Pharmacological examination of trifluoromethyl ring-substituted methcathinone analogs

Nicholas V. Cozzi[a,c,*], **Simon D. Brandt**[b], **Paul F. Daley**[c], **John S. Partilla**[d], **Richard B. Rothman**[d], **Andreas Tulzer**[e], **Harald H. Sitte**[e], and **Michael H. Baumann**[d]

[a]Neuropharmacology Laboratory, 2695 Medical Sciences Center, Department of Cell and Regenerative Biology, University of Wisconsin School of Medicine and Public Health, 1300 University Avenue, Madison, WI 53706, USA

[b]School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK

[c]Alexander Shulgin Research Institute, 1483 Shulgin Road, Lafayette, CA 94549, USA

[d]Translational Pharmacology Section, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, 333 Cassell Drive, Suite 4500, Baltimore, MD 21224, USA

[e]Center for Physiology and Pharmacology, Institute of Pharmacology, Waehringerstrasse 13a, Vienna A-1090, Austria

Abstract

Cathinones are a class of drugs used to treat various medical conditions including depression, obesity, substance abuse, and muscle spasms. Some "designer" cathinones, such as methcathinone, mephedrone, and methylone, are used nonclinically for their stimulant or entactogenic properties. Given the recent rise in nonmedical use of designer cathinones, we aimed to improve understanding of cathinone pharmacology by investigating analogs of methcathinone with a CF_3 substituent at the 2-, 3-, or 4-position of the phenyl ring (TFMAPs). We compared the TFMAPs with methcathinone for effects on monoamine uptake transporter function *in vitro* and *in vivo*, and for effects on locomotor activity in rats. At the serotonin transporter (SERT), 3-TFMAP and 4–TFMAP were 10-fold more potent than methcathinone as uptake inhibitors and as releasing agents, but 2-TFMAP was both a weak uptake inhibitor and releaser. At the norepinephrine and dopamine transporters (NET and DAT), all TFMAP isomers were less potent than methcathinone as uptake inhibitors and releasers. *In vivo*, 4-TFMAP released 5-HT, but not dopamine, in rat nucleus accumbens and did not affect locomotor activity, whereas methcathinone increased both 5-HT and dopamine and produced locomotor stimulation. These experiments reveal that TFMAPs are substrates for the monoamine transporters and that phenyl ring substitution at the 3- or 4-position increases potency at SERT but decreases potency at NET and DAT, resulting in selectivity for SERT. The TFMAPs might have a therapeutic value for a variety of medical and psychiatric conditions and may have lower abuse liability compared to methcathinone due to their decreased DAT activity.

[*]Corresponding author at: University of Wisconsin School of Medicine and Public Health, Department of Cell and Regenerative Biology, Neuropharmacology Laboratory, 2695 Medical Sciences Center, 1300 University Avenue, Madison, WI 53706, USA. Tel.: +1 608 262 7596. cozzi@wisc.edu (N.V. Cozzi).

Keywords

Bath salts; Cathinone; Designer drug; Dopamine; Mephedrone; Methylone; Monoamine transporter; Norepinephrine; Serotonin

Introduction

Cathinones are phenylisopropylamine compounds with a carbonyl oxygen at the benzylic position of the molecule. The prototype drug in this class is cathinone itself, a naturally-occurring substance that has been used for centuries in plant form for its stimulant effects (Alles et al., 1961). Other drugs in this class include the psychostimulant methcathinone (Fig. 1), the antidepressant and stop-smoking aid bupropion, antispasmodic agents eperisone and tolperisone, and the anorectic agent diethylpropion. Cathinones with a wide range of activities have been synthesized and some may be useful as new therapeutic agents with improved adverse effect profiles for treating depression, obesity, cocaine and nicotine addiction, and as centrally acting muscle relaxants (Carroll et al., 2009, 2010; Cozzi et al., 2005; Foley and Cozzi, 2003; Lukas et al., 2010; Nisijima et al., 1998; Shiozawa et al., 1995). Identified biological targets for cathinones include acetylcholine, serotonin, dopamine, norepinephrine, histamine, and sigma-1 receptors, voltage-gated sodium and calcium ion channels, plasma membrane transporters for serotonin, norepinephrine, and dopamine (SERT, NET, and DAT, respectively) and the vesicle monoamine transporter 2 (VMAT2) (Baumann et al., 2012; Carroll et al., 2009, 2010; Cozzi and Foley, 2003; Cozzi et al., 1999, 2005, 2007; Foley and Cozzi, 2003; Fujioka and Kuriyama, 1985; Hofer et al., 2006; Kehr et al., 2011; Kocsis et al., 2005; Slemmer et al., 2000).

Lately, psychoactive “designer” cathinones, marketed as “bath salts”, have become commercially available for purchase over the Internet and in retail shops. These compounds are used outside of medical settings for personal experimentation, mood elevation, and for entertainment purposes as “legal highs”. Some of the most popular designer cathinones include methylone (3,4-methylene-dioxymethcathinone), mephedrone (4-methylmethcathinone), and 3,4-methylenedioxypyrovalerone (MDPV) (Brandt et al., 2010, 2011; EMCDDA, 2011; Kelly, 2011; Vardakou et al., 2011). The effects of these agents range from amphetamine-like stimulation (alertness, loss of fatigue, increased locomotor activity) to entactogenic effects (increased empathy, reduced fear) resembling those produced by 3,4-methylenedioxymethamphetamine (MDMA) (Mithoefer et al., 2011; Nichols et al., 1986). The emergence of designer cathinones on the recreational drug market represents an approach to scientific data mining of the existing patent literature and contemporary medicinal chemistry literature. This approach is certainly not new, but it is worth noting that a large number of cathinone derivatives have appeared very recently, especially in the European Union. An annual monitoring exercise, carried out by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), revealed that synthetic cathinones represent a key substance class, with 34 new designer cathinones reported between 2005 and 2011 (EMCDDA, 2012). In the United States, mephedrone, methylone, and MDPV were classified as Schedule I controlled substances in October 2011 (Anonymous, 2011).

We recently prepared three trifluoromethyl ring-substituted positional isomers of methcathinone (trifluoromethyl-methylami-nopropiophenones; TFMAPs) as reference standards for the analytical characterization of new cathinones for forensic and clinical purposes (Brandt et al., 2012). These new compounds incorporate a CF_3 substituent at the 2-, 3-, or 4-position of the phenyl ring (2-TFMAP, 3-TFMAP, and 4-TFMAP, respectively; Fig. 1). Here, we examine the TFMAPs *in vitro* and *in vivo* for their neurochemical and

behavioral effects. We tested the TFMAPs for their abilities to inhibit neurotransmitter uptake *via* the human transporters hSERT, hNET, and hDAT. We then tested the TFMAPs and methcathinone as releasing agents at the rat SERT (rSERT) using rat brain synaptosomes preloaded with [^{3}H]5-HT, and at the rNET and rDAT using synaptosomes preloaded with [^{3}H]1-methyl-4-phenylpyridin-1-ium ([^{3}H]MPP$^+$), a model catecholamine transporter substrate (Wall et al., 1995). Lastly, we used *in vivo* microdialysis to compare the effects of intravenously-administered 4-TFMAP and methcathinone on extracellular dopamine and 5-HT levels in nucleus accumbens, while simultaneously monitoring spontaneous locomotor activity in awake, freely-moving rats.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 300–350 g were housed under conditions of controlled temperature (22 ± 2°C) and humidity (45 ± 5%) with food and water available *ad libitum* and under a 12 h light–dark cycle. Rats were maintained in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, and procedures were carried out in accordance with the Animal Care and Use Committee of the NIDA IRP. Rats were housed in pairs upon receipt and were allowed at least two weeks for acclimatization to the vivarium conditions before being used in experiments.

2.2. Drugs and reagents

Methcathinone was prepared *via* the permanganate oxidation of racemic ephedrine as described (Zhingel et al., 1991). To synthesize the new TFMAP compounds (Fig. 1), commercially available 2-, 3-, or 4-positional isomers of trifluoromethylpropiophenones (Sigma-Aldrich, St. Louis, MO, USA) were used as precursors to prepare 2-trifluoromethylmethcathinone (2-TFMAP; 2-(methylamino)-1-(2-trifluoromethylphenyl)propan-1-one), 3-trifluoromethylmethcathinone (3-TFMAP; 2-(methylamino)-1-(3-trifluoromethylphenyl)propan-1-one), and 4-trifluoromethylmethcathinone (4-TFMAP; 2-(methylamino)-1-(4-trifluoromethylphenyl)propan-1-one), respectively. The TFMAPs were synthesized using a modification of the method of Schmidt and Eberhard (1915). This consists of side-chain α-bromination of the appropriate trifluoromethylpropiophenone with elemental bromine in dichloromethane, followed by displacement of the bromine with a 20% solution of methylamine in 1:1 water:ethanol to generate the respective trifluoromethyl-substituted methylamino-propiophenone. After work-up, all target compounds were isolated as the hydrochloride salts. Chemical purity and identity of these compounds were verified by melting-point determination, thin-layer chromatography, proton nuclear magnetic resonance, and mass spectrometry. All analytical data were consistent with the assigned structures and analytical details have been published elsewhere (Brandt et al., 2012). [^{3}H]Serotonin ([^{3}H]5-HT, specific activity=20–30 Ci/mmol), [^{3}H]dopamine ([^{3}H]DA, specific activity=27.5 Ci/mmol), and [^{3}H]norepinephrine ([^{3}H]NE, specific activity=71.7 Ci/mmol) were purchased from Perkin-Elmer (Shelton, CT, USA). [^{3}H]1-methyl-4-phenylpyridin-1-ium acetate ([^{3}H]MPP$^+$, specific activity=85 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Cell culture medium and antibiotics were obtained from Life Technologies (Gaithersburg, MD, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). Citalopram, desipramine, GBR12935, nomifensine, pargyline, buffer components, and other miscellaneous chemicals were acquired from multiple commercial sources.

2.3. Inhibition of hSERT-mediated [^{3}H]5-HT uptake into human platelets

Human platelets, which naturally express the human SERT (hSERT), were used to assess drug activity on [^{3}H]5-HT uptake. Outdated human platelets were obtained from the blood bank at the University of Wisconsin Clinical Sciences Center (Madison, WI, USA). Platelets from 5 to 10 donors were pooled, 10% dimethylsulfoxide was added, and aliquots were stored frozen at −80 °C until use. For assays, 5 mL of platelets were thawed and added to 20 mL ice-cold Krebs-Ringer-HEPES (KRH) buffer containing (mM): NaCl (124.0), KCl (2.9), $MgSO_4$ (1.3), KH_2PO_4 (1.2), $CaCl_2$ (2.4), D-glucose (5.2), HEPES (25.0), sodium ascorbate (0.1), pargyline (0.1), pH=7.4. The platelet suspension was subjected to centrifugation (4340 ×*g*, 4 °C, 10 min) and the supernatant was discarded. The pellet was washed twice by resuspension in KRH followed by centrifugation. The final pellet was suspended in 20 mL ice-cold KRH and stored on ice until use. The ability of platelets to accumulate [^{3}H]5-HT was measured in the absence and presence of various concentrations of TFMAP isomers and methcathinone as follows: a 400 μL aliquot of the platelet suspension was added to glass tubes containing 50 μL test drugs (dissolved in KRH) or 50 μL KRH (for total and nonspecific determinations). The assay tubes were preincubated in a 37 °C shaking water bath for 5 min. The tubes were then returned to the ice bath and chilled for 15 min. [^{3}H]5-HT was added (50 μL of stock solution; final concentration, 10 nM), giving a total incubation volume of 500 μL. All tubes except nonspecific tubes were returned to the 37 °C shaking water bath for 10 min to initiate neurotransmitter uptake. Uptake was terminated by chilling the test tubes in the ice bath. The assay tubes were vacuum filtered through glass fiber filters (Whatman GF/B) pretreated with 0.1% polyethyleneimine) using a Brandel 24-channel cell harvester (Brandel, Gaithersberg, MD, USA). Filters were washed with 2 × 3 mL ice-cold 300 mM NaCl, allowed to dry briefly under vacuum, then placed in liquid scintillation vials. Scintillation cocktail (3 mL) was added and the vials were sealed, vortexed, and allowed to stand overnight. Filters were assessed for tritium content by liquid scintillation counting (Packard Tri-Carb 1600; Perkin-Elmer, Waltham, MA, USA). Specific uptake was defined as uptake at 37 °C minus uptake at 0 °C in the absence of drugs. Under these conditions, specific [^{3}H]5-HT uptake was typically greater than 90%. The IC_{50} ±S.E.M. for each test drug was determined from at least three displacement curves per test drug, using six drug concentrations, each run in triplicate. Data from each experiment were transformed from dpm to % specific uptake and fitted to a four-parameter logistic curve to obtain an IC_{50} value. Curve fitting was accomplished using commercial computer software (GraphPad Prism; GraphPad Software, La Jolla, CA, USA).

2.4. Inhibition of hDAT-mediated [^{3}H]DA uptake into transfected cells

HEK293 cells stably expressing the human dopamine transporter (HEK293-hDAT) were maintained in a humidified atmosphere (5% CO_2 in air) in selective culture medium: Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL geneticin). Uptake inhibition of [^{3}H]DA by TFMAP isomers was measured essentially as described recently (Bulling et al., 2012). In brief, HEK293-hDAT cells were seeded onto 48-well plates precoated with poly-D-lysine (5×10^4 cells/well) 24 h prior to the experiment. For assays, each well was washed with 500 μL of Krebs-HEPES buffer (KHB) containing (mM): NaCl (1 3 0), KH_2PO_4 (1.3), $CaCl_2$ (1.5), $MgSO_4$ (0.5), HEPES (10), pH=7.4. Cells were pre-incubated with various concentrations of individual TFMAP isomers in KHB at room temperature for 15 min, then uptake was assessed by adding [^{3}H]DA (final concentration, 100 nM) for 1 min. Nonspecific uptake was defined with 10 μM mazindol. The assay was ended by discarding the incubation buffer and washing the cells with 0.5 mL ice-cold KHB. Finally, cells were lysed with 0.5 mL of 1% sodium dodecyl sulfate (SDS) and the well contents were transferred into 2 mL of scintillation fluid (Rotiszint eco plus LSC, Art.

0016.3) and counted in a Packard 2300TR Tri-Carb Liquid Scintillation Analyzer (Perkin-Elmer, Waltham, MA, USA). Data were processed as described in Section 2.3.

2.5. Inhibition of hNET-mediated [^{3}H]NE uptake into transfected cells

C6 glioma cells stably expressing the human norepinephrine transporter (C6-hNET) were maintained in a humidified atmosphere (5% CO_2 in air) in selective DMEM as described for HEK293-hDAT cells. For assays, 3 × 100 mm dishes of confluent cells were washed with phosphate buffered saline, pH=7.1. The cells were then treated with trypsin/EDTA and split into 4 × 6-well (35 mm) plates in 2 mL/well culture medium minus geneticin. The cells were allowed to grow overnight and were used for uptake assays the following day. The ability of test drugs to inhibit [^{3}H]NE uptake was measured as follows: the DMEM was aspirated from the 6-well plates containing confluent C6-hNET cells. The cells were washed with 2 × 2 mL 37 °C KRH, then 490 μL 37 °C KRH was added to each well. This was followed by the addition of 5 μL KRH (for total determinations), 5 μL of 10 mM desipramine hydrochloride stock solution (for nonspecific determinations; final concentration, 100 μM), or 5 μL of test drug solution. The plates were preincubated at 37 °C for 15 min, then 5 μL of [^{3}H]NE (final concentration, 20 nM) was added to each well to initiate uptake. Uptake was allowed to proceed at 37 °C for 20 min, then the incubation buffer was discarded and the cells were washed with 3 × 2 mL ice-cold KRH. The cells were lysed in 500 μL 37 °C 1% SDS, then the solubilized well contents were transferred to liquid scintillation vials containing 3 mL scintillation cocktail for radioactivity measurement (Packard Tri-Carb 1600; Perkin-Elmer, Waltham, MA, USA). Data were processed as described in Section 2.3.

2.6. Drug-evoked release via rSERT, rNET, and rDAT

The abilities of TFMAP isomers and methcathinone to evoke release *via* the rat monoamine transporters (rSERT, rNET, and rDAT) were examined in rat brain synaptosomes essentially as described (Rothman et al., 2003). Rats were euthanized with CO_2, decapitated, and the brains were rapidly removed and dissected on ice. Rat whole brain minus cerebellum (for rSERT and rNET release assays) or rat striatum (for rDAT release assays) was homogenized in ice-cold 10% sucrose containing 1 μM reserpine. For rSERT release assays, 100 nM nomifensine and GBR12935 were added to the sucrose solution to block uptake of [^{3}H]5-HT into NE and DA terminals. For the rNET release assays, 100 nM GBR12935 and citalopram were added to block [^{3}H]MPP$^+$ uptake into DA and 5-HT terminals. For the rDAT release assays, 100 nM desipramine and citalopram were added to block [^{3}H]MPP$^+$ uptake into NE and 5-HT terminals. Tissues were homogenized with a Potter-Elvehjem homogenizer (12 strokes), then the homogenates were subjected to centrifugation at 1000 ×*g* for 10 min at 4 °C. The pellet was discarded and the supernatant containing the synaptosomes was retained on ice until use. Synaptosomes were preloaded with 5 nM [^{3}H]5-HT to test for release *via* rSERT or with 5 nM [^{3}H]MPP$^+$ to test for release *via* rNET and rDAT. To load, synaptosomes were incubated in a polypropylene beaker with stirring at 25 °C for 60 min with 5 nM [^{3}H]5-HT or [^{3}H]MPP$^+$ in Krebs-phosphate buffer containing (mM): NaCl (154.4), KCl (2.9), $CaCl_2$ (1.1), $MgCl_2$ (0.83), D-glucose (5), sodium ascorbate (5.7), pargyline (0.05), reserpine (0.001), pH=7.4. The appropriate combination of transporter blockers (citalopram, desipramine, nomifensine, or GBR12935; 100 nM) was present depending on the transporter under study. After incubation to steady state, 850 μL of preloaded synaptosomes were added to 12 × 75 mm polystyrene test tubes or 96-well polypropylene plates that contained 150 μL test drug in uptake buffer plus 1 mg/mL bovine serum albumin. Incubations were maintained at 25 °C. After 5 min (rSERT assays) or 30 min (rNET and rDAT assays), the release reaction was terminated by dilution with 4 mL wash buffer (10 mM Tris–HCl containing 0.9% NaCl at 25 °C, pH=7.4) followed by rapid vacuum filtration through glass fiber filters (Whatman GF/B) using a cell harvester (Brandel, Gaithersberg, MD, USA). Filters were rinsed twice with 4 mL wash buffer, dried

briefly under vacuum, then transferred to 24-well plates. Scintillation cocktail (0.6 mL) was added to each well, and after an overnight incubation, the retained tritium was assessed at 40% efficiency by liquid scintillation counting. EC_{50} values for transporter assays were determined using the nonlinear least-squares curve fitting program MLAB-PC (Civilized Software, Bethesda, MD, USA).

2.7. In vivo microdialysis of nucleus accumbens

For *in vivo* microdialysis experiments, rats received sodium pentobarbital (60 mg/kg, i.p.) for surgical anesthesia. Indwelling catheters made of Silastic Medical Grade tubing (Dow Corning, Midland, MI, USA) were implanted into the right jugular vein. Immediately thereafter, intracerebral guide cannulae (CMA 12; CMA/Microdialysis, Acton, MA, USA) were implanted above the nucleus accumbens, according to stereotaxic coordinates: 1.6 mm lateral and 1.6 mm anterior to bregma, and 6.0 mm below the surface of the dura. Guide cannulae were secured to the skull using stainless steel anchor screws and dental acrylic. Animals were individually housed post-operatively and allowed 7–10 days for recovery. *In vivo* microdialysis sampling was carried out as described, with minor modifications (Baumann et al., 2011). On the evening before an experiment, rats were moved to the testing room. A plastic collar was placed around the neck of each rat, CMA/12 dialysis probes (probe exchange surface was 2 × 0.5 mm) were inserted into the previously implanted guide cannulae, and extension tubes were attached to the indwelling jugular catheters. Each rat was placed into its own locomotor activity monitor (Coulbourn Instruments, Allentown, PA, USA) and connected to a tethering system that allowed movement within the activity monitor. Probes were perfused overnight with artificial cerebrospinal fluid (Harvard Bioscience, Holliston, MA, USA) containing the following ion concentrations (mM): Na (150), K (3), Ca (1.4), Mg (0.8), P (1.0), Cl (155) and pumped at a flow rate of 0.6 μL/min. The next morning, dialysate samples were collected at 20 min intervals. Samples were immediately assayed for dopamine and 5-HT by high-pressure liquid chromatography with electrochemical detection (HPLC-ECD) as described below. Rats were randomly assigned to control or drug groups. After three stable baseline samples were obtained, rats received two sequential intravenous injections of either saline (1 mL/kg; controls) or a test drug at time zero, followed by another injection 60 min later. Drugs were dissolved in saline to a concentration that allowed dosing volumes of 1 mL/kg to deliver the appropriate test dose. Methcathinone was administered at doses of 0.3 mg/kg and 1.0 mg/kg while 4-TFMAP was administered at 1.0 mg/kg and 3.0 mg/kg. Microdialysis samples were collected every 20 min throughout the post-injection period for 120 min. At the end of the experiments, rats were euthanized with CO_2 and decapitated. Brain sections were examined to verify placement of microdialysis probe tips at the nucleus accumbens. Only those rats with correct placements were included in data analyses, resulting in six rats in the saline control group and seven rats in each of the methcathinone- and 4-TFMAP-treated groups.

2.8. Drug effects on horizontal locomotor activity

Locomotor activity was monitored during microdialysis testing as previously described (Baumann et al., 2011). During the overnight acclimation period and while undergoing microdialysis, each rat was housed within a cubic Plexiglass arena (43 cm × 43 cm × 43 cm) that was equipped with an activity monitoring system (Tru Scan; Coulbourn Instruments, Allentown, PA, USA). A sensor ring, lined with photobeams spaced 2.54 cm apart, was positioned in the horizontal plane to allow for real-time monitoring of motor parameters. Activity was monitored in 20-min bins during microdialysis testing, beginning 60 min before intravenous drug injections and continuing for 120 min thereafter. Horizontal locomotor activity was defined as the total distance traveled (cm) in the horizontal plane as measured by photobeam breaks.

2.9. High-pressure liquid chromatography with electrochemical detection (HPLC-ECD) of dialysate dopamine and 5-HT

Aliquots of dialysate (5 μL) were analyzed by HPLC-ECD by injection onto a microbore C18 column that was coupled to a Model LC-4C amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN, USA) with a glassy carbon electrode set at a potential of +650 mV relative to the Ag/AgCl reference. Aqueous mobile phase (150 mM monochloroacetic acid, 150 mM sodium hydroxide, 2.5 mM sodium octanesulfonic acid, 250 μM disodium EDTA, 6% methanol, 6% acetonitrile, pH=5.3) was pumped at 60 μL/min using a Model 260D syringe pump (ISCO, Lincoln, NE, USA). Chromatographic data were acquired on-line and exported to an Empower software system (Waters Associates, Milford, MA, USA) for peak amplification, integration, and analysis. A monoamine standard mix containing dopamine, 5-HT, and their respective acid metabolites was injected before and after the experiment to insure validity of constituent retention times. Peak heights of unknowns were compared to peak heights of standards. The lower limit of assay sensitivity (3 × baseline noise) was 50 fg/5 μL sample.

2.10. Data analysis

All values are expressed as the mean ±S.E.M. Discrete best-fit IC_{50} or EC_{50} values, obtained from nonlinear regression analysis of the *in vitro* uptake inhibition or release data from three to nine separate experiments, were treated as individual "n" and statistically processed to generate a mean value ±S.E.M. Pairwise comparisons of IC_{50} and EC_{50} values for the various drugs were made using one-way ANOVA followed by the Newman-Keuls post-test. Dialysate neurotransmitter levels and behavioral data from individual rats were normalized to percent control values (i.e., % basal) using the averaged raw data from three preinjection time points for each rat as basal; each rat thus served as its own control. Normalized group data in the microdialysis experiments and locomotor activity assay were then compared to vehicle controls using two-way ANOVA with drug and time as the two factors. When significant differences were observed, a Bonferroni post-test was used to compare treatment effects at each time point. $P < 0.05$ was considered significant for all statistical tests.

3. Results

Uptake of radiolabeled [^{3}H]5-HT into human platelets, [^{3}H]DA into HEK293-hDAT cells, and [^{3}H]NE into C6-hNET cells was robust and was typically greater than 90% specific. All of the test drugs inhibited accumulation *via* the hSERT, hDAT, and hNET. Uptake inhibition curves for TFMAP isomers at hSERT, hNET, and hDAT are shown in Fig. 2. All inhibition curves had slope coefficients of unity, indicating that the test drugs interacted with a single site on the transporter proteins. IC_{50} values ±S.E.M. for all drugs and transporter systems tested are summarized in Table 1. The 4-trifluoromethylmethcathinone analog (4-TFMAP) was the most potent hSERT uptake inhibitor in the series whereas 2-TFMAP was the weakest. At the catecholamine transporters, all the TFMAP isomers were less potent than methcathinone as uptake inhibitors and exhibited especially low potency at the hDAT.

As *in vitro* releasing agents, all of the TFMAP isomers and methcathinone were active at the rSERT, rNET, and rDAT. Release curves are depicted in Fig. 3 and EC_{50} values are listed in Table 1. 4-TFMAP was the most potent releaser at the rSERT, with an EC_{50} for [^{3}H]5-HT release of 186 nM, while methcathinone was the most potent releaser at the rNET and rDAT, with an EC_{50} for [^{3}H]MPP^+ release of 20 nM at both transporters. Among the TFMAP isomers, 4-TFMAP was the most potent releaser at rNET, with an EC_{50} for [^{3}H]MPP^+ release of 867 nM, but the 3-TFMAP isomer was the most potent [^{3}H]MPP^+ releaser at the rDAT with an EC_{50} of 610 nM (Table 1).

Methcathinone produced large dose-dependent increases in extracellular levels of 5-HT and dopamine in rat nucleus accumbens (Fig. 4). There was also an increase in horizontal locomotor activity that corresponded temporally to the increase in dopamine levels but not to 5-HT levels (Fig. 4). In contrast, 4-TFMAP increased extracellular 5-HT, but had no effect on dopamine or locomotor activity (Fig. 5). 4-TFMAP had approximately 1/3 the potency of methcathinone on dialysate 5-HT.

4. Discussion

Cathinones include drugs with important therapeutic applications such as pharmacotherapy for depression, obesity, muscle spasms, and tobacco smoking-cessation programs. Some designer cathinones have also appeared outside of medical circles in recent years, for purchase as “legal highs” or “bath salts”. A number of biological targets for cathionones have been identified, but incomplete data and complex structure-activity relationships for cathinones that do not always match those of amphetamine analogs (Dal Cason et al., 1997) necessitate the pharmacological characterization of new cathinones on a case-by-case basis to discover their psychological and physiological effects and to predict adverse drug reactions. This study aims to contribute to these efforts.

A major finding was that 3- or 4-CF_3 substitution on the methcathinone phenyl ring enhanced *in vitro* uptake inhibition and releasing activity at SERT but decreased activity at NET and DAT, regardless of whether the transporter was derived from human or rat (Table 1). On the other hand, 2-CF_3 ring-substitution decreased activity at all of these transporters. These results parallel earlier studies where, compared to cathinones lacking phenyl ring substituents, compounds with 3- or 4-position substituents were 30- to 60-fold more potent as SERT uptake inhibitors (Cozzi et al., 1999, 2005, 2007; Foley, 2002). For example, the most potent SERT inhibitors in these earlier studies were 3-bromomethcathinone (IC_{50}=2.0 μM), 4-bromomethcathinone (IC_{50}=1.8 μM), and 4-iodomethcathinone (IC_{50}=1.0 μM). Similarly, in the present study, it is the TFMAPs with ring substitutions at the 3- or 4-positions which are the most potent SERT inhibitors, with IC_{50} values in the low micromolar range (11.3 and 4.5 μM, respectively; Table 1). Both 3-TFMAP and 4-TFMAP are also more potent than methcathinone (10- to 20-fold) as releasing agents (Table 1). Again, 2-TFMAP was considerably weaker than the other compounds in this assay, which may reflect steric or electrostatic interference within the SERT substrate binding site for this particular isomer.

When compared to methcathinone, all CF_3 ring-substituted isomers had decreased potency in uptake inhibition and releasing activity at the catecholamine transporters. At the NET, both uptake inhibition (6- to 19-fold) and releasing activity (43- to 136-fold) were weaker (Fig. 3 and Table 1). The decreased potency (~400- to 1900-fold) was especially noticeable for [^{3}H]DA uptake inhibition *via* the DAT, where the TFMAPs had IC_{50} values of several hundred micromolar (Fig. 2 and Table 1). The TFMAP isomers were also less potent at stimulating [^{3}H]MPP^+ release (30- to 400-fold) than methcathinone at the DAT. Clearly, the presence of the ring trifluoromethyl group has a large deleterious effect on catecholamine transporter uptake inhibition and releasing potency. These results match earlier studies in which ring-substituted amphetamines and cathinones such as MDMA, *para*-chloroamphetamine, *meta*- and *para*-fluoroamphetamine, *meta*- and *para*-methylamphetamine, mephedrone, and methylone were always less potent as uptake inhibitors and releasers at the catecholamine transporters than drugs such as amphetamine, methamphetamine, and methcathinone, which do not contain ring-substituents (Baumann et al., 2011, 2012; Cozzi et al., 1999; Wall et al., 1995). These data predict that the TFMAPs should have fewer psychostimulant effects and lower abuse liability relative to methcathinone in behavioral assays.

Based on the *in vitro* data, we selected 4-TFMAP for *in vivo* testing and comparison to methcathinone. The SERT selectivity and 5-HT releasing action displayed by 4-TFMAP *in vitro* was confirmed by the drug's effect *in vivo*. As anticipated, 4-TFMAP produced a dose-related increase in 5-HT efflux, but did not stimulate dopamine release from cells in the nucleus accumbens, nor did it stimulate locomotor activity (Fig. 5). These results are similar to those from an earlier study in which both 3-bromomethcathinone and 4-bromomethcathinone were selective for SERT *in vitro* and produced fewer locomotor effects *in vivo* compared to methcathinone (Foley and Cozzi, 2003). These effects contrast with the response to methcathinone in the present study (Fig. 4) and with the response to the closely-related cathinone, mephedrone, recently reported by ourselves (Baumann et al., 2012) and by Kehr et al. (2011). In those studies, mephedrone stimulated both 5-HT and DA release in nucleus accumbens and produced dose-related locomotor stimulation at 1 and 3 mg/kg.

Early studies of methcathinone pharmacology demonstrated its ability to release dopamine from rat caudate nucleus (Glennon et al., 1987). We later extended these findings by showing that racemic [^{3}H]methcathinone is a SERT substrate with a K_M of 244 nM at the human SERT (Cozzi and Foley, 2003) and that in rat brain tissue, the *S*-isomer of methcathinone is a potent, nanomolar-potency releaser at NET and DAT, with lower, micromolar potency as a SERT releaser (Rothman et al., 2003). The *in vitro* results in the present study compare well with and extend these earlier findings by showing that methcathinone is a potent (nanomolar) and specific releaser at the catecholamine transporters, with lesser potency as a SERT substrate (Fig. 3 and Table 1). Methcathinone-induced [^{3}H]MPP$^+$ release *via* the catecholamine transporters had an EC_{50} of 20 nM but the drug was almost 200-fold less potent as a SERT releaser. The response to intravenous methcathinone in the *in vivo* microdialysis experiments (Fig. 4) parallels the *in vitro* results, albeit not with the 200-fold observed separation of potencies between dopamine and 5-HT release *in vitro* (Table 1); *in vivo*, methcathinone was only about twice as potent as a dopamine releasing agent in nucleus accumbens as it was as a 5-HT releaser. Here we note a divergence between the *in vitro* and *in vivo* results with methcathinone: while this drug is very selective for the catecholamine transporters *in vitro*, it elevates both dopamine and 5-HT *in vivo*. We have observed this phenomenon before with methamphetamine, a drug that is selective for NET and DAT *in vitro* but causes parallel increases in extracellular dopamine and 5-HT *in vivo* (Rothman and Baumann, 2003). There are at least two factors that contribute to this outcome: (1) the *in vitro* assays are controlled situations in which conditions for selectivity at a single transporter are optimized by adding selective blockers to prevent interactions at other transporters and (2) when administered *in vivo*, drugs like methamphetamine and methcathinone rapidly increase extracellular dopamine to sufficiently high concentrations such that dopamine interacts with, and becomes a substrate for, the SERT, thereby causing 5-HT release *via* reverse transport. A similar effect does not occur with SERT-selective drugs in the nucleus accumbens because here, 5-HT concentrations are at least 10-fold lower than dopamine concentrations. Associated with the rise in extracellular nucleus accumbens dopamine was a matching increase in spontaneous locomotor activity, in agreement with earlier studies (Baumann et al., 2008, 2011).

In summary, the present data indicate that all three isomeric TFMAPs are substrates for the monoamine transporters and that phenyl ring substitution at the 3- or 4-positions dramatically increases potency at the SERT compared to DAT and NET, resulting in selectivity for SERT *vs.* the catecholamine transporters. On the other hand, the TFMAPs are much weaker than methcathinone both as uptake inhibitors and releasers at the NET and DAT (Fig. 3 and Table 1). The ability of these substances to release preloaded [^{3}H]5-HT and [^{3}H]MPP$^+$ is an indicator that these drugs, like methcathinone, are substrates for the uptake transporters and differentiates them from pure uptake inhibitors such as cocaine and uptake-blocking antidepressants (Blakely et al., 1997; Ritz et al., 1990). These data suggest that the

TFMAPs might provide access to drugs with therapeutic value while having greatly reduced cardiovascular, locomotor, and psychostimulant effects, as well as diminished abuse liability in humans, compared to methcathinone. Furthermore, the trifluoromethyl group is metabolically stable (Hiyama, 2000), a property that could prove advantageous in developing potent, extended-action drugs. The 3- and 4-CF_3-substituted isomers may be worth investigating as potential antidepressants or antiobesity agents with an improved adverse reaction profile compared to existing cathinones in therapeutic use, such as bupropion and diethylpropion.

Acknowledgments

This work was supported by funding from the National Institute on Drug Abuse (Grant DA017675 to NVC), the Austrian Science Foundation (grant SFB3506-B11 to HHS), and by the Intramural Research Program of the National Institutes of Health, National Institute on Drug Abuse. We thank Mr. Darin Evans for excellent technical assistance.

References

Alles GA, Fairchild MD, Jensen M. Chemical pharmacology of Catha edulis. J Med Pharm Chem. 1961; 3:323–352. [PubMed: 13860593]

Anonymous. Schedules of controlled substances: temporary placement of three synthetic cathinones into schedule I. Fed Regist. 2011; 76:65371–65375. [PubMed: 22016903]

Baumann MH, Ayestas MA, Partilla JS, Sink JR, Shulgin AT, Daley PF, Brandt SD, Rothman RB, Ruoho AE, Cozzi NV. The designer methcathinone analogs, mephedrone and methylone, are substrates for monoamine transporters in brain tissue. Neuropsychopharmacology. 2012; 37:1192–1203. [PubMed: 22169943]

Baumann MH, Clark RD, Rothman RB. Locomotor stimulation produced by 3,4-methylenedioxymethamphetamine (MDMA) is correlated with dialysate levels of serotonin and dopamine in rat brain. Pharmacol Biochem Behav. 2008; 90:208–217. [PubMed: 18403002]

Baumann MH, Clark RD, Woolverton WL, Wee S, Blough BE, Rothman RB. *In vivo* effects of amphetamine analogs reveal evidence for serotonergic inhibition of mesolimbic dopamine transmission in the rat. J Pharmacol Exp Ther. 2011; 337:218–225. [PubMed: 21228061]

Blakely, RD.; Ramamoorthy, S.; Qian, Y.; Schroeter, S.; Bradley, C. Regulation of antidepressant-sensitive serotonin transporters. In: Reith, ME., editor. Neurotransmitter Transporters: Structure, Function, and Regulation. Humana Press: Totowa, NJ; 1997. p. 29-72.

Brandt SD, Daley PF, Cozzi NV. Analytical characterization of three trifluoromethyl-substituted methcathinone isomers. Drug Test Anal. 2012; 4:525–529. [PubMed: 22362591]

Brandt SD, Freeman S, Sumnall HR, Measham F, Cole J. Analysis of NRG 'legal highs' in the UK: identification and formation of novel cathinones. Drug Test Anal. 2011; 3:569–575. [PubMed: 21960541]

Brandt SD, Sumnall HR, Measham F, Cole J. Analyses of second-generation 'legal highs' in the UK: initial findings. Drug Test Anal. 2010; 2:377–382. [PubMed: 20687197]

Bulling S, Schicker K, Zhang YW, Steinkellner T, Stockner T, Gruber CW, Boehm S, Freissmuth M, Rudnick G, Sitte HH, Sandtner W. The mechanistic basis for noncompetitive ibogaine inhibition of serotonin and dopamine transporters. J Biol Chem. 2012; 287:18524–18534. [PubMed: 22451652]

Carroll FI, Blough BE, Abraham P, Mills AC, Holleman JA, Wolckenhauer SA, Decker AM, Landavazo A, McElroy KT, Navarro HA, Gatch MB, Forster MJ. Synthesis and biological evaluation of bupropion analogues as potential pharmacotherapies for cocaine addiction. J Med Chem. 2009; 52:6768–6781. [PubMed: 19821577]

Carroll FI, Blough BE, Mascarella SW, Navarro HA, Eaton JB, Lukas RJ, Damaj MI. Synthesis and biological evaluation of bupropion analogues as potential pharmacotherapies for smoking cessation. J Med Chem. 2010; 53:2204–2014. [PubMed: 20158204]

Cozzi NV, Foley KF. Methcathinone is a substrate for the serotonin uptake transporter. Pharmacol Toxicol. 2003; 93:219–225. [PubMed: 14629733]

Cozzi NV, Foley KF, Fontanilla D, Gopalakrishnan A, Ruoho AE. A novel amphetamine-related photoaffinity probe. FASEB J. 2007; 21:715.2.

Cozzi NV, Marona-Lewicka D, Nichols DE, Gokin A, Foley KF. Novel aminopropiophenones as antiobesity agents. Soc Neurosci Abs. 2005; 31:533.5.

Cozzi NV, Sievert MK, Shulgin AT, Jacob P III, Ruoho AE. Inhibition of plasma membrane monoamine transporters by β-ketoamphetamines. Eur J Pharmacol. 1999; 381:63–69. [PubMed: 10528135]

Dal Cason TA, Young R, Glennon RA. Cathinone: an investigation of several N-alkyl and methylenedioxy–substituted analogs. Pharmacol Biochem Behav. 1997; 58:1109–1116. [PubMed: 9408221]

EMCDDA. Report on the Risk Assessment of Mephedrone in the Framework of the Council Decision on New Psychoactive Substances. Publications Office of the European Union, European Monitoring Centre for Drugs and Drug Addiction; Lisbon: 2011. p. 200

EMCDDA. EMCDDA-Europol 2011 Annual Report on the Implementation of Council Decision 2005/387/JHA. European Monitoring Centre for Drugs and Drug Addiction & EUROPOL; Lisbon: 2012. p. 29

Foley, KF. Ph D Thesis. East Carolina University; Greenville, NC: 2002. Aminopropiophenones at the Norepinephrine Transporter: Structure-activity Relationships and Behavioral Effects of Methcathinone Analogs; p. 130

Foley KF, Cozzi NV. Novel aminopropiophenones as potential antidepressants. Drug Dev Res. 2003; 60:252–260.

Fujioka M, Kuriyama H. Eperisone, an antispastic agent, possesses vasodilating actions on the guinea-pig basilar artery. J Pharmacol Exp Ther. 1985; 235:757–763. [PubMed: 3935775]

Glennon RA, Yousif M, Naiman N, Kalix P. Methcathinone: a new and potent amphetamine-like agent. Pharmacol Biochem Behav. 1987; 26:547–551. [PubMed: 3575369]

Hiyama, T. Organofluorine Compounds: Chemistry and Applications. Springer-Verlag; Berlin: 2000. p. 284

Hofer D, Lohberger B, Steinecker B, Schmidt K, Quasthoff S, Schreibmayer W. A comparative study of the action of tolperisone on seven different voltage dependent sodium channel isoforms. Eur J Pharmacol. 2006; 538:5–14. [PubMed: 16650844]

Kehr J, Ichinose F, Yoshitake S, Goiny M, Sievertsson T, Nyberg F, Yoshitake T. Mephedrone, compared to MDMA (ecstasy) and amphetamine, rapidly increases both dopamine and serotonin levels in nucleus accumbens of awake rats. Br J Pharmacol. 2011; 164:1949–1958. [PubMed: 21615721]

Kelly JP. Cathinone derivatives: a review of their chemistry, pharmacology and toxicology. Drug Test Anal. 2011; 3:439–453. [PubMed: 21755607]

Kocsis P, Farkas S, Fodor L, Bielik N, Than M, Kolok S, Gere A, Csejtei M, Tarnawa I. Tolperisone-type drugs inhibit spinal reflexes via blockade of voltage-gated sodium and calcium channels. J Pharmacol Exp Ther. 2005; 315:1237–1246. [PubMed: 16126840]

Lukas RJ, Muresan AZ, Damaj MI, Blough BE, Huang X, Navarro HA, Mascarella SW, Eaton JB, Marxer-Miller SK, Carroll FI. Synthesis and characterization of *in vitro* and *in vivo* profiles of hydroxybupropion analogues: aids to smoking cessation. J Med Chem. 2010; 53:4731–4748. [PubMed: 20509659]

Mithoefer MC, Wagner MT, Mithoefer AT, Jerome L, Doblin R. The safety and efficacy of (+/−) 3,4-methylenedioxymethamphetamine-assisted psychotherapy in subjects with chronic, treatment-resistant posttraumatic stress disorder: the first randomized controlled pilot study. J Psychopharmacol. 2011; 25:439–452. [PubMed: 20643699]

Nichols DE, Hoffman AJ, Oberlender RA, Jacob P 3rd, Shulgin AT. Derivatives of 1-(1,3-benzodioxol-5-yl)-2-butanamine: representatives of a novel therapeutic class. J Med Chem. 1986; 29:2009–2015. [PubMed: 3761319]

Nisijima K, Shimizu M, Ishiguro T. Treatment of tardive dystonia with an antispastic agent. Acta Psychiatry Scand. 1998; 98:341–343.

Ritz MC, Cone EJ, Kuhar MJ. Cocaine inhibition of ligand binding at dopamine, norepinephrine and serotonin transporters: a structure–activity study. Life Sci. 1990; 46:635–645. [PubMed: 2308472]

Rothman RB, Baumann MH. Monoamine transporters and psychostimulant drugs. Eur J Pharmacol. 2003; 479:23–40. [PubMed: 14612135]

Rothman RB, Vu N, Partilla JS, Roth BL, Hufeisen SJ, Compton-Toth BA, Birkes J, Young R, Glennon RA. *In vitro* characterization of ephedrine-related stereoisomers at biogenic amine transporters and the receptorome reveals selective actions as norepinephrine transporter substrates. J Pharmacol Exp Ther. 2003; 307:138–145. [PubMed: 12954796]

Schmidt E, Eberhard A. Ueber das ephedrin und verwandte verbindungen. Arch Pharm (Weinheim). 1915; 253:62–91.

Shiozawa A, Narita K, Izumi G, Kurashige S, Sakitama K, Ishikawa M. Synthesis and activity of 2-methyl-3-aminopropiophenones as centrally acting muscle relaxants. Eur J Med Chem. 1995; 30:85–94.

Slemmer JE, Martin BR, Damaj MI. Bupropion is a nicotinic antagonist. J Pharmacol Exp Ther. 2000; 295:321–327. [PubMed: 10991997]

Vardakou I, Pistos C, Spiliopoulou C. Drugs for youth via Internet and the example of mephedrone. Toxicol Lett. 2011; 201:191–195. [PubMed: 21187132]

Wall SC, Gu H, Rudnick G. Biogenic amine flux mediated by cloned transporters stably expressed in cultured cell lines: amphetamine specificity for inhibition and efflux. Mol Pharmacol. 1995; 47:544–550. [PubMed: 7700252]

Zhingel KY, Dovensky W, Crossman A, Allen A. Ephedrone: 2-methylamino-1-phenylpropan-1-one (Jeff). J Forensic Sci. 1991; 36:915–920.

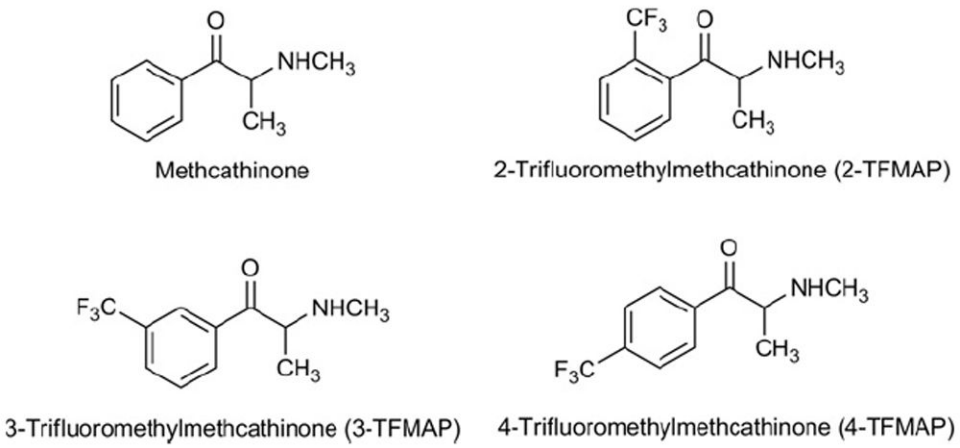

Fig. 1.
Chemical structures of methcathinone and trifluoromethylmethcathinone (TFMAP) positional isomers.

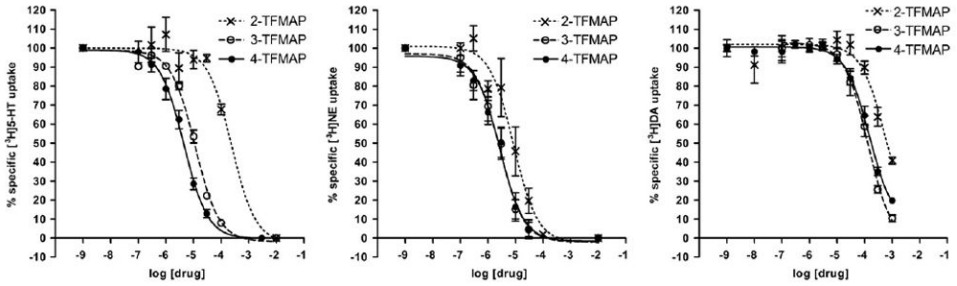

Fig. 2.

Inhibition of transporter-mediated uptake by TFMAP isomers. Panel A: inhibition of hSERT-mediated uptake of [^{3}H]5-HT in human platelets. Panel B: inhibition of [^{3}H]NE uptake in C6 glioma cells expressing the hNET. Panel C: inhibition of [^{3}H]DA uptake in HEK-293 cells expressing the hDAT. Data are the mean ±S.E.M. of 3–9 experiments, each run in triplicate. Symbols: 2-TFMAP, crosses and dotted line; 3-TFMAP, open circles and dashed line; 4-TFMAP, solid circles and solid line.

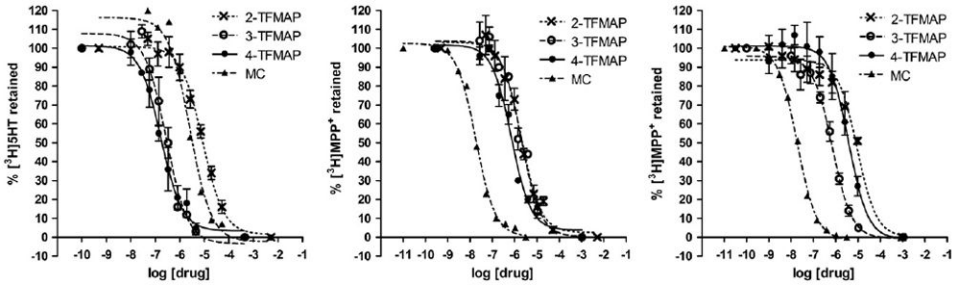

Fig. 3.

Stimulation of transporter-mediated release by methcathinone (MC) and TFMAP isomers. The ability of MC and TFMAP isomers to evoke release of [^{3}H]5-HT *via* rSERT (Panel A) or [^{3}H]MPP$^+$ release *via* rNET (Panel B) and rDAT (Panel C) was examined in rat brain synaptosomes. Data are the mean ±S.E.M. of three determinations, each run in triplicate. Symbols: MC, solid triangles and broken line; 2-TFMAP, crosses and dotted line; 3-TFMAP, open circles and dashed line; 4-TFMAP, solid circles and solid line.

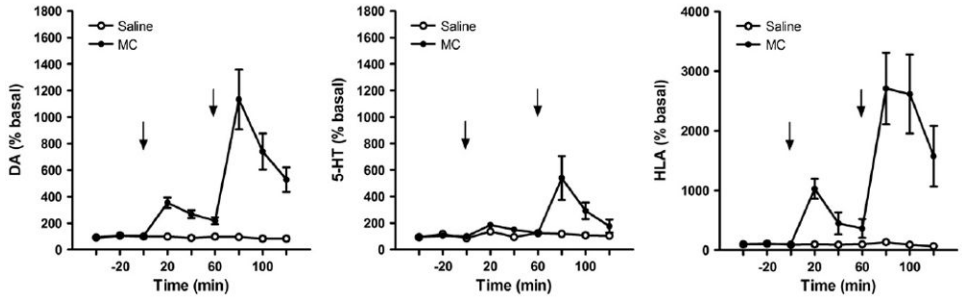

Fig. 4.

Effect of intravenous methcathinone (MC) on neurotransmitter levels and behavior in rats. A dose of 0.3 mg/kg MC was given at time=0 min, followed by 1 mg/kg at time=60 min. Extracellular DA (Panel A) and 5-HT (Panel B) levels were measured in dialysate from nucleus accumbens. Horizontal motor activity (HLA) was measured simultaneously (Panel C). Data are expressed as % basal and are the mean ±S.E.M. of six (saline) or seven (MC) animals. Symbols: saline, open circles; MC solid circles; arrows indicate intravenous injections.

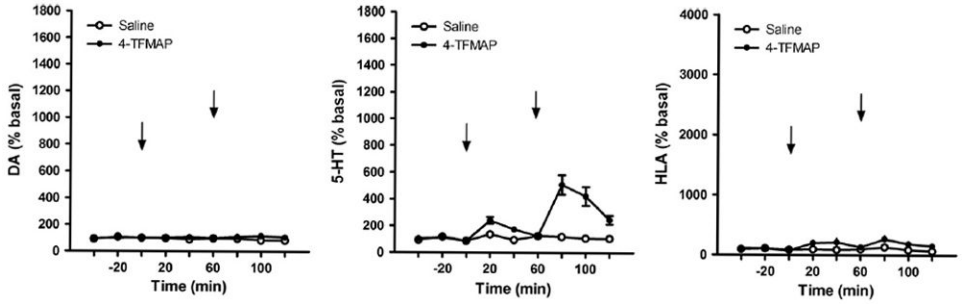

Fig. 5.

Effect of intravenous 4-TFMAP on neurotransmitter levels and behavior in rats. A dose of 1 mg/kg 4-TFMAP was given at time=0 min, followed by a dose of 3 mg/kg at time=60 min. Extracellular DA (Panel A) and 5-HT (Panel B) levels were measured in dialysate from nucleus accumbens. Horizontal motor activity (HLA was measured simultaneously (Panel C). Data are expressed as % basal and are the mean ±S.E.M. of six (saline) or seven (4-TFMAP) animals. Symbols: saline, open circles; 4-TFMAP, solid circles; arrows indicate intravenous injections.

Table 1

Summary table of the effects of methcathinone and trifluoromethylmethcathinone isomers (TFMAPs) on monoamine uptake inhibition and release. Uptake inhibition data are IC_{50} values and release data are EC_{50} values, in μM. All values are the mean ±S.E.M. of 3–9 separate experiments, each performed in triplicate.

Drug	Monoamine uptake transporter					
	SERT		NET		DAT	
	[^{3}H]5-HT uptake	[^{3}H]5-HT release	[^{3}H]NE uptake	[^{3}H]MPP$^+$ release	[^{3}H]DA uptake	[^{3}H]MPP$^+$ release
Methcathinone	35±5[a,c]	4±1[c]	0.5±0.1[a,c]	0.020±0.003	0.36±0.06[a]	0.020±0.001
2-TFMAP	239±13[b]	8.4±0.8[b]	10±3[b]	2.2±0.3[b]	689±160[b]	8.0±0.9[b]
3-TFMAP	11.3±0.4[b,c]	0.38±0.06[b,c]	3.0±0.8[b]	2.7±0.8[b]	136±8[b,c]	0.61±0.09[b,c]
4-TFMAP	4.5±0.6[b,c]	0.19±0.03[b,c]	3.0±0.9[b]	0.9±0.3[d]	200±50[b,c]	2.7±0.3[b,c,d]

[a] Uptake inhibition at hSERT, hNET, and rDAT (Cozzi et al., 1999).

[b] $P<0.05$ *vs.* methcathinone.

[c] $P<0.05$ *vs.* 2-TFMAP.

[d] $P<0.05$ *vs.* 3-TFMAP.