 (VMAT-2) are key regulators of both the abuse and neurotoxic potential of structurally- and behaviorally-related agents, the impact of MDPV on these transporters was investigated. Results revealed that a single *in vivo* MDPV administration rapidly (within 1 h) and reversibly increased both rat striatal DAT and VMAT-2 activity, as assessed via [^{3}H]DA uptake in synaptosomes and synaptic vesicles, respectively, prepared from treated rats. There was no evidence of an MDPV-induced increase in plasmalemmal membrane DAT surface expression. Plasma concentrations of MDPV increased dose-dependently as assessed 1 h after 2.5 and 5.0 mg/kg administration (s.c.) and returned to levels less than 10 ng/ml by 18 h after 2.5 mg/kg (s.c.). Neither pretreatment with a D1 receptor (SCH23390), a D2 receptor (eticlopride), nor a nicotinic receptor (mecamylamine) antagonist attenuated the MDPV-induced increase in DAT activity. In contrast, eticlopride pretreatment attenuated both the MDPV-induced increase in VMAT-2-mediated DA uptake and an associated increase in cytoplasmic-associated vesicle VMAT-2 immunoreactivity. SCH23390 did not attenuate the MDPV-induced increase in VMAT-2 activity. Repeated MDPV injections did not cause persistent DAergic deficits, as assessed 7 – 8 d later. The impact of MDPV on striatal and hippocampal serotonergic assessments was minimal. Taken together, these data contribute to a growing pharmacological rubric for evaluating the ever-growing list of designer cathinone-related stimulants. The profile of MDPV compared with related psychostimulants is discussed.

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Significance Statement

Pharmacological characterization of the synthetic cathinone, 3,4-methylenedioxypyrovalerone (MDPV), (commonly referred to as a “bath salt”) is critical for understanding the abuse liability and neurotoxic potential of this and related agents. Accordingly, the impact of MDPV on monoaminergic neurons is described and compared with that of related psychostimulants.

Introduction

3,4-Methylenedioxypyrovalerone (MDPV) is part of an expanding class of illicit psychostimulants, commonly referred to as “bath salts.” Most of these “bath salts” are structural derivatives of the natural plant-derived product, cathinone (Baumann et al., 2013). “Bath salts” can cause agitation, combative violent behavior, tachycardia, hallucinations, and death (Spiller et al., 2011). Approximately 1% of high school age students use “bath salts,” including MDPV, annually (Johnston et al., 2019).

MDPV has been described as a potent reuptake inhibitor at the dopamine (DA) transporter (DAT) (for review see Baumann et al., 2017). *In vitro*, MDPV potently decreases DAT activity in rat synaptosomes with weaker activity at the serotonin transporter (SERT) (Baumann et al., 2013). In mouse striatal slices, DAT-mediated clearance is inhibited by MDPV as measured by fast-scan cyclic voltammetry (Baumann et al., 2013). Additionally, and consistent with other DAT reuptake inhibitors, MDPV is not a substrate for the transporter (Cameron et al., 2013) and does not evoke transporter-mediated release (Eshleman et al., 2013; Simmler et al., 2013).

Preclinical self-administration studies suggest that MDPV is reinforcing, as evidenced by escalating consumption when consumed orally (Gannon et al., 2017b) or administered intravenously (Aarde et al., 2013; Watterson et al., 2014; Schindler et al., 2016; Gannon et al., 2017a; Gannon et al., 2018). MDPV self-administration, like non-contingent MDPV administration, decreases reward threshold, indicative of reward potentiation; this effect likely involves cholinergic neurotransmission as it is blocked by a nicotinic acetylcholine receptor (nAChR) antagonist (Geste et al., 2018). Of note, MDPV self-administration induces locomotor hyperactivity while increasing extracellular DA levels in the nucleus accumbens, indicating that DA likely contributes prominently to the behavioral and reinforcing effects of MDPV (Schindler et al., 2016).

Two transporters primarily regulate the extracellular and intracellular distribution of DA: the plasmalemmal membrane DAT and the vesicular monoamine transporter-2 (VMAT-2). Many psychostimulants exert neurochemical and behavioral effects by altering DAT and VMAT-2 function. Further, dysregulation of these transporters contributes to the amphetamine-related long-term deficits in central dopaminergic neuronal function. Of note, others and we have reported that the abuse of amphetamines is linked to development of basal ganglia and cerebellar disorders, including Parkinson's disease (Garwood et al., 2006; Callaghan et al., 2010; Christine et al., 2010; Callaghan et al., 2012; Curtin et al., 2015). Consequently, the primary purpose of the study was to investigate the impact of *in vivo* MDPV administration on DAT and VMAT-2 function and subcellular distribution.

Results elucidate the pharmacological profile of MDPV. First, despite directly decreasing DAT function *in vitro*, MDPV treatment rapidly and reversibly *increases* striatal DAT function, as assessed in striatal synaptosomes prepared from treated rats. Second, and although having little effect on VMAT-2 *in vitro*, *in vivo* MDPV administration rapidly and reversibly increases striatal VMAT-2 function, as assessed in non-membrane-associated (presumably cytoplasmic) vesicles prepared from treated rats. This effect involves an increase in cytoplasmic VMAT-2 immunoreactivity and is D2-receptor mediated. Third, repeated MDPV injections (administered in a "binge-like" paradigm) cause neither persistent striatal nor hippocampal serotonergic or DAergic deficits. Taken together, these data contribute to an expanding literature assessing the pharmacologic and toxicologic properties of the ever-growing list of "bath salts," and may contribute to more efficient medical and legal management of these agents.

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Materials and Methods

Reagents, Drugs, and Animals. All chemicals, unless otherwise specified, were purchased from Sigma Aldrich (St. Louis, MO). Male and female Sprague-Dawley rats (Charles River, Raleigh, NC; average weights between 350 – 450 g for males and 277 g for females) were housed in a temperature- (22° C) and light-controlled (14/10 light/dark cycle) environment with food and water provided *ad libitium.* All experiments were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Utah Institutional Animal Care and Use Committee. Ambient room temperature for animal handling and treatment was generally maintained at 23 – 24° C. Core body temperatures were recorded using a rectal probe (RET-2; Physitemp, Clifton NJ), and rats were sacrificed via decapitation. (±)-MDPV hydrochloride, (±)-methamphetamine hydrochloride (METH) and cocaine hydrochloride were synthesized by Research Triangle Institute (Research Triangle Park, NC) and provided by National Institute on Drug Abuse. MDPV and METH doses were calculated as respective free base; SCH23390 (*R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride), eticlopride (*S*-(−)-3-chloro-5-ethyl-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxybenzamide hydrochloride), and mecamylamine (N,2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride) were calculated as salt. Sterile saline (0.9% NaCl, pH 7.4) was used as the vehicle for all *in vivo* experiments.

Synaptosome Isolation. Two striata or two hippocampi were placed into 2 ml ice-cold sucrose buffer (0.32 M sucrose, 3.75 mM NaH_2PO_4, 12.7 mM Na_2HPO_4) and homogenized (20 strokes) using a Dounce homogenizer. Samples were then centrifuged (800 x g, 12 min, 4 °C). The resulting supernatant was centrifuged (22,000 x g, 15 min, 4 °C). For synaptosomal [^{3}H]DA uptake or [^{3}H]5-hydroxytryptamine ([^{3}H]5HT) assays, the resulting whole synaptosomal pellets were resuspended in assay buffer (126 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.4 mM $MgSO_4$, 11 mM glucose, 1 mM ascorbic acid, 3.75 mM NaH_2PO_4, 12.7 mM Na_2HPO_4, pH 7.4). To minimize

the possibility of residual drug introduced by the MDPV injection impacting results, synaptosomal pellets were washed by repeated centrifugation for all plasmalemmal [^{3}H]monoamine uptake assays occurring within 18 h of the MDPV treatment. Specifically, synaptosomal pellets were washed by centrifuging (22,000 x g, 15 min, 4°C) and resuspending in assay buffer: this process was repeated 4 times prior to the [^{3}H]monoamine uptake assays. To remove residual MDPV from the synaptosomal preparation after *in vitro* MDPV preincubation, synaptosomal pellets were incubated with MDPV and washed by repeated centrifugation 4 or 6 times. Synaptosomal [^{3}H]monoamine assays were conducted as described below.

Synaptosomal [^{3}H]DA and [^{3}H]5HT Uptake Assays. [^{3}H]Monoamine uptake assays were performed on striatal or hippocampal crude synaptosomal pellets largely as described (Fleckenstein et al., 1997; Hadlock et al., 2009). Briefly, each assay tube contained synaptosomal tissue (1.5 mg of tissue wet weight/ml for [^{3}H]DA uptake in the striatum, 7.5 mg of tissue wet weigh/ml for [^{3}H]5HT uptake in the striatum, and 10.0 mg tissue wet weight/ml for [^{3}H]5HT uptake in the hippocampus) in assay buffer (126 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.4 mM $MgSO_4$, 11 mM glucose, 1 mM ascorbic acid, 3.75 mM NaH_2PO_4, 12.7 mM Na_2HPO_4, pH 7.4) containing 1 µM pargyline. After preincubation of assay tubes for 10 min at 37°C, assays were initiated by addition of [^{3}H]DA or [^{3}H]5HT (0.5 or 5 nM final concentrations, respectively). Samples were incubated at 37°C for 3 min and then filtered through Whatman GF/B filters (Whatman; Clifton, NJ) soaked previously in 0.05% polyethylenimine. Filters were washed rapidly 3 times with 5 ml ice-cold 0.32 M sucrose using a Brandel filtering manifold. Radioactivity trapped in filters was counted using a liquid scintillation counter and normalized to sample protein as determined by Bradford Protein Assay (Bio-Rad Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories; Hercules, CA).

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Vesicular [^{3}H]DA Uptake Assays. Synaptosomes from rat striatum were lysed by resuspension in ice-cold ddH_2O, trituration with a 1 ml pipette, and homogenization with 5 strokes in a Dounce homogenizer. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and potassium tartrate (KT) were then added (final concentration of 25 and 100 mM, respectively, pH 7.5). Samples were centrifuged (20,000 x g, 20 min, 4 °C) to remove synaptosomal membranes. The pellet (containing the synaptosomal membranes) was resuspended in assay buffer (final concentration: 100 mM KT, 25 mM HEPES, 1.7 mM ascorbic acid, 0.05 mM EGTA, 0.01 mM EDTA, 2 mM $MgSO_4$; pH 7.5) and saved for use in Western blot. $MgSO_4$ (1 mM final concentration) was added to the supernatant, which was centrifuged for 100,000 x g for 45 min at 4 °C. The resulting pellet was resuspended in VMAT-2 assay buffer at a concentration of 50 mg/ml (original tissue wet weight) to obtain a vesicle-enriched (presumably cytoplasmic) preparation. Some sample was retained for Western blotting. For [^{3}H]DA uptake, synaptic vesicles were incubated in VMAT-2 assay buffer containing 2 mM ATP-Mg^{2+} and 30 nM [^{3}H]DA for 3 min (30 °C). Non-specific [^{3}H]DA uptake was defined by measuring [^{3}H]DA uptake in wash buffer (i.e., in the absence of ATP), with samples remaining on wet-ice. Following incubation, all samples were filtered through 0.5% polyethylenimine-soaked GF/F filter paper (Whatman) and washed three times with ice-cold VMAT-2 assay buffer. Radioactivity captured in filters was counted using a liquid scintillation counter and normalized to sample protein as determined by Bradford protein assay.

Subcellular Fractionation for DAT Localization. Subcellular fractions were prepared as described by German et al. (2012). Briefly, striata were placed into 2 ml ice-cold 0.32M sucrose buffer (0.32 M sucrose, 3.75 mM NaH_2PO_4, 12.7 mM Na_2HPO_4) containing protease and phosphatase inhibitors (final concentration: 1 mM phenylmethylsulfonyl fluoride, 1.5 µM aprotinin, 2.3 µM leupeptin, 1 mM sodium orthovanadate, 1 mM sodium fluoride) and synaptosomes were obtained as described above. The synaptosomal pellet was resuspended in 450 µl ice-cold water.

Then 50 µl of buffer (1 M KT, 0.25 M HEPES) was added to stabilize solution. The lysate was centrifuged at 1,500 x g for 15 min at 4 °C. Protease and phosphatase inhibitors (final concentration: 1 mM phenylmethylsulfonyl fluoride, 1.5 µM aprotinin, 2.3 µM leupeptin, 1 mM sodium orthovanadate, 1 mM sodium fluoride) were added to the supernatant (S3). The pellet (P3) was resuspended in RIPA Lysis Buffer (EMD Millipore Corp., Billerica, MD) containing protease and phosphatase inhibitors (final concentration: 1 mM phenylmethylsulfonyl fluoride, 1.5 µM aprotinin, 2.3 µM leupeptin, 1 mM sodium orthovanadate, 1 mM sodium fluoride). The P3 and S3 fractions were analyzed via Western blot.

Subcellular Fractionation for VMAT-2 Localization. Striata were placed in 2 ml ice cold 0.32M sucrose phosphate buffer and synaptosomes were obtained as described above. Synaptosomes were lysed in ice-cold water at a concentration of 75 mg/ml tissue wet weight. The lysate was centrifuged at 22,000 x g for 20 min at 4 °C. The resulting supernatant was the non-membrane associated fraction. The resulting pellet was resuspended in ice-cold water at a concentration of 75 mg/ml tissue wet weight and was the membrane associated fraction. The non-membrane and membrane associated fractions were analyzed via Western blot.

Western Blotting. Samples (for DAT localization: 5 µg of protein from P3 and 10 µg of protein from S3; for VMAT-2 localization: 20 µl of the non-membrane fraction containing 9 – 12 µg of protein, 20.5 µl of the membrane-associated fraction containing and 12 – 18 µg protein, and 1 µg protein for the vesicle enriched fraction) were mixed with lithium dodecyl sulfate sample buffer (Bolt LDS Sample Buffer; ThermoFisher Scientific, Rockford, IL) and dithiothreitol (50mM) and heated at 70°C for 10 min. Samples were then loaded onto Bolt 4-12% Bis-Tris Plus Gels (ThermoFisher Scientific) and electrophoresed for 1 – 1.5 h (200 V constant). Samples were transferred to polyvinylidene difluoride membranes (Perkin Elmer; Waltham, MA) for 2 – 2.5 h (35

V constant). Membranes were blocked with StartingBlock T20 (ThermoFisher Scientific) for 20 - 30 min, and then probed with primary antibody overnight (4 °C). The DAT primary antibody was produced by Sigma Genosys (Sigma Aldrich, St. Louis MO) raised against N-terminal amino acids 42-59 (1:1,000) (Vaughan, 1995). The VMAT-2 primary antibody was produced by Bethyl Labs and raised against a 20-amino acid c-terminal sequence of human VMAT-2 (1:2,000). Following primary antibody incubation, membranes were then probed with secondary antibody (Peroxidase AffiniPure Goat Anti-Rabbit, Jackson Immunoresearch Labs (1:10,000)) for 1 h with gentle agitation at room temperature. Membranes were developed using SuperSignal West Pico PLUS (ThermoFisher Scientific) and imaged with an Alpha Innotech FluorChem HD2 imager. Band density was determined using the FluorChem software and quantification. Protein levels were determined by Bradford assay or by Pierce BCA Protein Assay (ThermoFisher Scientific). For Fig. 3, data were normalized to sample protein concentrations.

High Performance Liquid Chromatography. One striatum or hippocampus from each treated rat was flash frozen on dry ice immediately following decapitation and was stored at -80°C until assay. On the day of assay, tissues were thawed and sonicated for 3–5 s at a concentration of 20 mg striata/ml or 25 mg hippocampus/ml wet weight in tissue buffer (50 mM sodium phosphate, 30 mM citric acid, pH = 2.5, containing 10% methanol), and centrifuged (30,000 x g, 1 h). The resulting pellet was sonicated in 2 ml tissue buffer and assayed for protein by Bradford assay. For hippocampal analysis, 40 µl of the resulting supernatant was injected onto a C-18 reverse-phase analytical column (Partisphere, 5-µm spheres; 250 X 4.6 mm; Whatman, Clifton, NJ, USA). For striatal analysis, supernatant was further diluted to 5 mg/ml original wet weight in tissue buffer and 12 µl was injected onto a C-18 reverse-phase analytical column (Partisphere, 5-µm spheres; 250 X 4.6 mm; Whatman, Clifton, NJ, USA). DA, dihydroxyphenylacetic acid (DOPAC), norepinephrine, 5HT, and 5-hydroxyindoleacetic acid (5HIAA) content was determined using an amperometric electrochemical detector with a flow cell using a three-electrode configuration

(Waters 2465 Electrochemical Detector; Milford, MA). The glassy carbon working electrode potential was set at +0.70 V relative to a salt-bridge Ag+/AgCl reference electrode. The mobile phase consisted of 0.05 M sodium phosphate, 0.03 M citrate buffer containing 0.12 mM EDTA, 0.032% sodium octyl sulfate, and 20% methanol (pH= 2.8). Monoamine and metabolite content were determined by comparing sample peak areas with those recorded from standards run on the same day (recorded by Waters Breeze 2 HPLC system).

MDPV Assessment Utilizing Basified Liquid/Liquid extraction Followed by Ultra-High-Performance Liquid Chromatography (UHPLC) - Electrospray Ionization (ESI) - Tandem Mass Spectrometry (UHPLC-ESI-MS/MS). Trunk blood was collected into 4.5 ml collection tube with gel and lithium heparin on ice (BD Vacutainer, Franklin Lakes, NJ). Blood samples were centrifuged at 3,000 x g for 15 min at 4 °C to obtain plasma. The rat plasma sample aliquot volume was 0.1 mL, and MDPV-d_8 was added as the internal standard (50 μl of a 200 ng/ml working solution). Reference materials for MDPV and the internal standard were obtained from Cerilliant (Round Rock, TX). Prior to extraction, the pH of the rat plasma was adjusted by addition of 0.5 mL of 10% (v/v) ammonium hydroxide (Fisher Chemical, Fair Lawn, NJ), and the samples were extracted with 1-chlorobutane (EMD Millipore OmniSolv, Billerica, MA). The organic layer was isolated and evaporated to dryness, and the extract was reconstituted in mobile phase for analysis by UHPLC-ESI-MS/MS. The liquid chromatographic system was a Waters (Milford, MA) Acquity UPLC® autosampler and pumping system. Chromatographic separation was performed on a Phenomenex (Torrance, CA) Luna® Omega C18 column (2.1 x 50 mm, 1.6 μm particle size) using gradient elution with a cycle time of approximately 6 minutes per injection. The mobile phases were 5 mM ammonium acetate and methanol (Honeywell Burdick & Jackson, Muskegon, MI). The MS was a Waters (Milford, MA) Quattro Premier XE™ triple quadrupole mass spectrometer that was operated in the Selected Reaction Monitoring (SRM) mode under positive ionization conditions. For MDPV and MDPV-d8, the SRM transitions were mass/charge (m/z)

276.2 to 126.1 and m/z 284.2 to 134.1, respectively. The concentration of MDPV in the samples was determined from the ratio of the peak area of MDPV to the peak area of MDPV-d8, and comparison of this ratio with the calibration curve that was generated from the analysis of rat plasma fortified with known concentrations of MDPV and the internal standard.

Duplicate calibrators were prepared fresh for each analytical run in rat plasma at nominal concentrations for MDPV of 10, 25, 50, 100, 250, 500, 750, and 1000 ng/ml. Two double blanks (matrix without internal standard) and two zero samples (matrix with internal standard) were prepared for each analytical batch the same lot of rat plasma used for the calibrators. Quality Control samples (QCs) were prepared in advance at nominal concentrations for MDPV of 30, 150, and 700 ng/ml. Aliquots of the QCs were placed into polypropylene tubes and stored at -80 ˚C until thawing for analysis in triplicate with each analytical run of study samples.

For the analysis of samples in synaptosomal matrix, the following modifications were made to the rat plasma method. Duplicate calibrators were prepared fresh for each analytical run in rat plasma, to conserve synaptosomal matrix, at nominal concentrations for MDPV of 0.25, 0.5, 1, 5, 10, 40, 80, and 100 ng/ml. QCs were prepared by 10-fold dilution of the higher concentration range rat plasma QCs at nominal concentrations for MDPV of 3, 15, and 70 ng/mL and analyzed in triplicate for each analytical run. In addition, QCs in synaptosomal matrix at concentrations for MDPV of 3 and 70 ng/ml were prepared for each analytical run for analysis in triplicate. Finally, the volume of mobile phase used for reconstitution and the mass spectrometer tuning parameters were optimized for the reduced concentration range.

Food Training and Self-Administration. Food training and self-administration was accomplished using an operant chamber (30.5 cm x 25.5 cm x 30.5 cm; Coulbourn Instruments, Whitehall, PA USA) in a room maintained at 26.9 ± 0.7 º C as described previously (McFadden

et al., 2012). Prior to surgery, each rat was trained to press for a 45-mg food pellet on a fixed-ratio 1 schedule of reinforcement during four overnight 14-h sessions. During these sessions, the active lever was counterbalanced across rats and alternated each session such that each lever was reinforced for 2 training sessions. Following food training, rats were anesthetized, and an indwelling catheter was implanted. The catheter was constructed as described previously (Frankel et al., 2011). Each rat received Prevail (flunixin meglumine; 1.1 mg/kg, s.c.) on the day of and following the surgery. Immediately following surgery and daily thereafter until self-administration, each rat was infused with 0.1 ml of cefazolin (10 mg/ml). The animals' food was reduced to 25 g the day before self-administration began. On all following days, animals were free-fed.

Rats underwent 7 d of self-administration (90 min/session; FR1; 0.0175 mg/infusion MDPV or 10 µl saline during the light cycle). For each active lever press, an infusion pump connected to a liquid swivel (Coulbourn Instruments) delivered 10 µl of MDPV or saline per infusion over a 5-s duration through a polyethylene tube located within a spring leash (Coulbourn Instruments) tethered to the rat. During this period, both levers were retracted. Following the infusion, the levers remained retracted for an additional 20 s. The active lever was counterbalanced within each group. Pressing the inactive lever resulted in no programmed consequences. MDPV self-administering rats were included in analysis if they: 1) pressed an average of more than 10 active lever presses per d; and 2) the ratio of active/inactive lever presses was ≥ 2:1 (Brennan et al., 2010). Core body temperatures were collected at the end of the last self-administration session.

Statistical Analysis. All samples within a given experiment were processed concurrently unless stated otherwise. Statistical analysis was conducted in GraphPad Prism 7 (San Diego, CA). Statistical outliers were identified using a Grubb's test (i.e. extreme studentized deviate method) and excluded from analysis. Statistical analyses between 2 groups were conducted by using a *t* test. Analyses among more than two groups were conducted using a one- or two-way analysis

of variance (with repeated measures if appropriate) followed by Bonferroni *post hoc* analyses. In cases requiring a non-parametric test due to significantly different variance among groups, a Mann Whitney test was conducted in place of a *t* test and a Kruskal-Wallis followed by Dunn's multiple comparisons test was conducted in place of a one-way ANOVA. Data are expressed as mean ± S.E.M.

Results

MDPV Self-administration. Results presented in Fig. 1 demonstrate that MDPV (0.0175 mg/10 μl infusion; 90-min daily sessions for 7 d) is readily self-administered by male, Sprague Dawley rats. Rats discriminated between the active and inactive levers at a ratio of at least 2:1 beginning on 3rd d. Core body temperatures, as assessed within 30 min of the final self-administration session, were did not differ between MDPV- and saline-administering groups (saline, 38.8 ± 0.2˚C; MDPV, 38.9 ± 0.2˚C, n = 5/group; $t_8 = 0.367$, $p = .724$). Cumulative MDPV intake over the course of the 7 sessions ranged from 2.10 mg to 6.56 mg (mean = 3.72 ± 0.75, n = 5/group). MDPV plasma concentrations ranged from 20.9 ng/ml to 79.5 ng/ml (mean = 50.1 ± 10.3), as assessed 1 h after the final self-administration session.

Effects of *In Vivo* Exposure to MDPV on VMAT-2. Results presented in Fig. 2A demonstrate that *in vivo* administration of MDPV (2.5 – 5.0 mg/kg, s.c.) increased vesicular [^{3}H]DA uptake, as assessed in the striatal vesicle-enriched preparation from male rats 1 h after injection ($F_{2,18} = 27.75$, $p < 0.0001$; *post hoc* comparison indicated significant differences between 0 and 2.5 mg/kg, and between 0 and 5.0 mg/kg, ($p < 0.0001$)). The increase in VMAT-2 function caused by 2.5 mg/kg (s.c.) was reversed 6 h later (Fig. 2B; Kruskal-Wallis statistic = 15.43, $p = 0.0039$; *post hoc* comparison reveals differences between 0 and 1 h ($p < 0.005$) and between 0 and 3 h ($p < 0.05$)).

Results presented in Fig. 3A and 3B demonstrate that MDPV (2.5 mg/kg, s.c.) concurrently increased and decreased striatal VMAT-2 immunoreactivity in the non-membrane-associated fraction ($t_{14} = 2.382$, $p = 0.032$) and the membrane-associated fraction ($t_{14} = 2.198$, $p = 0.045$) prepared from male rats, respectively, compared to saline (1 ml/kg, s.c.) vehicle controls, as assessed 1 h after treatment. Western blots display band localization, and the associated graphs represent band density per μg protein.

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Results presented in Fig. 4A demonstrate that a single administration of the D1 antagonist, SCH-23390 (0.5 mg/kg, i.p.), 15 min prior to MDPV (2.5 mg/kg, s.c.) or saline (1 ml/kg, s.c.) was without effect on [^{3}H]DA uptake (MDPV main effect: $F_{1,20} = 26.37$, $p < 0.0001$; SCH-23390 main effect: $F_{1,20} = 4.017$, $p = 0.059$; interaction: $F_{1,20} = 0.498$, $p = 0.489$). In contrast, results presented in Fig. 4B demonstrate that a single administration of the D2 antagonist, eticlopride (0.5 mg/kg i.p.), 15 min prior to MDPV (2.5 mg/kg, s.c.) or saline (1 ml/kg, s.c.), attenuated the MDPV-induced increase in striatal vesicular [^{3}H]DA uptake in male rats, as assessed 1 h after MDPV or saline treatment (MDPV main effect: $F_{1,26} = 33.95$, $p < 0.0001$; eticlopride main effect: $F_{1,26} = 5.235$, $p = 0.031$; interaction $F_{1,26} = 10.95$, $p = 0.0028$). Additionally, this same eticlopride pretreatment (a single administration 0.5 mg/kg, i.p., 15 min prior to a single administration of MDPV 2.5 mg/kg s.c.) also attenuated the MDPV-induced redistribution of VMAT-2 (Fig. 4C; MDPV main effect: $F_{1,26} = 15.34$, $p < 0.0006$; eticlopride main effect: $F_{1,26} = 6.916$, $p = 0.014$; interaction: $F_{1,26} = 12.33$, $p = 0.002$).

Effects of *In Vivo* Exposure to MDPV on DAT. *In vivo* administration of MDPV (2.5 mg/kg, s.c.) rapidly and reversibly increased striatal [^{3}H]DA uptake, as assessed in striatal synaptosomes prepared from male rats (Fig. 5; $F_{4,33} = 3.098$, $p = 0.0286$; *post hoc* comparisons indicate significant increase at 1 h, $p = 0.0275$). No alterations in DAT localization between the subcellular fractions, P3 ($t_{14} = 0.208$, $p = 0.838$) and S3 (Mann Whitney U = 21.5, $p = 0.291$, n = 8/group) were detected 1 h after MDPV treatment (2.5 mg/kg, s.c.; Fig. 6).

The MDPV-induced increase in DAT activity was not attenuated by pretreatment with a single administration of either the D1 antagonist, SCH23390 (0.5 mg/kg, i.p.; Fig. 7A; MDPV main effect: $F_{1,28} = 75.29$, $p < 0.0001$; SCH23390 main effect: $F_{1,28} = 1.242$, $p = 0.275$; interaction $F_{1,28} = 0.233$, $p = 0.633$), or the D2 antagonist, eticlopride (0.5 mg/kg, i.p.; Fig. 7B; MDPV main effect: $F_{1,22} =$

41.67, $p < 0.0001$; eticlopride main effect: $F_{1,22} = 0.100$, $p = 0.756$; interaction $F_{1,28} = 1.904$, $p = 0.182$), administered 15 min prior to MDPV (2.5 mg/kg, s.c.). Of note, pretreatment with the D1 antagonist SCH23390 prevented the MDPV-induced increase in temperature (saline/saline = 38.0 ± 0.1˚C, saline/MDPV = 38.7 ± 0.2˚C, SCH23390/saline = 38.2 ± 0.1˚C, SCH/MDPV = 38.1 ± 0.2˚C).

Although studies have demonstrated that nAChR blockade attenuate some behavioral effects of MDPV (Geste et al., 2018), the MDPV-induced increase in DAT activity was not attenuated by pretreatment with a single administration of the non-selective nAChR antagonist mecamylamine (3 mg/ml, s.c.), administered 10 min prior to MDPV (2.5 mg/kg, s.c.; Fig. 7C; MDPV main effect $F_{1,27} = 53.77$, $p < 0.0001$; mecamylamine main effect $F_{1,27} = 1.468$, $p = 0.236$; interaction $F_{1,27} = 1.668$, $p = 0.208$).

Effects of *In Vivo* MDPV Administration on Monoamine Content. Despite concurrent alterations in VMAT-2 and DAT and function, results presented in Table 1 demonstrate that MDPV (2.5 or 5.0 mg/kg, s.c.) had no effect on content of striatal DA ($F_{2,21} = 2.85$, $p = 0.080$), and modestly increased content of the DA metabolite, DOPAC ($F_{2,21} = 6.678$, $p = 0.006$), as assessed 1 h after treatment. *Post hoc* comparisons reveal that compared to saline treated rats, DOPAC is increased in rats that received 2.5 ($p < 0.01$) or 5.0 mg/kg ($p < 0.05$) MDPV.

In contrast to effects on DAT*, in vivo* administration of MDPV (0.5, 2.5, or 5.0 mg/kg, s.c.) was without effect on plasmalemmal [^{3}H]5HT uptake in the striatum or the hippocampus of male rats, as assessed 1 h after administration (Supplemental Table 1). Further, *in vivo* administration of MDPV (2.5, or 5.0 mg/kg, s.c.) was without effect on norepinephrine, 5HIAA, or 5HT in the hippocampus at this 1 h timepoint (Supplemental Table 2).

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Effects of MDPV on Core Body Temperature. A single 2.5 or 5.0 mg/kg injection (s.c.) of MDPV increased core body temperatures in male rats compared with saline controls (1 ml/kg); saline, 37.4 ± 0.2 ˚C, MDPV 2.5 mg/kg, 39.2 ± 0.3 ˚C, MDPV 5.0, = 38.6 ± 0.2 ˚C, as assessed 30 min after a single administration ($F_{2,21}$ = 15.62, p < 0.0001; *post hoc* comparison revealed differences between 0 and 2.5 mg/kg, and between 0 and 5.0 mg/kg; p < 0.0001). The effect on core body temperature was reversible as temperatures returned to baseline 2.5 h after a single 2.5 mg/kg (s.c.) injection (saline = 37.6 ± 0.1 ˚C, 30 minutes = 38.5 ± 0.2 ˚C*, 2.5 h = 38.1 ± 0.1 ˚C, 5.5 h = 37.5 ± 0.2 ˚C, 17.5 h = 37.0 ± 0.2 ˚C; $F_{4,33}$ = 10.39, p < .0001; * indicates significant difference from saline, p = 0.008)

MDPV Pharmacokinetics. Plasma concentrations of MDPV, as assessed 1 h after administration of 2.5 and 5.0 mg/kg (s.c.) in male rats, were 367 ± 22 and 823 ± 56 ng/ml, respectively. Pharmacokinetic analysis 1, 3, 6, and 18 h after administration of 2.5 mg/kg (s.c.) reveals that plasma MDPV concentrations declined sharply after 1 h, and were below the lower limit of quantification of 10 ng/ml in 8 out of 9 samples by 18 h (1 h, 325 ± 28 ng/ml; 3 h, 80.4 ± 11.9 ng/ml; 6 h, 13.1 ± 3.3 ng/ml).

Impact *In Vivo* of Repeated MDPV Administrations on Monoaminergic Neurons. Results presented in Fig. 8 demonstrate that despite causing moderate hyperthermia (Fig. 8B; drug x time interaction F = 14.56, p < 0.0001), and in contrast to METH (3 injections, 2-h intervals, 7.5 mg/kg/injection), repeated administrations of MDPV (3 injections, 2-h intervals, 2.5 or 5.0 mg/kg/injection) did not alter [^{3}H]DA uptake in male rats as assessed 7 d after treatment (Fig. 8A; $F_{3,48}$ = 4.642, p = 0.0063; *post hoc* comparisons revealed no differences between saline and MDPV treated animals, p > 0.05), and decreased [^{3}H]DA uptake in METH-treated rats compared to saline treated rats (p < 0.01). This finding was extended to female rats. Specifically, repeated MDPV administrations (3 injections, 2-h intervals, 2.5 or 5.0 mg/kg/injection) did not alter [^{3}H]DA

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uptake in the striatum as assessed 8 d after treatment (3 x 0 mg/kg MDPV, 3.31 ± 0.16 fmol [^{3}H]DA/µg protein; 3 x 2.5 mg/kg MDPV, 2.94 ± 0.15 fmol [^{3}H]DA/µg protein; 3 x 5.0 mg/kg MDPV = 3.31 ± 0.21 fmol [^{3}H]DA/µg protein; $F_{2,18}$ = 1.771, p = 0.199).

Repeated MDPV administrations (3 injections, 2-h intervals, 2.5 or 5.0 mg/kg) did not affect plasmalemmal striatal or hippocampal [^{3}H]5HT uptake, as assessed 8 d after MDPV treatment (Supplemental Table 3). In the hippocampus, repeated administrations of MDPV did not affect norepinephrine, 5HIAA, or 5HT content (Supplemental Table 4).

IC_{50} of MDPV on VMAT-2 and DAT. Direct *in vitro* application of MDPV, at concentrations as large as 10 µM, was without effect on vesicular [^{3}H]DA uptake, as assessed in a vesicle-enriched (presumably cytoplasmic) subcellular fraction prepared from male rat striata (data not shown). Direct application of MDPV (3 min, 37˚C, as described in Methods in Synaptosomal [^{3}H]DA and [^{3}H]5HT Uptake Assays) inhibited striatal plasmalemmal [^{3}H]DA uptake with an IC_{50} of 5.4 ± 0.9 nM.

Effects of 1-h *In Vitro* Incubation with MDPV on DAT. Similar to direct application of MDPV for 3 min, incubation of synaptosomes with MDPV for 1 h decreased [^{3}H]DA uptake (data not shown). In contrast to the direct effects of MDPV on DAT, Table 2 demonstrates this direct decrease induced by MDPV incubation was no longer observed when synaptosomes were "washed" 4 – 6 times (see Methods; 4 washes: $F_{3,20}$ = 0.840, p = 0.488; 6 washes: $F_{3,20}$ = 0.959, p = .427). Resulting MDPV concentrations at the time of the [^{3}H]DA uptake assay, all of which at least one order of magnitude less than the IC_{50} of 5.4 nM, are reported in Table 2.

Discussion

As demonstrated previously, MDPV is readily self-administered by male rats (Aarde et al., 2013; Watterson et al., 2014; Aarde et al., 2015a; Aarde et al., 2015b; Schindler et al., 2016; Gannon et al., 2017a; Geste et al., 2018). The present study extends these findings by reporting that plasma MDPV concentrations 1 h after the final self-administration session range from 20.9 to 79.5 ng/ml. In contrast, concentrations found 1 h after 2.5 mg/kg (s.c; 367 and 325 ng/ml) were approximately 7-fold greater. The latter results are consistent with circulating drug concentrations reported after a single administration of MDPV (0.5 – 2.0 mg/kg, s.c.) (Anizan et al., 2016). These levels resemble plasma concentrations in human MDPV abusers (i.e., 24-241 ng/ml; Spiller et al., 2011), with the important caveat of interspecies differences in drug metabolism. The average "bath salts" dose in human abusers ranges from 5 to 20 mg (Ross et al., 2012), and the dose primarily used in this study (2.5 mg/kg) is equivalent to a 24 mg dose in a 60 kg human (Reagan-Shaw et al., 2008).

A second novel finding described herein is that MDPV administration rapidly and reversibly increases VMAT-2 function, as assessed in a non-membrane-associated (presumably cytoplasmic) subcellular fraction. This effect is associated with increases and decreases in levels of non-membrane-associated and plasmalemmal membrane-associated VMAT-2, respectively. We hypothesize that this shift represents a rapid redistribution of VMAT-2, and presumably associated vesicles, within striatal nerve terminals. Of note, D2 antagonist (eticlopride) pretreatment attenuated both the increase in cytoplasmic [^{3}H]DA uptake and VMAT-2 immunoreactivity. In contrast, D1 antagonist (SCH23390) pretreatment did not attenuate the MDPV-induced increase in [^{3}H]DA uptake.

Findings of concurrent increased VMAT-2 function and VMAT-2 immunoreactivity in a cytoplasmic subcellular fraction are consistent with the rapid *in vivo* effects of several "classical" DA reuptake

inhibitors. For example, cocaine and methylphenidate redistribute VMAT-2 (and presumably associated vesicles) from a synaptosomal membrane-associated fraction to a cytoplasmic fraction. Further, and similar to cocaine (Farnsworth et al., 2009) and methylphenidate (Volz et al., 2008), eticlopride pretreatment prevents the MDPV-induced increase in VMAT-2 activity. Of note, these VMAT-2 data stand in contrast to drugs classically referred to as DA "releasing agents" such as METH. For example, METH redistributes VMAT-2 to a subcellular neuronal fraction that is not retained in the preparation of synaptosomes (Riddle et al., 2002). Activation of D2 autoreceptors by extracellular DA may be key to the MDPV-induced redistribution of VMAT-2 (Truong et al., 2004).

The impact of MDPV administration on striatal DAT was also investigated. There is one report of rapid DAT upregulation following MDPV exposure, and it was suggested it may be due increased DAT cell surface expression (as determined by [^{3}HWIN 35428 binding to differentiated PC12 cells) (Lopez-Arnau et al., 2018). In contrast, another report indicated that MDPV causes rapid internalization (albeit after incubation of human embryonic kidney cells), as well as a decreased total striatal DAT and altered subcellular localization 24 h after *in vivo* administration (Colon-Perez et al., 2018). Our results confirmed findings by Lopez-Arnau (2018) that MDPV rapidly and reversibly increased DAT function, as assessed in synaptosomes prepared from treated rats. However, and in contrast to both Lopez-Arnau et al. (2018) and Colon-Perez et al. (2018), we did not find evidence of altered DAT surface expression.

Reports of rapid increases in DAT function after *in vivo* stimulant administration are sparse. Cocaine treatment reportedly increases DAT function, as assessed *ex vivo* in synaptosomes prepared from treated rats (Fleckenstein et al., 1999; Daws et al., 2002; Lopez-Arnau et al., 2018) and *in vivo* (Daws et al., 2002). Thus, and while studies noted above demonstrate that MDPV acts via a cocaine-like mechanism (Baumann et al., 2013), additional studies are needed to

investigate a potential dual action of these stimulants: that is, one to inhibit and a second to enhance DAT function.

To further investigate the impact of MDPV on DAT, *in vitro* studies were conducted wherein striatal synaptosomes from naïve rats were pre-incubated for 1 h with MDPV *in vitro*. In some experiments, the uptake assays were conducted directly after the pre-incubation (i.e. synaptosomes were not "washed") and results revealed a *decrease* in [^{3}H]DA uptake, consistent with other reuptake inhibitors. In separate studies, synaptosomes were "washed" 4 – 6 times prior to assessment of [^{3}H]DA uptake owing to discussion by Lopez-Arnau et al. (2018) regarding the "high affinity of MDPV for DAT which is probably accompanied by a very slow dissociation rate." The resulting MDPV concentrations in these latter studies at the time of [^{3}H]DA uptake were evaluated and were all at least one order of magnitude less than the IC_{50} of 5.4 nM (i.e., as reported in Table 2). Further, in these latter studies, MDPV incubation did not alter DAT activity. These data stand in contrast to Lopez-Arnau (2018), who reported that *in vitro* incubation with MDPV at concentrations of 1 μM increased [^{3}H]DA uptake, albeit using concentrations of the transmitter (i.e., 200 nM) that were 400-fold greater than concentrations used in the present studies (i.e., 0.5 nM).

Our *in vitro* incubation data confirm that effects of psychostimulants on DAT *in vitro* do not always mimic effects following *in vivo* exposure (German et al., 2012). One explanation for a lack of *in vitro* effect is that neuronal circuitry beyond that present in the synaptosomal preparation may be required to reveal the *in vivo* MDPV-induced increase in plasmalemmal [^{3}H]DA uptake. Thus, and because D2 receptor activation can regulate DAT function (Dickinson et al., 1999; Bolan et al., 2007), the impact of eticlopride pretreatment was investigated. Results revealed that eticlopride did not attenuate the MDPV-induced increase in DAT function. While we cannot rule out the possibility that the dose and/or timing of eticlopride administration may not have been

optimal, we observed eticlopride-mediated blockade of MDPV-induced stereotypy and locomotion (data not shown). Of note, this eticlopride dose and timing was selected as it attenuates or prevents DAT inhibitor- or stimulant-induced alterations in VMAT-2 function (Brown et al., 2001; Sandoval et al., 2002; Truong et al., 2004; Rau et al., 2005), including the MDPV-induced increase in VMAT-2 function reported in the present manuscript. Similarly, pretreatment with SCH23390 did not attenuate the MDPV-induced increase in DAT function.

Because treatment with the nAChR antagonist, mecamylamine, reduced MDPV self-administration and blocked the reward enhancing effects of MDPV (Geste et al., 2018), the role of nAChRs in the effects of MDPV on DAT function was assessed. Results revealed that mecamylamine had no effect on the MDPV-induced increase in DAT activity. Thus, given these and the DA antagonist data, the subcellular mechanism underlying the impact of MDPV on DAT function remains to be elucidated.

Given that there is an upregulation in DA transport and sequestration by DAT and VMAT-2 respectively, DA and DOPAC tissue content were analyzed. Results revealed that despite the concurrent MDPV-induced increases in DAT and VMAT-2 function, tissue DA content is not altered, as assessed 1 h after treatment. In contrast, a modest increase in DOPAC was noted, which may suggest an increase in DA turnover/altered metabolism as suggested by Colon-Perez et al, 2018.

Of interest are findings that MDPV can increase vesicular DA release (Hoffman et al., 2016). Future studies as to how these data, coupled with alterations in DAT/VMAT-2 function, along with alterations in DA turnover/metabolism, are warranted.

To determine if the MDPV-induced transporter upregulation extended to other monoamine transporters, we examined SERT function after an *in vivo* MDPV administration. Upregulation of plasmalemmal transporters was specific to DAT as MDPV did not increase SERT function in either the striatum or hippocampus, nor did it alter norepinephrine, 5HIAA, or 5HT content in the hippocampus.

Synthetic cathinone users often will repeatedly self-administer within a single drug-taking session to prevent the “come-down” associated with a single administration (Ross et al., 2012). To model this pattern and assess the long-term impacts of MDPV, rats were exposed to a “binge” regimen and evaluated 7 – 8 d later. Results revealed that repeated MDPV administrations (3 injections, 2.5 or 5.0 mg/kg/injection, 2-h intervals) to rats (male or female) did not cause persistent deficits in striatal [^{3}H]DA or striatal [^{3}H]5HT uptake. These data are consistent with another report involving female mice of limited to no dopaminergic deficits 48 h after MDPV treatment (Anneken et al., 2015).

It is interesting to speculate that enhanced VMAT-2 capacity may contribute to the lack of persistent DAergic deficits caused by MDPV. Specifically, the increase in DAT activity may promote DA uptake into the cytoplasmic space, where it would otherwise be rapidly converted to reactive DA quinones and related species were it not for the capacity of MDPV to rapidly sequester DA owing to enhanced vesicular DA uptake.

In summary, MDPV has a pharmacological profile that in some ways resembles other reuptake inhibitors like methylphenidate or cocaine, but also causes an upregulation in DAT function. The effects of MDPV on both DAT and VMAT-2 likely contribute to its highly reinforcing properties while also mitigating persistent dopaminergic deficits due to aberrant DA transport.

Author Contributions

Participated in research design: Magee, German, Wilkins, Fleckenstein

Conducted experiments: Magee, German, Curtis, Siripathane, Anderson, Fleckenstein

Contributed new reagents or analytic tools:

Performed data analysis: Magee, Wilkins, Fleckenstein

Wrote or contributed to the writing of the manuscript: Magee, German, Hanson, Fleckenstein, Anderson, Wilkins

References

Aarde SM, Creehan KM, Vandewater SA, Dickerson TJ and Taffe MA (2015a) In vivo potency and efficacy of the novel cathinone alpha-pyrrolidinopentiophenone and 3,4-methylenedioxypyrovalerone: self-administration and locomotor stimulation in male rats. *Psychopharmacology* **232**:3045-3055.

Aarde SM, Huang PK, Creehan KM, Dickerson TJ and Taffe MA (2013) The novel recreational drug 3,4-methylenedioxypyrovalerone (MDPV) is a potent psychomotor stimulant: Self-administration and locomotor activity in rats. *Neuropharmacology* **71**:130-140.

Aarde SM, Huang PK, Dickerson TJ and Taffe MA (2015b) Binge-like acquisition of 3,4-methylenedioxypyrovalerone (MDPV) self-administration and wheel activity in rats. *Psychopharmacology* **232**:1867-1877.

Anizan S, Concheiro M, Lehner KR, Bukhari MO, Suzuki M, Rice KC, Baumann MH and Huestis MA (2016) Linear pharmacokinetics of 3,4-methylenedioxypyrovalerone (MDPV) and its metabolites in the rat: relationship to pharmacodynamic effects. *Addiction Biology* **21**:339-347.

Anneken JH, Angoa-Perez M and Kuhn DM (2015) 3,4-Methylenedioxypyrovalerone prevents while methylone enhances methamphetamine-induced damage to dopamine nerve endings: beta-ketoamphetamine modulation of neurotoxicity by the dopamine transporter. *Journal of Neurochemistry* **133**:211-222.

Baumann MH, Bukhari MO, Lehner KR, Anizan S, Rice KC, Concheiro M and Huestis MA (2017) Neuropharmacology of 3,4-Methylenedioxypyrovalerone (MDPV), Its Metabolites, and Related Analogs. *Current topics in behavioral neurosciences* **32**:93-117.

Baumann MH, Partilla JS, Lehner KR, Thorndike EB, Hoffman AF, Holy M, Rothman RB, Goldberg SR, Lupica CR, Sitte HH, Brandt SD, Tella SR, Cozzi NV and Schindler CW (2013) Powerful Cocaine-Like Actions of 3,4-Methylenedioxypyrovalerone (MDPV), a

Principal Constituent of Psychoactive 'Bath Salts' Products. *Neuropsychopharmacology* **38**:552-562.

Brennan KA, Colussi-Mas J, Carati C, Lea RA, Fitzmaurice PS and Schenk S (2010) Methamphetamine self-administration and the effect of contingency on monoamine and metabolite tissue levels in the rat. *Brain Research* **1317**:137-146.

Brown JM, Hanson GR and Fleckenstein AE (2001) Regulation of the vesicular monoamine transporter-2: A novel mechanism for cocaine and other psychostimulants. *Journal of Pharmacology and Experimental Therapeutics* **296**:762-767.

Callaghan RC, Cunningham JK, Sajeev G and Kish SJ (2010) Incidence of Parkinson's Disease Among Hospital Patients with Methamphetamine-Use Disorders. *Movement Disorders* **25**:2333-2339.

Callaghan RC, Cunningham JK, Sykes J and Kish SJ (2012) Increased risk of Parkinson's disease in individuals hospitalized with conditions related to the use of methamphetamine or other amphetamine-type drugs. *Drug and Alcohol Dependence* **120**:35-40.

Cameron KN, Kolanos R, Solis E, Glennon RA and De Felice LJ (2013) Bath salts components mephedrone and methylenedioxypyrovalerone (MDPV) act synergistically at the human dopamine transporter. *British Journal of Pharmacology* **168**:1750-1757.

Christine CW, Garwood ER, Schrock LE, Austin DE and McCulloch CE (2010) Parkinsonism in Patients with a History of Amphetamine Exposure. *Movement Disorders* **25**:228-231.

Colon-Perez LM, Pino JA, Saha K, Pompilus M, Kaplitz S, Choudhury N, Jagnarine DA, Geste JR, Levin BA, Wilks I, Setlow B, Bruijnzeel AW, Khoshbouei H, Torres GE and Febo M (2018) Functional connectivity, behavioral and dopaminergic alterations 24 hours following acute exposure to synthetic bath salt drug methylenedioxypyrovalerone. *Neuropharmacology* **137**:178-193.

Curtin K, Fleckenstein AE, Robison RJ, Crookston MJ, Smith KR and Hanson GR (2015) Methamphetamine/amphetamine abuse and risk of Parkinson's disease in Utah: A population-based assessment. *Drug and Alcohol Dependence* **146**:30-38.

Daws LC, Callaghan PD, Moron JA, Kahlig KM, Shippenberg TS, Javitch JA and Galli A (2002) Cocaine increases dopamine uptake and cell surface expression of dopamine transporters. *Biochemical and Biophysical Research Communications* **290**:1545-1550.

Eshleman AJ, Wolfrum KM, Hatfield MG, Johnson RA, Murphy KV and Janowsky A (2013) Substituted methcathinones differ in transporter and receptor interactions. *Biochemical Pharmacology* **85**:1803-1815.

Farnsworth SJ, Volz TJ, Hanson GR and Fleckenstein AE (2009) Cocaine Alters Vesicular Dopamine Sequestration and Potassium-Stimulated Dopamine Release: The Role of D2 Receptor Activation. *Journal of Pharmacology and Experimental Therapeutics* **328**:807-812.

Fleckenstein AE, Haughey HM, Metzger RR, Kokoshka JM, Riddle EL, Hanson JE, Gibb JW and Hanson GR (1999) Differential effects of psychostimulants and related agents on dopaminergic and serotonergic transporter function. *European Journal of Pharmacology* **382**:45-49.

Fleckenstein AE, Metzger RR, Wilkins DG, Gibb JW and Hanson GR (1997) Rapid and reversible effects of methamphetamine on dopamine transporters. *Journal of Pharmacology and Experimental Therapeutics* **282**:834-838.

Frankel PS, Hoonakker AJ, Alburges ME, McDougall JW, McFadden LM, Fleckenstein AE and Hanson GR (2011) Effect of Methamphetamine Self-Administration on Neurotensin Systems of the Basal Ganglia. *Journal of Pharmacology and Experimental Therapeutics* **336**:809-815.

Gannon BM, Galindo KI, Rice KC and Collins GT (2017a) Individual Differences in the Relative Reinforcing Effects of 3,4-Methylenedioxypyrovalerone under Fixed and Progressive Ratio

Schedules of Reinforcement in Rats. *Journal of Pharmacology and Experimental Therapeutics* **361**:181-189.

Gannon BM, Russell LN, Modi MS, Rice KC and Fantegrossi WE (2017b) Effects of orally self-administered bath salt constituent 3,4-methylenedioxypyrovalerone (MDPV) in mice. *Drug and Alcohol Dependence* **179**:408-415.

Gannon BM, Sulima A, Rice KC and Collins GT (2018) Inhibition of Cocaine and 3,4-Methylenedioxypyrovalerone (MDPV) Self-Administration by Lorcaserin Is Mediated by 5-HT2C Receptors in Rats. *The Journal of pharmacology and experimental therapeutics* **364**:359-366.

Garwood ER, Bekele W, McCulloch CE and Christine CW (2006) Amphetamine exposure is elevated in Parkinson's disease. *Neurotoxicology* **27**:1003-1006.

German CL, Hanson GR and Fleckenstein AE (2012) Amphetamine and methamphetamine reduce striatal dopamine transporter function without concurrent dopamine transporter relocalization. *Journal of Neurochemistry* **123**:288-297.

Geste JR, Pompilus M, Febo M and Bruijnzeel AW (2018) Self-administration of the synthetic cathinone MDPV enhances reward function via a nicotinic receptor dependent mechanism. *Neuropharmacology* **137**:286-296.

Hadlock GC, Baucum AJ, King JL, Horner KA, Cook GA, Gibb JW, Wilkins DG, Hanson GR and Fleckenstein AE (2009) Mechanisms Underlying Methamphetamine-Induced Dopamine Transporter Complex Formation. *Journal of Pharmacology and Experimental Therapeutics* **329**:169-174.

Hoffman AF, Spivak CE and Lupica CR (2016) Enhanced Dopamine Release by Dopamine Transport Inhibitors Described by a Restricted Diffusion Model and Fast-Scan Cyclic Voltammetry. *Acs Chemical Neuroscience* **7**:700-709.

Johnston LD, Miech RA, O'Malley PM, Bachman JG, Schulenberg JE and Patrick ME (2019) Monitoring the Future national survey results on drug use 1975-2018: Overview, key

findings on adolescent drug use., in, Ann Arbor: Institute for Social Research, University of Michigan.

Lopez-Arnau R, Duart-Castells L, Aster B, Camarasa J, Escubedo E and Pubill D (2018) Effects of MDPV on dopamine transporter regulation in male rats. Comparison with cocaine. *Psychopharmacology*.

McFadden LM, Hadlock GC, Allen SC, Vieira-Brock PL, Stout KA, Ellis JD, Hoonakker AJ, Andrenyak DM, Nielsen SM, Wilkins DG, Hanson GR and Fleckenstein AE (2012) Methamphetamine Self-Administration Causes Persistent Striatal Dopaminergic Alterations and Mitigates the Deficits Caused by a Subsequent Methamphetamine Exposure. *Journal of Pharmacology and Experimental Therapeutics* **340**:295-303.

Rau KS, Birdsall E, Hanson JE, Johnson-Davis KL, Carroll FI, Wilkins DG, Gibb JW, Hanson GR and Fleckenstein AE (2005) Bupropion increases striatal vesicular monoamine transport. *Neuropharmacology* **49**:820-830.

Reagan-Shaw S, Nihal M and Ahmad N (2008) Dose translation from animal to human studies revisited. *Faseb Journal* **22**:659-661.

Riddle EL, Topham MK, Haycock JW, Hanson GR and Fleckenstein AE (2002) Differential trafficking of the vesicular monoamine transporter-2 by methamphetamine and cocaine. *European Journal of Pharmacology* **449**:71-74.

Ross EA, Reisfield GM, Watson MC, Chronister CW and Goldberger BA (2012) Psychoactive "Bath Salts" Intoxication with Methylenedioxypyrovalerone. *American Journal of Medicine* **125**:854-858.

Sandoval V, Riddle EL, Hanson GR and Fleckenstein AE (2002) Methylphenidate redistributes vesicular monoamine transporter-2: Role of dopamine receptors. *Journal of Neuroscience* **22**:8705-8710.

Schindler CW, Thorndike EB, Goldberg SR, Lehner KR, Cozzi NV, Brandt SD and Baumann MH (2016) Reinforcing and neurochemical effects of the "bath salts" constituents 3,4-

methylenedioxypyrovalerone (MDPV) and 3,4-methylenedioxy-N-methylcathinone (methylone) in male rats. *Psychopharmacology* **233**:1981-1990.

Simmler LD, Buser TA, Donzelli M, Schramm Y, Dieu LH, Huwyler J, Chaboz S, Hoener MC and Liechti ME (2013) Pharmacological characterization of designer cathinones in vitro. *British Journal of Pharmacology* **168**:458-470.

Spiller HA, Ryan ML, Weston RG and Jansen J (2011) Clinical experience with and analytical confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. *Clinical Toxicology* **49**:499-505.

Truong JG, Newman AH, Hanson GR and Fleckenstein AE (2004) Dopamine D2 receptor activation increases vesicular dopamine uptake and redistributes vesicular monoamine transporter-2 protein. *European Journal of Pharmacology* **504**:27-32.

Vaughan RA (1995) Photoaffinity-Labeled Ligand-Binding Domains on Dopamine Transporters Identified by Peptide-Mapping. *Molecular Pharmacology* **47**:956-964.

Volz TJ, Farnsworth SJ, Rowley SD, Hanson GR and Fleckenstein AE (2008) Methylphenidate-induced increases in vesicular dopamine sequestration and dopamine release in the striatum: The role of muscarinic and dopamine D2 receptors. *Journal of Pharmacology and Experimental Therapeutics* **327**:161-167.

Watterson LR, Kufahl PR, Nemirovsky NE, Sewalia K, Grabenauer M, Thomas BF, Marusich JA, Wegner S and Olive MF (2014) Potent rewarding and reinforcing effects of the synthetic cathinone 3,4-methylenedioxypyrovalerone (MDPV). *Addiction Biology* **19**:165-174.

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Figure Legends

Fig. 1 MDPV is self-administered by male, Sprague Dawley rats. Rats self-administered MDPV (0.0175 mg/infusion; FR1; n = 5) or saline (10 µl/infusion; n = 5) for 7 d (90 min/d). Values represent means and vertical lines represent 1 S.E.M.

Fig. 2 MDPV administration rapidly and reversibly increases striatal vesicular [^{3}H]DA uptake. (A) Rats received a single injection (s.c.) of MDPV (2.5 or 5.0 mg/kg) or saline vehicle (1 ml/kg) and were sacrificed 1 h later. Values/columns represent means and vertical lines represent 1 S.E.M. (n = 6 - 8). *p < 0.05 from saline. (B) Rats received a single injection (s.c.) of MDPV (2.5 mg/kg) or saline (1 ml/kg; zero-time value) and were sacrificed 1 – 18 h later. Values/columns represent means and vertical lines represent 1 S.E.M. from two combined independent experiments (n = 11 - 14). *p < 0.05 from saline.

Fig. 3 VMAT-2 immunoreactivity decreases in the (A) membrane-associated fraction and increases in the (B) non-membrane-associated fraction in rats that received a single injection (s.c.) of MDPV (2.5 mg/kg) compared to rats that received a single injection (s.c.) of saline (1 ml/kg) and were sacrificed 1 h later. Western blots display band localization prior to normalization to protein content. Bar graphs represent data normalized to protein content (see Methods). Columns represent means and vertical lines represent 1 S.E.M, n = 8). *p < 0.05 from saline.

Fig. 4 D1 receptor antagonist pretreatment is without effect on the MDPV-induced increase in striatal vesicular [^{3}H]DA uptake whereas D2 receptor antagonist pretreatment attenuates the MDPV-induced increases in striatal vesicular [^{3}H]DA uptake and associated VMAT-2 redistribution. Rats received a single injection (i.p.) of (A) SCH23390 (0.5 mg/kg), (B-C) eticlopride (0.5 mg/kg) or saline (1 ml/kg), followed by a single injection (s.c.) of MDPV (2.5 mg/kg) or saline

(1 ml/kg) 15 min later. Rats were sacrificed 1 h following second injection. (A) [^{3}H]DA uptake, (B) [^{3}H]DA uptake, and (C) representative Western blot of VMAT-2 immunoreactivity in the vesicle-enriched fraction as described in Methods, and associated quantification. Columns represent means and vertical lines represent 1 S.E.M. (n = 6 - 8). *p < 0.05 from saline.

Fig. 5 MDPV administration rapidly and reversibly increases striatal plasmalemmal [^{3}H]DA uptake. Rats received a single injection (s.c.) of MDPV (2.5 mg/kg) or saline (1 ml/kg; zero-time value) and were sacrificed 1 – 18 h later. Symbols represent means and vertical lines represent 1 S.E.M. (n = 7 - 8). *p < 0.05 from saline.

Fig. 6 MDPV administration does not affect DAT localization in synaptosome subcellular fractions. Representative image of Western blots as described in Methods, and quantification of DAT in P3 and S3 obtained from rats that received a single injection (s.c.) of MDPV (2.5 mg/kg) or saline (1 ml/kg) and were sacrificed 1 h later. Values represent means and vertical lines represent 1 S.E.M. (n = 8).

Fig. 7 Neither D1, D2, nor nACh receptor antagonist pretreatment attenuates the MDPV-induced increase in striatal plasmalemmal [^{3}H]DA uptake. Rats received a single injection of (A) SCH23390 (0.5 mg/kg, i.p.) or saline (1 ml/kg, i.p.), (B) eticlopride (0.5 mg/kg, i.p.) or saline (1 ml/kg, i.p.), or (C) mecamylamine (3 mg/kg, s.c.) or saline (1 ml/kg, s.c.) followed by a single injection (s.c.) of MDPV (2.5 mg/kg) or saline vehicle (1 ml/kg) 15 or 10 min later for SCH23390/eticlopride or mecamylamine, respectively. Rats were sacrificed 1 h following the second injection. Columns represent means and vertical lines represent 1 S.E.M. (n = 8). *p < 0.05 from respective saline control.

Fig. 8 MDPV, unlike METH, does not alter plasmalemmal [^{3}H]DA uptake as assessed 7 d after treatment, despite causing hyperthermia. Male Rats received multiple injections (s.c.; 2-h intervals) of either MDPV (3 x 2.5 or 5.0 mg/kg/injection) or saline vehicle (3 x 1 ml/kg/injection). (A) [^{3}H]DA uptake as assessed 7 d after MDPV treatment. (B) Core body temperatures were assessed at times indicated by arrows. Values/columns represent means and vertical lines represent 1 S.E.M. (n = 11 – 14).

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Table 1. *In vivo* MDPV administration increases striatal DOPAC concentrations without effect on striatal DA concentrations 1 h after administration.

Drug Treatment	DA (ng/mg protein)	DOPAC (ng/mg protein)
Saline 1 ml/kg	134.9 ± 6.7	26.81 ± 1.2
2.5 mg/kg MDPV	149.2 ± 7.3	32.47 ± 1.5 *
5.0 mg/kg MDPV	129.0 ± 3.8	32.35 ± 1.0 *

Rats received a single injection (s.c.) of MDPV (2.5 or 5.0 mg/kg) or saline vehicle (1 ml/kg) and were sacrificed 1 h later. Values represent means ± S.E.M. (n = 8). *$p < 0.05$ from saline.

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Table 2. *In vitro* pre-incubation of striatal synaptosomes with MDPV is without effect on plasmalemmal [^{3}H]DA uptake.

Number of "Washes"	MDPV Preincubation (nM)	[^{3}H]DA uptake (fmol/μg protein)	MDPV in Synaptosome Preparation after Repeated Washing (nM)
4	0	1.74 ± 0.09	0
	10	1.95 ± 0.13	0
	100	1.92 ± 0.13	0.20 ± 0.11
	1000	1.96 ± 0.10	0.26 ± 0.11
6	0	1.27 ± 0.06	0
	10	1.34 ± 0.07	0
	100	1.25 ± 0.06	0.02 ± 0.18
	1000	1.39 ± 0.05	0.09 ± 0.18

Synaptosomes were pre-incubated with varying concentrations of MDPV (0 – 1000 nM) for 1 h at 37° C. Samples were then centrifuged (22,000 x g for 15 min at 4° C) and resuspended in assay buffer either 4 or 6 times and then assayed for [^{3}H]DA uptake as described in the Methods. MDPV concentrations represent the drug concentration at the time of [^{3}H]DA uptake assay. Values represent means ± S.E.M. (n = 6).

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Fig. 1

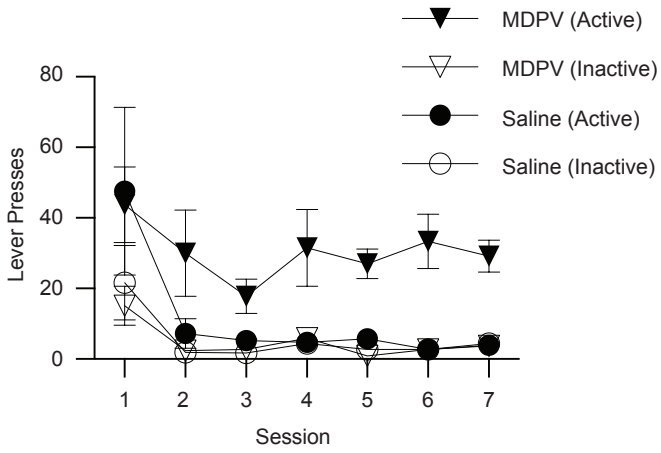

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Fig. 2

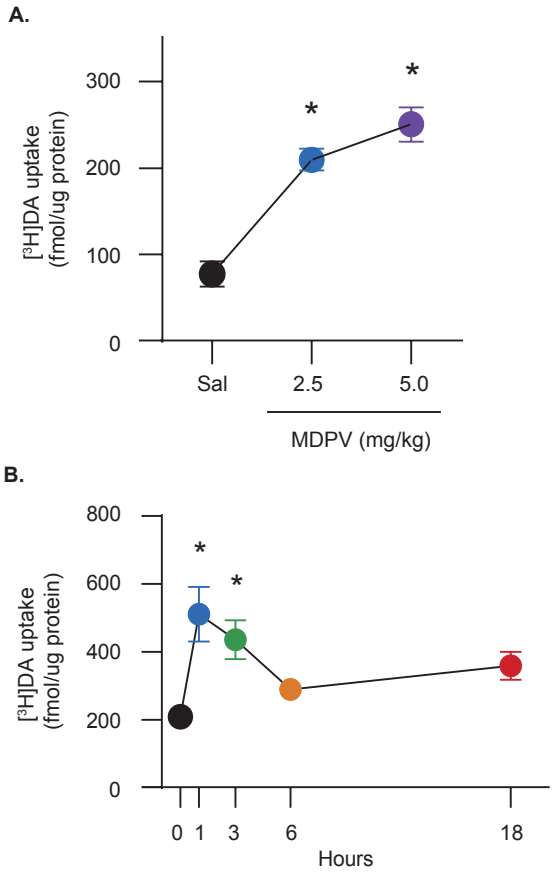

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Fig. 3

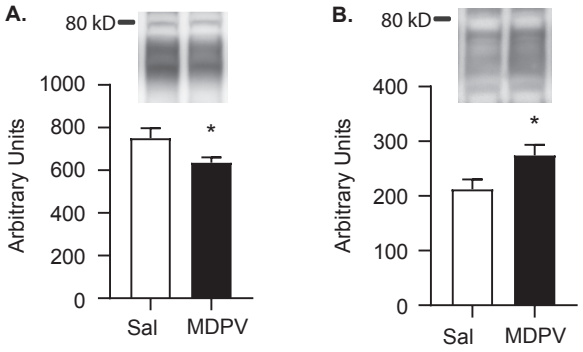

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Fig. 4

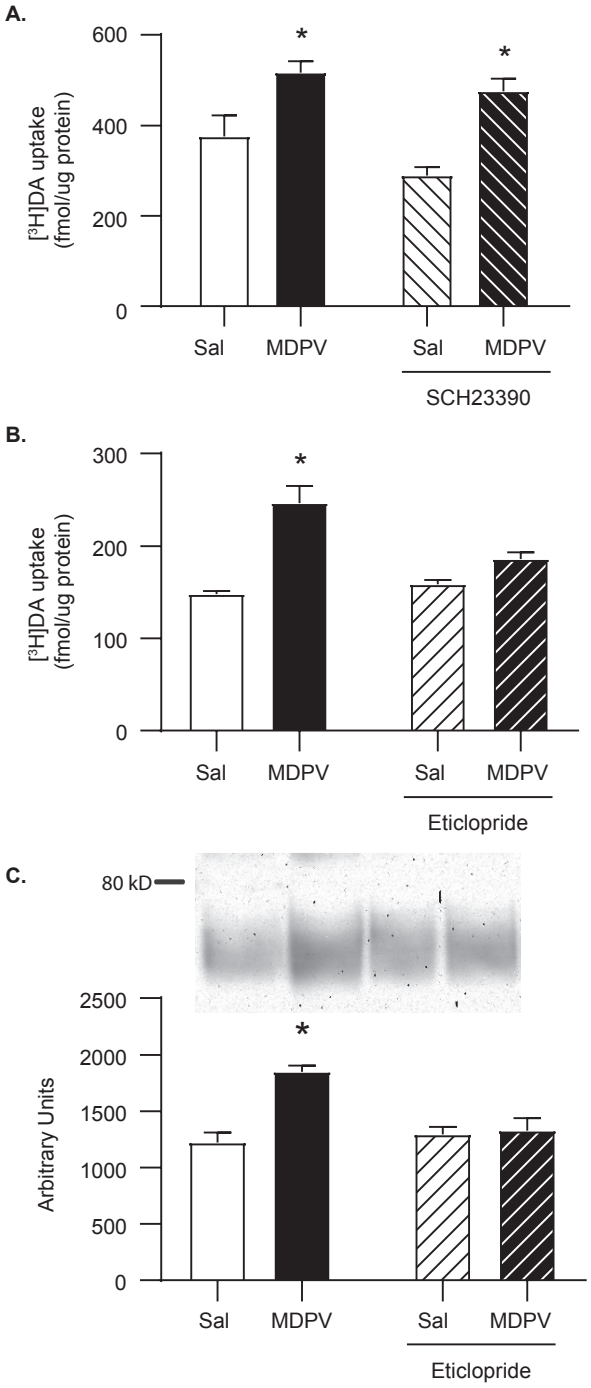

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Fig. 5

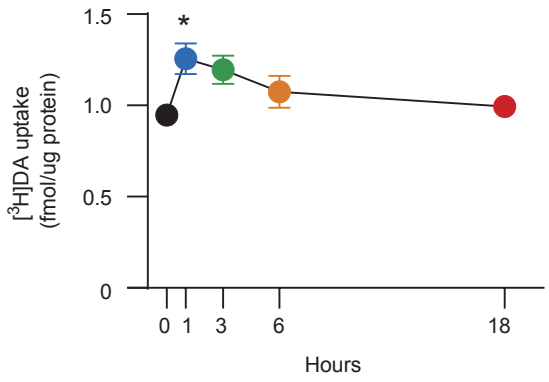

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Fig. 6

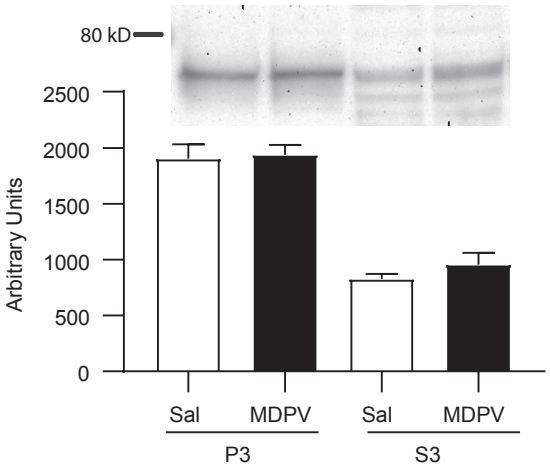

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Fig. 7

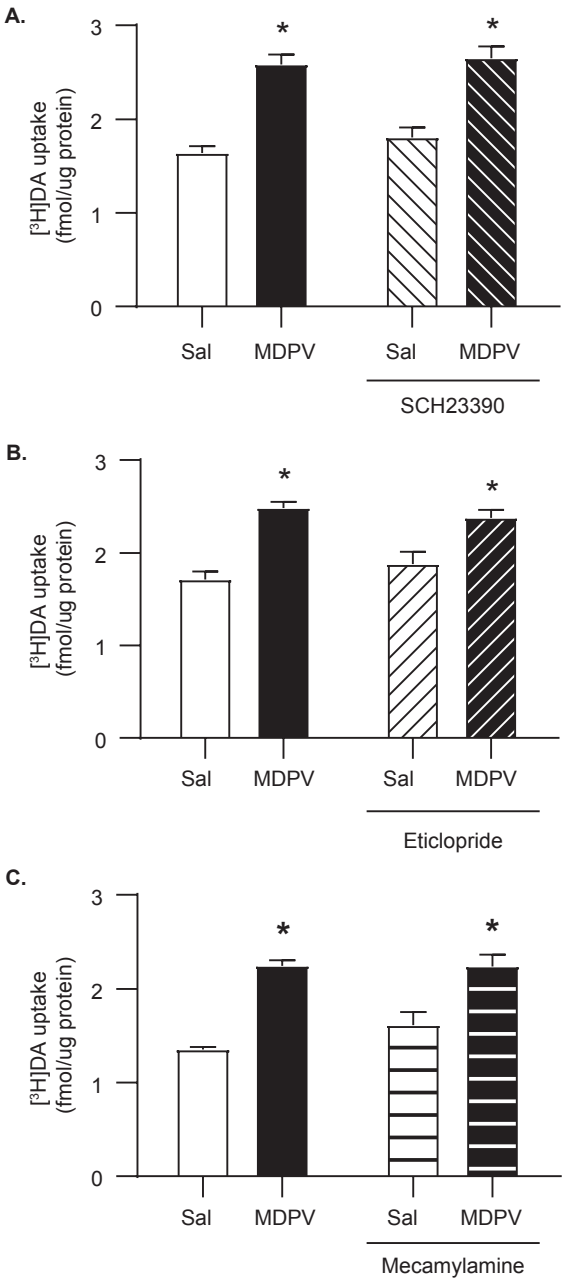

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Fig. 8