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The psychoactive aminoalkylbenzofuran derivatives, 5-APB and 6-APB, mimic the effects of 3,4-methylenedioxyamphetamine (MDA) on monoamine transmission in male rats

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Abstract

Rationale The non-medical use of new psychoactive substances (NPS) is a worldwide public health concern. The so-called “benzofury” compounds, 5-(2-aminopropyl)benzofuran (5-APB) and 6-(2-aminopropyl)benzofuran (6-APB), are NPS with stimulant-like properties in human users. These substances are known to interact with monoamine transporters and 5-HT receptors in transfected cells, but less is known about their effects in animal models.

Methods Here, we used *in vitro* monoamine transporter assays in rat brain synaptosomes to characterize the effects of 5-APB and 6-APB, together with their *N*-methyl derivatives 5-MAPB and 6-MAPB, in comparison to 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA). *In vivo* neurochemical and behavioral effects of 5-APB (0.3 and 1.0 mg/kg, i.v.) and 6-APB (0.3 and 1.0 mg/kg, i.v.) were assessed in comparison to MDA (1.0 and 3.0 mg/kg, i.v.) using microdialysis sampling in the nucleus accumbens of conscious male rats.

Results All four benzofuran derivatives were substrate-type releasers at dopamine transporters (DAT), norepinephrine transporters (NET) and serotonin transporters (SERT) with nanomolar potencies, similar to the profile of effects produced by MDA and MDMA. However, the benzofurans were at least 3-fold more potent than MDA and MDMA at evoking transporter-mediated release. Like MDA, both benzofurans induced dose-related elevations in extracellular dopamine and serotonin in the brain, but benzofurans were more potent than MDA. The benzofuran derivatives also induced profound behavioral activation characterized by forward locomotion which lasted for at least 2 h post-injection.

Conclusions Overall, benzofurans are more potent than MDA *in vitro* and *in vivo*, producing sustained stimulant-like effects in rats. These data suggest that benzofuran-type compounds may have abuse liability, and could pose risks for adverse effects, especially if used in conjunction with abused drugs or medications which enhance monoamine transmission in the brain.

Introduction

The non-medical (i.e., recreational) use of psychoactive substances that evade legislative control has become an issue of concern for law enforcement, clinicians, and policy makers (Baumann and Volkow 2016; Madras 2017). The structural and pharmacological diversity of these new psychoactive substances (NPS) gives rise to a rich array of drugs available in recreational drug markets worldwide (Hondebrink et al. 2018; Luethi and Liechti 2020), and this is reflected by intensive global monitoring measures of hundreds of such substances that emerged on the market in recent years (Evans-Brown and Sedefov 2018; Tettey et al. 2018). The evolution of novel compounds comes from various sources, such as structural analogs of illicit drugs of abuse and legitimate medication development efforts, but also newly-invented chemical entities without any history of scientific exploration (Brandt et al. 2014b).

1-(1-Benzofuran-5-yl)propan-2-amine (5-(2-aminopropyl)benzofuran or 5-APB) and 1-(1-benzofuran-6-yl)propan-2-amine (6-(2-aminopropyl)benzofuran, 6-APB), and their *N*-methyl derivatives 5-MAPB and 6-MAPB (Figure 1), first appeared on the recreational drug market almost a decade ago (King 2014). In Europe, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported the detection of 5-APB in 2010 (EMCDDA-Europol 2011) followed by 6-APB in 2011 (EMCDDA-Europol 2012), whereas both 5-MAPB and 6-MAPB were reported in 2013 (EMCDDA-Europol 2014). Anecdotal evidence from people who use such substances suggests that both 5-APB and 6-APB produce psychostimulant and entactogen-like effects similar those produced by 3,4-methylenedioxymethamphetamine (MDMA) (Greene 2013; Nugteren-van Lonkhuyzen et al. 2015). A number of intoxication cases with adverse effects, including fatalities, have been described where 5-APB and 6-APB were forensically confirmed (Adamowicz et al. 2014; Chan et al. 2013; Daveluy et al. 2016; Elliott and Evans 2014; McIntyre et al. 2015; Nugteren-van Lonkhuyzen et al. 2015), and recent data indicate these compounds are still used (Krpó et al. 2018). The Trans European Drug Information (TEDI) project, designed to carry out chemical analysis of drug samples submitted by recreational users as a harm reduction strategy, identified the appearance of 4-APB, 5-APB and 6-APB in samples and tablets circulating on the “ecstasy” market (Brunt et al. 2017). A case of acute toxicity following consumption of 5-MAPB has also been reported (Hofer et al. 2017). The synthetic preparation of both 5-APB and 6-APB was first published in 2000 as part of a research program designed for the development of selective 5-HT_{2C} receptor agonists (Briner et al. 2000; Briner et al. 2006) and the preparation of other isomers was reported for forensic purposes a decade later (Casale and Hays 2012; Stanczuk et al. 2013). When appearing on the NPS market, one commonly used term associated with 5-APB and 6-APB, which was originally related to the first branded products offered for sale, was “benzofury” (Nugteren-van Lonkhuyzen et al. 2015).

Initial studies examining the preclinical pharmacology of 5-APB and 6-APB showed they act as agonists at 5-HT_{2A} and 5-HT_{2B} receptors (Dawson et al. 2014; Iversen et al. 2013; Rickli et al. 2015; Shimshoni et al. 2017), whilst agonist or partial agonist properties have been reported for their interactions with trace amine-associated receptor 1 (TAAR1) (rat, mouse, and human) (Simmler et al. 2016). Additionally, both substances inhibit neurotransmitter uptake at dopamine transporters (DAT), norepinephrine transporters (NET) and serotonin (5-HT) transporters (SERT) in a dose-dependent manner (Iversen et al. 2013; Rickli et al. 2015; Zwartsen et al. 2017). Given the structural similarities between benzofuran compounds and 3,4-methylenedioxymethamphetamine (MDMA), it is tempting to speculate that they act as transportable substrates at monoamine transporters. Indeed, Rickli et al. (2015) first showed that 5-APB and 6-APB display transmitter releasing properties in HEK-293 cells transfected with human transporters, and the recent findings of Eshleman et al. (2019) confirm this observation. Application of 10 µM 5-APB to rat brain slices evokes dopamine efflux, consistent with substrate activity at DAT (Dawson et al. 2014). The *N*-methyl analog 5-MAPB was also reported to show DAT binding and inhibition of reuptake at DAT and SERT (Kim et al. 2019). The evaluation of cytotoxic effects of 5-APB and 5-MAPB in hepatocytes has also been reported (Nakagawa et al. 2018; Nakagawa et al. 2017).

In contrast to the abundance of *in vitro* data with 5-APB and 6-APB, only a few studies have examined the effects of these compounds *in vivo*. Microdialysis sampling in mouse striatum revealed that oral administration of 5-APB and 5-MAPB results in significant elevation of extracellular concentrations of dopamine and 5-HT with greater effects on 5-HT (Fuwa et al. 2016). Exposure to 5-MAPB was reported to result in increased levels of 5-HT and decreased concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) in microdialysate samples obtained from nucleus accumbens in unanesthetized rats (Kim et al. 2019).

In the present study, we sought to extend the findings from rodent models by comparing the pharmacology of the aminoalkylbenzofuran derivatives 5-APB, 5-MAPB, 6-APB and 6-MAPB with the structurally related club drugs, 3,4-methylenedioxyamphetamine (MDA) and MDMA. First, *in vitro* monoamine transporter assays in rat brain synaptosomes were used to examine the releasing effects of 5-APB, 5-MAPB, 6-APB, and 6-MAPB at DAT, NET and SERT. Next, *in vivo* microdialysis in rat nucleus accumbens was used to examine changes in extracellular dopamine and 5-HT produced by intravenous injection of 5-APB and 6-APB. Microdialysis sampling was carried out in arenas equipped with photobeam arrays to detect movement, so that neurochemistry and locomotor behaviors could be monitored simultaneously.

Material and methods

Drugs and chemicals

The four aminoalkylbenzofuran derivatives 5-APB, 5-MAPB, 6-APB and 6-MAPB were synthesized as HCl salts according to published procedures (Briner et al. 2000; Stanczuk et al. 2013) and were available from previous studies (Welter et al. 2015a; Welter et al. 2015b). (±)-3,4-Methylenedioxyamphetamine HCl (MDA) and (±)-3,4-methylenedioxymethamphetamine HCl (MDMA) were obtained from the Pharmacy at the National Institute on Drug Abuse (NIDA), Intramural Research Program (IRP), in Baltimore, MD. [³H]1-Methyl-4-phenylpyridinium ([³H]MPP⁺, specific activity = 85 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO, USA) whereas [³H]serotonin ([³H]5-HT, specific activity = 20 Ci/mmol) was purchased from Perkin Elmer (Shelton, CT, USA). All other chemicals and reagents used for the *in vitro* assays, microdialysis methods, and high-performance liquid chromatography with electrochemical detection (HPLC-ECD) were acquired from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified.

Animals and housing facilities

Male Sprague-Dawley rats (Envigo, Frederick, MD, USA) weighing 300–400g were housed under conditions of controlled temperature (22 ± 2 °C) and humidity (45 ± 5%), with food and water freely available. Animals 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. Lights were on from 0700–1900 h and experiments were carried out between 0900–1500 h.

***In vitro* transporter release assays in synaptosomes**

Synaptosomes were prepared from rat striatum for DAT assays, whereas synaptosomes were prepared from whole brain minus striatum and cerebellum for NET and SERT assays, as described previously (Rothman et al. 2001; Rothman et al. 2003). Reserpine (1 μ M) was added to the 10% sucrose solution during preparation of synaptosomes to block vesicular uptake of substrates. Radiolabeled substrates used for release assays were 9 nM [3 H]1-methyl-4-phenylpyridinium ([3 H]MPP⁺) (DAT and NET) and 5 nM [3 H]5-HT (SERT). Unlabeled transporter blockers were employed to prevent uptake of [3 H]substrates by competing transporters in some assays. For DAT assays, no unlabeled blockers were required. However, for NET assays, 100 nM GBR12935 and citalopram were added to the sucrose solution to block [3 H]MPP⁺ uptake by DAT and SERT. For SERT assays, 100 nM GBR12935 and nomifensine were added to sucrose to block [3 H]5-HT uptake by DAT and NET. Synaptosomes were preloaded with radiolabeled substrate in Krebs-phosphate buffer for 1 h to reach steady state. Release assays were initiated by addition of 850 μ L of preloaded synaptosomes to test tubes containing 150 μ L buffer solution with or without test drugs. Incubations lasted 30 min for DAT and NET assays, and 5 min for SERT assays. The release reaction was terminated by rapid vacuum filtration, followed by quantitative determination of radioactivity retained on filters by liquid scintillation counting.

***In vivo* microdialysis in conscious male rats**

Microdialysis sampling was carried out in conscious male rats as described previously (Baumann et al. 2012; Baumann et al. 2013). Each rat received a surgically-implanted jugular catheter and an intracranial guide cannula aimed at the nucleus accumbens under pentobarbital anesthesia (sodium pentobarbital, 60 mg/kg, i.p.). Rats were single-housed post-operatively and allowed at least one week to recover from surgery. On the evening before an experiment, rats were moved from their home cages and placed into microdialysis arenas which were equipped with photobeam arrays to detect locomotor activity (TruScan, Harvard Bioscience). For each rat, a microdialysis probe (2 \times 0.5 mm, CMA/12, Harvard Bioscience, Holliston, MA, USA) was inserted into the guide cannula, and an extension tube was attached to the jugular catheter. Rats were connected to a tether system that allowed movement within the arena. Ringers' salt solution was pumped at a flow rate of 0.6 μ L/min to perfuse the probes overnight. On the following morning, dialysate samples were collected at 20 min intervals, and dopamine and 5-HT levels were quantitatively determined using microbore HPLC-ECD as described elsewhere (Baumann et al. 2011). Rats were randomly assigned to groups receiving either drug or saline injections. Once three stable baseline samples were obtained, rats received two sequential intravenous (i.v.) injections of drug: one dose at time zero, followed by a threefold higher dose 60 min later. Control rats received sequential i.v. injections of saline (1 mL/kg) according to the same schedule. Microdialysis samples were collected every 20 min throughout the post-injection period of 120 min. Ambulation

within the arena, defined as movement in the horizontal plane, was quantified in 20 min bins that coincided with collection of dialysate samples. At the end of the experiments, rats were euthanized with CO₂ and decapitated. The brain sections were examined to verify placement of microdialysis probe tips within the nucleus accumbens. Only those rats with correct placements were included in data analyses.

Data analysis

All statistical analyses were carried out using GraphPad Prism (v. 7.0; GraphPad Scientific, San Diego, CA, USA). For synaptosome assays, EC₅₀ values for stimulation of release were calculated based on non-linear regression analysis. Release efficacy was expressed as a percentage of maximal release, which was determined using saturating concentrations of the non-selective releasing agent tyramine at 10 μM for DAT and NET, and 100 μM for SERT. For *in vivo* microdialysis experiments, neurotransmitter and behavioral data from individual rats were normalized to percent control values based on three preinjection samples. Normalized group data were expressed as mean ± SEM and were evaluated by a two-factor analysis of variance (ANOVA) (dose × time) where drug effects were compared with saline control. When significant main effects were noted, *post hoc* comparisons were carried out at each time point using Bonferroni's *post hoc* test.

Results

In vitro transporter release assays

Rat brain synaptosomes were preloaded with [³H]MPP⁺ or [³H]5-HT followed by incubation with various concentrations of test drug to assess the potential for transporter-mediated release. The dose-response effects for 5-APB, 6-APB and MDA to release [³H]MPP⁺ via NET and DAT and [³H]5-HT via SERT are depicted in [Figure 2](#). In order to compare the potency of test drugs to induce transporter-mediated release, non-linear regression analysis was employed to calculate potency in terms of EC₅₀ values. As shown in [Table 1](#), all tested substances were potent substrate-type releasers at DAT, NET and SERT. Compared to MDA, the benzofuran counterparts 5-APB and 6-APB were at least 3-fold more potent in their ability to induce release at DAT and SERT, whereas release evoked *via* NET was 2-3 times more potent than MDA. At DAT, for example, 5-APB (EC₅₀ = 31 nM) was 3.4 times more potent than MDA (EC₅₀ = 106 nM) while 6-APB (EC₅₀ = 10 nM) was 10-fold more potent than MDA. Potency values determined for SERT-mediated release of [³H]5-HT indicated that 5-APB (EC₅₀ = 19 nM) and 6-APB (EC₅₀ = 36 nM) were 8.5 and 4.5 times more potent than MDA. The findings for the *N*-methyl derivatives are illustrated in [Figure 3](#), and the comparison with MDMA showed that both 5-MAPB and 6-MAPB were at least 3 times more potent at DAT, 3.75 times more potent at NET, and up to 2.5 times more potent at SERT. The calculated DAT/SERT and DAT/NET ratios suggested that all six tested drugs were non-selective substrates across the three transporters. With regard to *N*-methylation of 5-APB and 6-APB, a difference in potency at NET was not observed whereas a slight reduction in potency was noticeable at DAT. At SERT, 5-APB remained 3.4 times more potent than 5-

MAPB ($EC_{50} = 64$ nM) although potency of 6-MAPB ($EC_{50} = 33$ nM) remained similar compared to 6-APB ($EC_{50} = 36$ nM).

***In vivo* microdialysis data**

Neurochemical and behavioral effects of acute i.v. administration of MDA to male rats ($n = 7$ rats per group) are shown in **Figure 4**. MDA treatment had a significant main effect on dialysate dopamine ($F[1,108]=132.2$, $p<0.0001$) and 5-HT ($F[1,108]=154.0$, $p<0.0001$) concentrations. *Post hoc* tests revealed that dopamine levels were significantly greater than saline control at all time points after the 3 mg/kg dose, whereas 5-HT levels were elevated at the 20 min time point after the 1 mg/kg dose, and at all time points after the 3 mg/kg dose (Figure 4). At the 80 min mark, i.e. 20 min following injection of the 3 mg/kg dose of MDA, dialysate dopamine levels increased 7.6-fold above baseline, which represented the maximum increase. The peak effect on 5-HT was 4.6-fold above baseline after 1 mg/kg and 11.2-fold after 3 mg/kg. MDA had a main effect on ambulation ($F[1,108]=102.2$, $p<0.0001$) when compared with saline controls. *Post hoc* analysis showed that MDA did not significantly increase locomotion after 1 mg/kg but did significantly stimulate activity ($p<0.0001$) at all times following administration of the 3 mg/kg dose. The peak effect of MDA on ambulation was 30-fold above baseline, which declined to about 18-fold when measured at the 120 min point.

The effects of 5-APB on neurochemistry and motor behavior ($n = 7$ rats per group) are displayed in **Figure 5**. 5-APB treatment had a significant main effect on dialysate levels of dopamine ($F[1,108]=92.1$, $p<0.0001$) and 5-HT ($F[1,108]=107.4$, $p<0.0001$). *Post hoc* tests showed that 5-APB failed to significantly increase dopamine levels at the 0.3 mg/kg dose, but a significant neurotransmitter elevation ($p<0.0001$) was detected at all time points after the 1.0 mg/kg dose. The peak increase of dopamine after the 1.0 mg/kg dose was 6.7-fold above baseline. 5-APB elevated dialysate 5-HT at 20 min after 0.3 mg/kg (5.7-fold, $p<0.05$), and at all time points after 1.0 mg/kg ($p<0.0001$). The peak effect on 5-HT was 17.7-fold above baseline after administration of 1.0 mg/kg. At the 120 min point, 5-HT dialysate levels remained elevated 11.3-fold above baseline. 5-APB administration at 1.0 mg/kg caused a main effect on ambulation ($F[1,108]=73.5$, $p<0.0001$) when compared with saline controls. *Post hoc* analysis also indicated that 5-APB significantly stimulated activity at all times following administration of the 1.0 mg/kg dose. The peak effect of 5-APB on ambulation was 21-fold above baseline, and when measured at the 120 min point, a 14.0-fold increase above baseline was still noticeable.

Figure 6 depicts the neurochemical and behavioral results obtained from acute i.v. administration of 6-APB to a group of male rats ($n = 7$ rats per group) and significant main effects on dialysate dopamine ($F[1,108]=385.8$, $p<0.0001$) and 5-HT ($F[1,108]=80.9$, $p<0.0001$) were noted. *Post hoc* tests revealed that 6-APB elevated dialysate dopamine at 20 min (2.6-fold, $p<0.01$) and 40 min (3.3-fold, $p<0.0001$) after 0.3 mg/kg, and at all-time points after 1.0 mg/kg ($p<0.0001$) when compared to saline. The peak increase in dopamine following 1 mg/kg 6-APB was 8.4-fold above baseline (Figure 6). 6-APB elevated dialysate 5-HT immediately after administration of 1.0 mg/kg ($p<0.0001$), a 16-fold increase relative to baseline, and this elevation

was still present at the last sampling period ($p < 0.0001$). 6-APB had a main effect on ambulation ($F[1,108]=75.4$, $p < 0.0001$) when compared with saline controls, as shown in Figure 6. *Post hoc* analysis showed that 6-APB administration induced a 24-fold increase in ambulation after the 1 mg/kg dose, whereas the 0.3 mg/kg dose failed to result in significant increases in locomotion.

Discussion

The present study investigated neurochemical and behavioral actions of the aminoalkylbenzofuran derivatives 5-APB and 6-APB, which together with their *N*-methylated counterparts 5-MAPB and 6-MAPB, appeared on the NPS market several years ago. As shown in Figures 2 and 3, the four benzofuran compounds are potent, non-selective, substrate-type releasing agents at DAT, NET, and SERT. In contrast to pure reuptake inhibitors such as cocaine, the benzofurans induce non-exocytotic transporter-mediated release of monoamines via reversal of normal transporter flux, consistent with the molecular mechanism of action for ring-substituted amphetamines (Baumann et al. 2013; Glennon 2014; Rothman and Baumann 2003; Sitte and Freissmuth 2015). Similar to what we have reported in previous studies, MDA and MDMA showed nM potencies as releasers at all three transporters in rat brain synaptosomes (Baumann et al. 2007; Sandtner et al. 2016). It is noteworthy that we found 5-APB and 6-APB to be substantially more potent than MDA and MDMA, and *N*-methylation of the benzofurans did not alter releasing potencies, which suggests this chemical modification is accommodated by the orthosteric binding site of monoamine transporters (Table 1).

The transporter-mediated releasing properties of 5-APB and 6-APB were first reported by Rickli et al. (2015) who examined [^3H]monoamine efflux after exposure to a single large dose (100 μM) in human embryonic kidney 293 (HEK) cells transfected with human transporters. Rickli et al. used uptake inhibition potencies from transfected cells to calculate DAT/SERT ratios of 0.05 and 0.29 for 5-APB and 6-APB, respectively; these values indicate substantial SERT selectivity and are much lower than the DAT/SERT ratios of 0.61 and 3.60 that we found using release assays in synaptosomes (see Table 1). The 10-fold lower DAT/SERT ratios reported for 5-APB and 6-APB in transfected cells may seem at odds with our findings in synaptosomes, but the DAT/SERT ratios for MDMA and MDA in transfected cells (i.e., 0.12 and 0.25) are also much lower than our findings in synaptosomes (Luethi et al. 2019). Thus, in both transfected cells and rat brain synaptosomes, the DAT/SERT ratios for benzofurans are similar to those reported for MDMA and MDA. Although 5-MAPB and 6-MAPB were not included in the study of Rickli et al., the *N*-ethyl derivative of 5-APB, i.e. 5-EAPB, was evaluated and exhibited a reduction in the magnitude of norepinephrine and dopamine release while maintaining 5-HT releasing properties (Rickli et al. 2015). More recently, Eshleman et al. (2019) examined the dose-response relationships for the releasing activity of 6-APB and 6-MAPB, and several other benzofurans in HEK cell transfected with human transporters. These investigators demonstrated that 6-APB and 6-MAPB display nM potencies for releasing [^3H]monoamines at human DAT, NET and SERT, in agreement with the data from rat synaptosomes reported here. Other studies show that 5-APB and 5-MAPB enhance electrically-induced dopamine efflux in slices of rat

nucleus accumbens (Dawson et al. 2014; Sahai et al. 2017) consistent with DAT-mediated dopamine release. It is noteworthy that 5-(2-aminopropyl)indole (5-IT) and 6-(2-aminopropyl)indole (6-IT), two structurally related NPS which differ from 5-APB and 6-APB by replacement of the heteroatom in the five-membered ring, also are reported to exhibit potent releasing activities at DAT and SERT (Luethi et al. 2018; Marusich et al. 2016).

Mechanistically, the non-selective transporter releasing activity of 5-APB, 6-APB, and related analogs is comparable to other NPS such as the ring-substituted cathinones mephedrone and methylone (Baumann et al. 2012), (\pm)-*cis-para*-methyl-4-methylaminorex (4,4'-DMAR) and (\pm)-*cis*-4-methylaminorex (Brandt et al. 2014a), and (\pm)-*cis*- and *trans*-3,4'-methylenedioxy-4-methylaminorex (MDMAR) (Maier et al. 2018; McLaughlin et al. 2015). By contrast, cathinone analogs which contain a pyrrolidine ring, such as α -pyrrolidinovalerophenone (α -PVP) and 3,4-methylenedioxypyrovalerone (MDPV), are pure uptake inhibitors devoid of substrate-type releasing activity (Baumann et al. 2013; Kolanos et al. 2015). It is tempting to speculate that the bulky pyrrolidine ring moiety of α -PVP and MDPV renders these substances too large to fit through the transporter permeation pore, confining their interaction to the extracellular face of the protein.

Once the potent monoamine releasing properties of 5-APB and 6-APB were confirmed using synaptosome assays *in vitro*, we predicted that an elevation of neurotransmitters would be observed under *in vivo* conditions. To this end, we examined extracellular levels of dopamine and 5-HT using microdialysis in rat nucleus accumbens, a brain region associated with the reward circuitry underlying drug abuse liability and dependence (Bonci et al. 2003; Willuhn et al. 2010). Here we show that MDA, 5-APB, and 6-APB significantly elevate extracellular dopamine levels in rats undergoing microdialysis. Importantly, MDA was about 3-fold less potent than the benzofuran compounds, in agreement with the *in vitro* findings from rat brain synaptosomes. It is noteworthy that only 6-APB induced a significant elevation of dopamine after the low dose (0.3 mg/kg), and this observation is consistent with the more potent releasing effects of this drug at DAT ($EC_{50} = 10$ nM). 6-APB also caused the highest peak and most sustained dopamine increase, though all drugs induced substantial elevations in dopamine. The *in vivo* microdialysis data also reveal that administration of MDA, 5-APB and 6-APB give rise to robust increases in dialysate 5-HT levels, but MDA is 3-fold less potent in this respect. It is interesting that the low dose of 5-APB increased dialysate 5-HT while the same dose of 6-APB did not. Again, the differences in potency with regard to 5-HT release were also observed in the *in vitro* data where 5-APB ($EC_{50} = 19$ nM) was slightly more potent than 6-APB ($EC_{50} = 36$ nM), and much more potent than MDA ($EC_{50} = 162$ nM) to release 5-HT from synaptosomes.

A previous microdialysis study revealed that oral administration of 5-APB (0.08 mmol/kg, equivalent to ~17 mg/kg) led to an 8-fold increase in dialysate dopamine, and a 23-fold increase in dialysate 5-HT, in the corpus striatum of male CD-1 mice (Fuwa et al. 2016). In the Fuwa et al. study, the massive rise in extracellular 5-HT produced by 5-APB was maintained for up to 3 h post-injection. The extended time course of effects for 5-APB observed by Fuwa et al. (2016) might be explained by the

larger dose administered or the oral route, which would be predicted to afford slower drug kinetics. Fuwa et al. also reported that oral administration of 5-MAPB (0.08 mmol/kg, equivalent to ~18 mg/kg) resulted in a peak maximum elevation in 5-HT which was much more pronounced than that produced by an equivalent dose of MDMA (0.08 mmol/kg, equivalent to ~18.4 mg/kg) (Fuwa et al. 2016). Interestingly, oral administration of either 1-(1-benzofuran-2-yl)-*N*-methylpropan-2-amine (2-MAPB) or 5-EAPB also produced large elevations in 5-HT levels, but the effects were smaller when compared to 5-MAPB. Overall, the present microdialysis studies, and those carried out in mice, show that 5-APB and 6-APB produce robust increases in extracellular dopamine and 5-HT, and both benzofuran compounds are more potent than MDA and MDMA. In the study reported by Kim et al. (2019), 5-MAPB induced an increase in 5-HT after administration of 1 mg/kg and a decrease of DOPAC after injection of lower doses (0.1 and 0.3 mg/kg) following analysis of microdialysates collected from rat nucleus accumbens.

In the present study, we found that MDA, 5-APB, and 6-APB induce profound dose-related behavioral activation characterized by forward ambulation, effects that last for at least 2 h post-injection. 6-APB produced a somewhat greater increase in activity (24-fold) than 5-APB (20-fold), perhaps due to higher elevations in extracellular dopamine produced by 6-APB. Our previous studies in rats have shown a significant positive correlation between the extent of motor activation and dialysate dopamine levels detected in the nucleus accumbens *in vivo* (Baumann et al. 2011; Zolkowska et al. 2009). The present behavioral findings in rats agree with the report of Dolan et al. (2017), who showed that intraperitoneal (i.p.) injection of 5-APB in mice increases forward locomotion with an ED₅₀ of 3.27 mg/kg. The potency of 5-APB to stimulate activity in mice is at least 3-fold greater than that of MDMA (e.g. Gatch et al. 2019) Dolan et al. (2017) also investigated the discriminative stimulus effects of 5-APB in groups of rats trained to recognize either methamphetamine (1 mg/kg, i.p.), MDMA (1.5 mg/kg, i.p.), or cocaine (10 mg/kg, i.p.). The studies revealed that 5-APB fully substitutes for the discriminative stimulus effects of MDMA, but not methamphetamine or cocaine. Moreover, the ED₅₀ value for 5-APB to substitute for the MDMA stimulus cue (i.e., ED₅₀ = 0.27 mg/kg, i.p.) is substantially lower than the training dose of MDMA, indicating greater potency for 5-APB. Taken together, the behavioral results with 5-APB in rodents are fully consistent with neurochemical findings reported here that benzofuran compounds mimic the effects of MDMA but are substantially more potent.

Reported clinical features associated with 5-APB, 6-APB, or 5-MAPB overdose include cardiovascular and neurological effects consistent with a stimulant toxidrome (Chan et al. 2013; Hofer et al. 2017; Kamour et al. 2014; McIntyre et al. 2015; Nugteren-van Lonkhuyzen et al. 2015), which could be mediated by potent monoamine releasing properties identified *in vitro* and *in vivo*. Additionally, it has been reported that 5-APB and 6-APB act as agonists at the 5-HT_{2A} and 5-HT_{2B} receptor (Dawson et al. 2014; Iversen et al. 2013; Rickli et al. 2015), which might be clinically relevant for adverse cardiovascular events, especially after prolonged drug use (Setola et al. 2003). Appreciable affinity toward subtypes of α_1 -adrenoceptors (α_{1A} , α_{2A}) (Rickli et al. 2015) and α_2 -adrenoceptors (α_{2A} , α_{2B} , α_{2C}) (Iversen et al. 2013) has also been observed, which could be a contributing factor for vasoconstriction and

prolonged systolic and diastolic hypertension observed in a 5-MAPB intoxication case (Hofer et al. 2017).

In the present study, 5-APB and 6-APB were more potent than MDA *in vitro* and *in vivo*, and produced sustained stimulant-like effects in rats. The extent to which the potent and non-selective releasing properties of benzofurans translate into abuse potential warrants further investigation in animal models. In the only study to examine the abuse liability of benzofuran compounds, Cha et al. (2016) showed that 5-APB induces conditioned place preference but does not support intravenous drug self-administration. These intriguing findings suggest that 5-APB is rewarding yet does not possess substantial reinforcing effects. Nevertheless, the potency of the aminoalkylbenzofurans studied here could pose risks for adverse effects in humans, especially if used in conjunction with abused drugs or medications which enhance overall monoamine transmission in the brain.

In summary, we found that 5-APB, 6-APB, and their *N*-methylated counterparts act as potent substrate-type releasers at DAT, NET, and SERT, which mimics the molecular mechanism of action of MDA and MDMA. Importantly, the benzofuran compounds are more potent than MDA *in vitro* and *in vivo*, and they produce sustained stimulant-like effects in rats. The preclinical data suggest that benzofuran-type compounds may have abuse potential and could pose risks for adverse effects, especially if used in conjunction with abused drugs or medications which enhance overall monoamine transmission in the brain.

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Compliance with ethical standards

Animal use procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Care and Use Committee of the Intramural Research Program of the National Institute on Drug Abuse (Baltimore, MD, USA).

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Potency (EC_{50}) and efficacy (%Emax) of psychoactive benzofuran compounds to release [3H]MPP $^+$ via DAT and NET, or [3H]5-HT via SERT, in rat brain synaptosomes.

Drug	DAT release EC_{50} (nM) [%Emax]	NET release EC_{50} (nM) [%Emax]	SERT release EC_{50} (nM) [%Emax]	DAT/SERT ratio	DAT/NET ratio
MDA	106 ± 7 [104%]	47 ± 7 [91%]	162 ± 28 [109%]	1.53	0.43
5-APB	31 ± 3 [103%]	21 ± 4 [103%]	19 ± 2 [104%]	0.61	0.67
6-APB	10 ± 1 [101%]	14 ± 2 [98%]	36 ± 5 [100%]	3.60	1.40
MDMA	120 ± 8 [101%]	90 ± 13 [99%]	85 ± 16 [104%]	0.71	0.75
5-MAPB	41 ± 3 [103%]	24 ± 3 [99%]	64 ± 7 [103%]	1.56	0.58
6-MAPB	20 ± 2 [106%]	14 ± 2 [100%]	33 ± 4 [106%]	1.65	0.70

Data are mean +/- SD for N=3 experiments performed in triplicate. Values in brackets are % of maximal release, as defined in Materials and Methods. DAT/SERT ratios are calculated as $(DAT\ EC_{50})^{-1}/(SERT\ EC_{50})^{-1}$, where larger number indicates higher DAT selectivity. DAT/NET ratios are calculated as $(DAT\ EC_{50})^{-1}/(NET\ EC_{50})^{-1}$, where larger number indicates higher DAT selectivity.

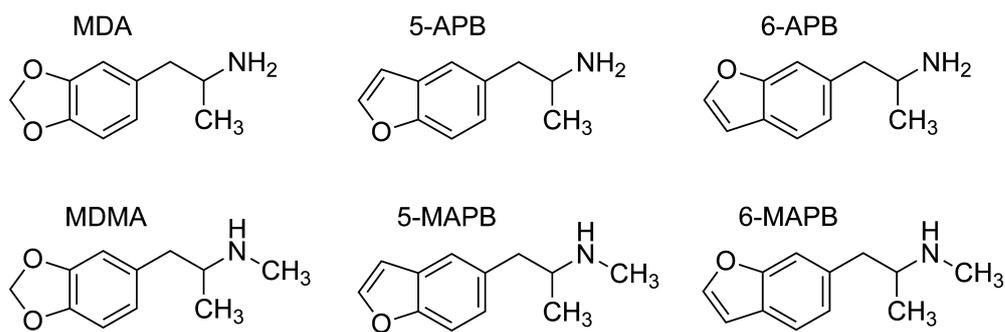


Figure 1. Chemical structures of 5-APB, 6-APB, and their *N*-methylated counterparts, as compared to MDA and MDMA.

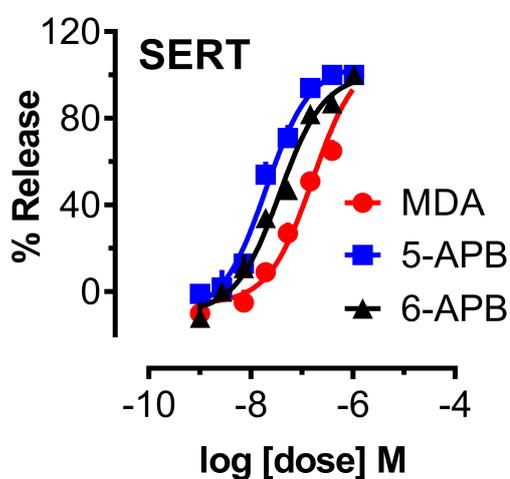
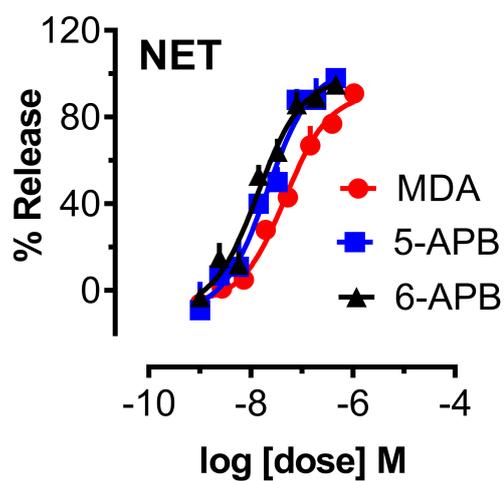
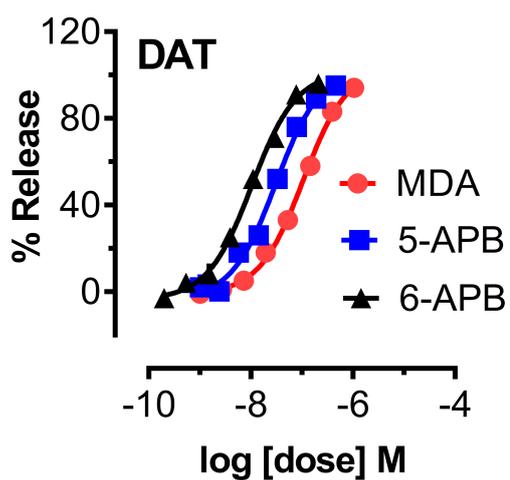


Figure 2. Dose-response effects of MDA, 5-APB, and 6-APB to induce release of [³H]MPP⁺ via DAT and NET, or [³H]5-HT via SERT, in rat brain synaptosomes. Data are mean +/- SD for N=3 experiments, expressed as a percent of maximal release.

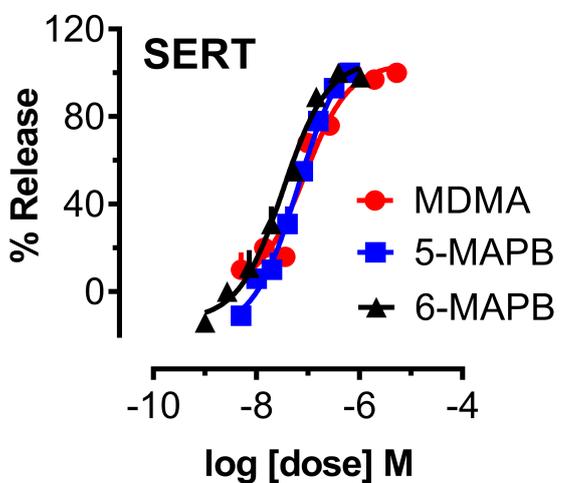
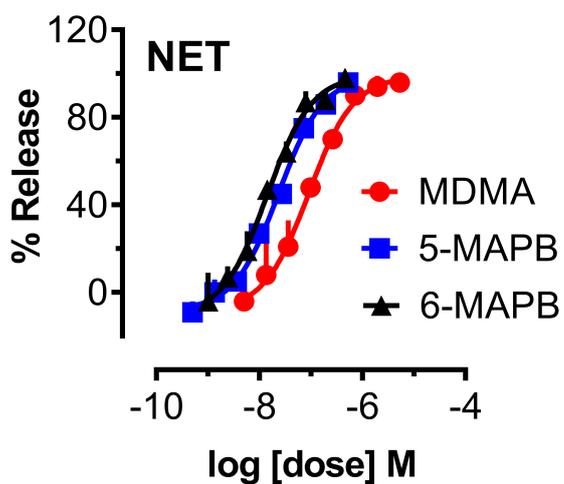
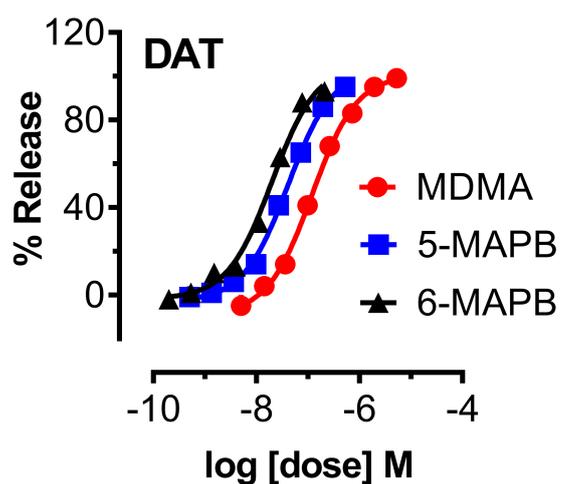


Figure 3. Dose-response effects of MDMA, 5-MAPB, and 6-MAPB to induce release of [³H]MPP⁺ via DAT and NET, or [³H]5-HT via SERT, in rat brain synaptosomes. Data are mean +/- SD for N=3 experiments, expressed as a percent of maximal release.

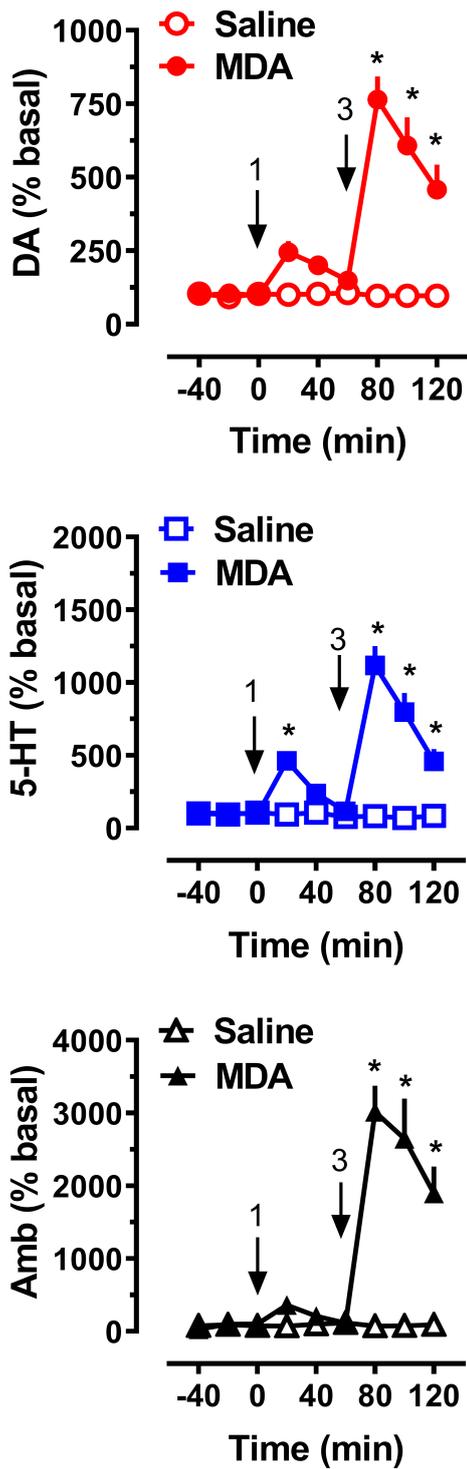


Figure 4. Effects of MDA administration on extracellular dopamine (DA) and 5-HT concentrations, and horizontal ambulation (Amb), in rats undergoing microdialysis in nucleus accumbens. Rats received i.v. drug injections at t=0 and 60 min, at the doses indicated, as shown by arrows. Data are mean \pm SEM for N=7 rats/group, expressed as percent of pre-injection basal values for each endpoint. * = significant effect compared to saline at corresponding time point.

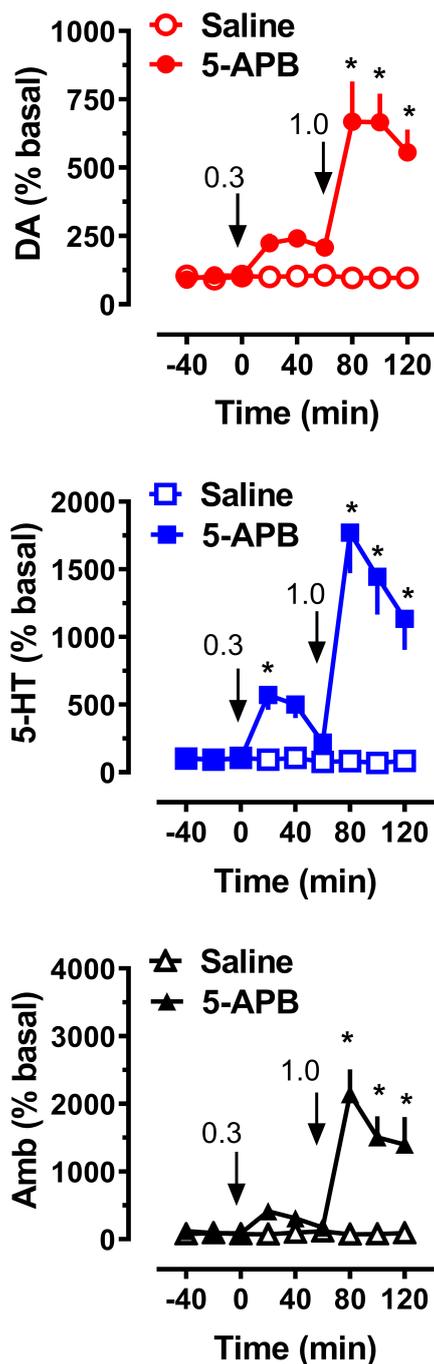


Figure 5. Effects of 5-APB administration on extracellular dopamine (DA) and 5-HT concentrations, and horizontal ambulation (Amb), in rats undergoing microdialysis in nucleus accumbens. Rats received i.v. drug injections at t=0 and 60 min, at the doses indicated, as shown by arrows. Data are mean \pm SEM for N=7 rats/group, expressed as percent of pre-injection basal values for each endpoint. * = significant effect compared to saline at corresponding time point.

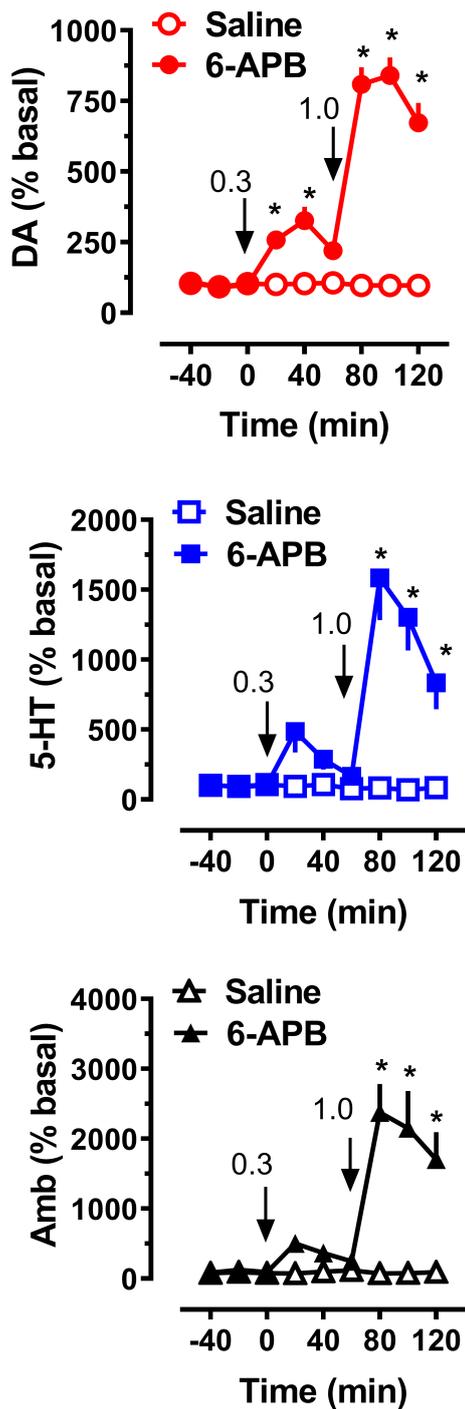


Figure 6. Effects of 6-APB administration on extracellular dopamine (DA) and 5-HT concentrations, and horizontal ambulation (Amb), in rats undergoing microdialysis in nucleus accumbens. Rats received i.v. drug injections at t=0 and 60 min, at the doses indicated, as shown by arrows. Data are mean \pm SEM for N=7 rats/group, expressed as percent of pre-injection basal values for each endpoint. * = significant effect compared to saline at corresponding time point.