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N-Alkylated Analogs of 4-Methylamphetamine (4-MA) Differentially Affect Monoamine Transporters and Abuse Liability

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Clandestine chemists synthesize novel stimulant drugs by exploiting structural templates known to target monoamine transporters for dopamine, norepinephrine, and serotonin (DAT, NET, and SERT, respectively). 4-Methylamphetamine (4-MA) is an emerging drug of abuse that interacts with transporters, but limited structure–activity data are available for its analogs. Here we employed uptake and release assays in rat brain synaptosomes, voltage-clamp current measurements in cells expressing transporters, and calcium flux assays in cells coexpressing transporters and calcium channels to study the effects of increasing *N*-alkyl chain length of 4-MA on interactions at DAT, NET, and SERT. In addition, we performed intracranial self-stimulation in rats to understand how the chemical modifications affect abuse liability. All 4-MA analogs inhibited uptake at DAT, NET, and SERT, but lengthening the amine substituent from methyl to ethyl, propyl, and butyl produced a stepwise decrease in potency. *N*-methyl 4-MA was an efficacious substrate-type releaser at DAT that evoked an inward depolarizing current and calcium influx, whereas other analogs did not exhibit these effects. N-methyl and *N*-ethyl 4-MA were substrates at NET, whereas *N*-propyl and *N*-butyl 4-MA were not. All analogs acted as SERT substrates, though *N*-butyl 4-MA had very weak effects. Intracranial self-stimulation in rats showed that elongating the *N*-alkyl chain decreased abuse-related effects *in vivo* that appeared to parallel reductions in DAT activity. Overall, converging lines of evidence show that lengthening the *N*-alkyl substituent of 4-MA reduces potency to inhibit transporters, eliminates substrate activity at DAT and NET, and decreases abuse liability of the compounds.
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INTRODUCTION

New psychoactive substances (NPSs) are synthetic alternatives to more traditional drugs of abuse that are engineered to circumvent drug control laws. Most NPSs that produce stimulant-like behavioral effects are derived from the structure of amphetamine or its β-keto analog cathinone (Baumann *et al*, 2013a; De Felice *et al*, 2014; Simmler *et al*, 2014). Subtle chemical modifications to these structural templates have created a vast number of potentially dangerous stimulants sold via the internet, in brick-and-mortar shops, and by street drug dealers (Baumann *et al*, 2014b). Like traditional stimulants, newly emerging stimulants interact with plasma membrane transporters for dopamine (DAT), norepinephrine (NET), and 5-HT (SERT) to increase synaptic concentrations of these neurotransmitters in the brain. Drugs targeting transporters can be divided into two types based on their mode of action: (1) 'inhibitors' bind to the orthosteric site on the transporter to block neurotransmitter uptake, whereas (2) 'substrates' bind to the orthosteric site, are subsequently transported into cells, and evoke release of neurotransmitters by reverse transport (Kahlig *et al*, 2005; Khoshbouei *et al*, 2003; Rothman and Baumann, 2006; Scholze *et al*, 2000). Importantly, transporter substrates and inhibitors can be discriminated based on their electrophysiological signatures, whereby substrates induce transporter-mediated inward sodium currents and inhibitors induce an apparent outward current (Cameron *et al*, 2013; Solis, 2017; Solis *et al*, 2012). Evidence shows that transporter-mediated inward currents and subsequent accumulation of substrate-type drugs in cells may be responsible for producing intracellular deficits in monoamine neurons such as inhibition of neurotransmitter synthesis and disruption of vesicular storage, leading to long-term

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neurotransmitter depletions (Baumann *et al*, 2014a; Fleckenstein *et al*, 2007).

4-Methylamphetamine (4-MA) is an example of a stimulant-like NPS that was resurrected from older biomedical literature. In the 1950s, 4-MA was investigated as a potential anorectic medication, but its development was abandoned before reaching the clinical market. 4-MA remained in relative obscurity until 2009, when it first appeared on the European street drug market in powders mixed with amphetamine and caffeine, being sold as 'speed' (EMCDDA, 2012). Since that time, 4-MA has been associated with many serious intoxications and fatalities in European countries (Blanckaert *et al*, 2013; EMCDDA, 2014). Preclinical studies from our laboratory and others show that 4-MA is a nonselective transporter substrate capable of releasing dopamine and serotonin from neurons *in vitro* and *in vivo* (Baumann *et al*, 2011; Wee *et al*, 2005). When compared with the parent drug amphetamine, 4-MA has much greater potency as a substrate at SERT, and a recent study confirms that locomotor and hypothermic effects produced by 4-MA in rats depend upon serotonergic systems (Rubio *et al*, 2015). Older literature indicates that 4-MA is also a potent inhibitor of monoamine oxidase (MAO) A, the principal enzyme responsible for the breakdown of monoamine neurotransmitters (Ross *et al*, 1977).

To date, few structure–activity studies have examined the pharmacology of 4-MA analogs. In the present investigation, we examined the effects of lengthening the amine substituent of 4-MA on drug interactions at DAT, NET, and SERT. We focused on the amine group because it is a primary site for modification of NPS based on the amphetamine and cathinone templates (Rothman *et al*, 2012; Saha *et al*, 2015; Simmler *et al*, 2014). In a representative study, Saha *et al* (2015) showed that elongating the amine substituent of 4-methyl-*N*-methylcathinone (mephedrone) by one carbon, which forms 4-methyl-*N*-ethylcathinone (4-MEC), produces marked changes in pharmacology. Whereas mephedrone is a nonselective transporter substrate, 4-MEC is an inhibitor at DAT but a substrate at SERT. Thus, 4-MEC displays so-called 'hybrid' transporter activity, characterized by a different molecular mechanism at DAT *vs* SERT. Here we synthesized *N*-methyl, *N*-ethyl, *N*-propyl, and *N*-butyl analogs of 4-MA (see Supplementary Figure 1), and hypothesized that increasing *N*-alkyl chain length would generate hybrid transporter ligands (Blough *et al*, 2014; Saha *et al*, 2015). The mechanism of transporter activity for 4-MA analogs was evaluated using a variety of *in vitro* methods including uptake and release assays in rat brain synaptosomes, measurement of ionic currents in cells expressing monoamine transporters, and detection of calcium influx in cells coexpressing transporters and calcium channels. The effects of 4-MA analogs were also tested *in vivo* using intracranial self-stimulation (ICSS) in rats to measure abuse-related and abuse-limiting effects of drugs (Negus and Miller, 2014). Overall, our findings demonstrate that increasing the length of the *N*-alkyl substituent of 4-MA causes a decrease in potency to inhibit monoamine transporters, a loss of substrate activity at DAT and NET, and a profound reduction in the rewarding effects of the compounds.

MATERIALS AND METHODS

Drugs and Reagents

N-methyl 4-MA was prepared as its hydrobromide salt, whereas *N*-ethyl, *N*-propyl, and *N*-butyl analogs of 4-MA were synthesized as their hydrochloride salts and analytically characterized to ensure purity. Their syntheses and characterization are provided in the Supplementary Methods section. [^{3}H]5-HT (specific activity = 30 Ci/mmol) was purchased from Perkin Elmer (Shelton, CT). [^{3}H]1-Methyl-4-phenylpyridinium ([^{3}H]MPP$^+$, specific activity = 85 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO). All other chemicals and reagents were acquired from Sigma-Aldrich (St Louis, MO) unless otherwise noted. For the ICSS studies, compounds were dissolved in 0.9% sterile saline and administered intraperitoneally (i.p.) at a volume of 1 ml/kg. Drug doses are expressed as the salt forms mentioned above.

Animals

Animal facilities were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, and all animal procedures were carried out in accordance with the Institutional Animal Care and Use Committees and the National Institutes of Health guidelines on care and use of animal subjects (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *et al*, 2011).

Adult male Sprague-Dawley rats (Harlan, Frederick, MD) were used for the synaptosome assays (rats weighing 250–350 g) and for the ICSS procedures (rats weighing 313–360 g at the time of surgery). Rats were group housed with free access to food and water, under a 12 h light/dark cycle with lights on from 0700 to 1900 h. Oocytes for the electrophysiology experiments were harvested and prepared from adult female *Xenopus laevis* females following standard procedures (Hatcher-Solis *et al*, 2014; Koren *et al*, 1990).

Transporter Uptake and Release in Rat Brain Synaptosomes

Rats were killed by CO_2 narcosis, and synaptosomes were prepared from brains as previously described (Rothman *et al*, 2003). Synaptosomes were prepared from caudate tissue for DAT assays, whereas synaptosomes were prepared from whole brain minus caudate and cerebellum for NET and SERT assays. For uptake inhibition assays, 5 nM [^{3}H]dopamine, 10 nM [^{3}H]norepinephrine, and 5 nM [^{3}H]5-HT were used for DAT, NET, and SERT, respectively. To optimize uptake for a single transporter, unlabeled blockers were included that prevented uptake of [^{3}H]transmitter by competing transporters. Uptake inhibition was initiated by incubating synaptosomes with test compound and [^{3}H]transmitter in Krebs-phosphate buffer. Uptake assays were terminated by rapid vacuum filtration and retained radioactivity was quantified with liquid scintillation counting (Baumann *et al*, 2013b). For release assays, 9 nM [^{3}H]MPP$^+$ was used as the radiolabeled substrate for DAT and NET, whereas 5 nM [^{3}H]5-HT was used for SERT. All buffers used in the release assay contained 1 μM reserpine to block vesicular uptake of substrates. The selectivity of release

assays was optimized for a single transporter by including unlabeled blockers to prevent the uptake of $[^3H]MPP^+$ or [^{3}H]5-HT by competing transporters. Synaptosomes were preloaded with radiolabeled substrate in Krebs-phosphate buffer for 1 h. Release assays were initiated by incubating preloaded synaptosomes and test drug. Release was terminated by vacuum filtration and retained radioactivity was quantified by liquid scintillation counting. Effects of test drugs on release were expressed as % maximum release, with maximum release (ie, 100% Emax) defined as the release produced by tyramine at doses that evoke the efflux of all 'releasable' tritium by synaptosomes (10 nM tyramine for DAT and NET assay conditions, and 100 nM tyramine for SERT assay conditions). Effects of test drugs on uptake inhibition and release were analyzed by nonlinear regression using GraphPad Prism 6 (GraphPad Scientific, San Diego, CA). Dose–response values for the uptake inhibition and release were fit to the equation, $Y(x) = Y_{min} + (Y_{max} - Y_{min}) / (1 + 10\exp[(\log P_{50} - \log x)] \times n)$, where x is the concentration of the compound tested, $Y(x)$ is the response measured, Y_{max} is the maximal response, P_{50} is either IC_{50} (the concentration that yields half-maximal uptake inhibition response) or EC_{50} (the concentration that yields half-maximal release), and n is the Hill slope parameter.

Transporter-Mediated Ionic Currents in DAT- or SERT-Expressing Oocytes

Adult *X. laevis* females were anesthetized with 1.5 mg/ml tricaine and 1.5 mg/ml $NaHCO_3$. Oocytes were removed and digested at 22 °C with collagenase type 2 (4 mg/ml, Worthington Biochemical Corporation, Lakewood, NJ), and dissolved in OR2 solution (in mM: 85 NaCl, 5 HEPES, 5 KCl, 5 NaOH, 1 $MgCl_2$-$6H_2O$, pH 7.4) until oocytes were defolliculated. Stage V–VI oocytes were selected for transporter cRNA injection within 24 h of isolation. The cRNA was transcribed from the pOTV vector with the mMessage Machine T7 kit (Ambion, Austin, TX) and oocytes were injected with 18–41.4 ng of either hDAT or hSERT cRNA (Nanoject AutoOocyteInjector, Drummond Scientific, Broomall, PA) and incubated at 18 °C for 4–12 days in Ringers solution supplemented with NaPyruvate (550 μg/ml), streptomycin (100 μg/ml), tetracycline (50 μg/ml), and 5% dialyzed horse serum. All two-electrode voltage-clamp (TEVC) recordings were performed at room temperature (23–25 °C). Electrodes with resistances from 1 to 5 MΩ were filled with 3 M KCl. As previously reported (Rodriguez-Menchaca *et al*, 2012; Solis *et al*, 2012), *X. laevis* oocytes expressing hSERT or hDAT were voltage-clamped to −60 mV and the holding current was recorded using a GeneClamp 500 TEVC amplifier (Axon Instruments, Sunnyvale, CA) and the Clampex 10 software (Molecular Devices, Sunnyvale, CA). External solution consisted of (in mM): 120 NaCl, 7.5 HEPES, 5.4 K Gluconate, 1.2 Ca Gluconate, pH 7.4. Compounds were perfused for durations indicated by solid horizontal lines on traces.

Intracellular Ca^{2+} Flux in Cells Coexpressing Monoamine Transporters and Ca_v Channels

The stable HEK cell lines expressing hDAT or hSERT were previously described (Cameron *et al*, 2015; Ruchala *et al*, 2014), whereas cells expressing hNET (accession number M65105.1) were created using the Flp-In T-REx 293 expression system (Life Technologies, Carlsbad, CA). Cells expressing hDAT, hNET, or hSERT were plated in 96-well imaging plates and were cotransfected with the cardiac isoform of the voltage-gated Ca^{2+} channel, $Ca_V1.2$, its auxiliary subunits, and the EGFP expression plasmid (as the transfection marker) in the following proportion: α_1:β_3:$\alpha_2\delta_1$:EGFP = 1:1:1:0.2. Ca^{2+} measurements were performed 3 days after induction. Acquisition of intracellular Ca^{2+} signals was performed using the Ca^{2+}-sensitive dye Fura-2AM and visualized in an epifluorescence microscope following procedures described previously (Cameron *et al*, 2015; Ruchala *et al*, 2014). In all experiments, a 5 s pulse of dopamine, norepinephrine, and 5HT at a saturating concentration (5–10 μM) was used as the internal positive control for DAT, NET, and SERT, respectively. Peak Ca^{2+} signals induced by test compounds that behaved as substrates were normalized to the peak signal of the positive control. For blockers, the peak signal induced by the transporter's endogenous transmitter mixed with the test drug was normalized to the peak value of the positive control signal in the absence of the test drug. Dose–response values for drugs eliciting transporter-mediated Ca^{2+} signals and for drugs inhibiting Ca^{2+}-evoked responses by transmitters were fit to the equation $Y(x) = Y_{min} + (Y_{max} - Y_{min}) / (1 + 10\exp[(\log P_{50} - \log x)] \times n)$ using GraphPad Prism 6, where x is the concentration of the compound tested, $Y(x)$ is the response measured, Y_{max} is the maximal response, P_{50} is either EC_{50} (the concentration that yields half-maximal Ca^{2+} response) or IC_{50} (the concentration yielding half-maximal inhibition of the transmitter-elicited Ca^{2+} signal), and n is the Hill slope parameter. All Ca^{2+} data are presented as mean ± SEM, except in Figures 3c–e that are presented as mean ± SD.

Intracranial Self-Stimulation in Rats

ICSS studies were conducted as described previously (Bonano *et al*, 2015; Negus and Miller, 2014). Briefly, rats were anesthetized with 3% isoflurane (Webster Veterinary, Phoenix, AZ) and stainless steel electrodes (Plastics One, Roanoke, VA) were stereotaxically implanted into the left medial forebrain bundle and fixed to the skull (Bonano *et al*, 2015; Negus and Miller, 2014). Ketoprofen (5 mg/kg) was used for postoperative analgesia, and animals were single-housed after surgery for at least 7 days of recovery. After recovery, rats were trained to press a lever for pulses of electrical brain stimulation under a frequency-rate procedure (frequencies of 1.75–2.2 log Hz, in ten 0.05 log increments). The drugs and doses tested were *N*-methyl 4-MA (0.1–3.2 mg/kg), *N*-ethyl 4-MA (0.32–3.2 mg/kg), *N*-propyl 4-MA (3.2–32 mg/kg), and *N*-butyl 4-MA (0.32–10 mg/kg). The effects of vehicle (0.9% sterile saline) were determined before and after each drug. Raw reinforcement rates during availability of each brain stimulation frequency were normalized to percent maximum control rate (%MCR) within each rat on each test day, and then averaged across rats. Group mean frequency-rate curves were analyzed by repeated measures two-way ANOVA with ICSS frequency and drug dose as factors. A significant ANOVA was followed by Holm–Sidak multiple comparisons *post hoc* test, and the criterion for statistical significance was $P < 0.05$. As an

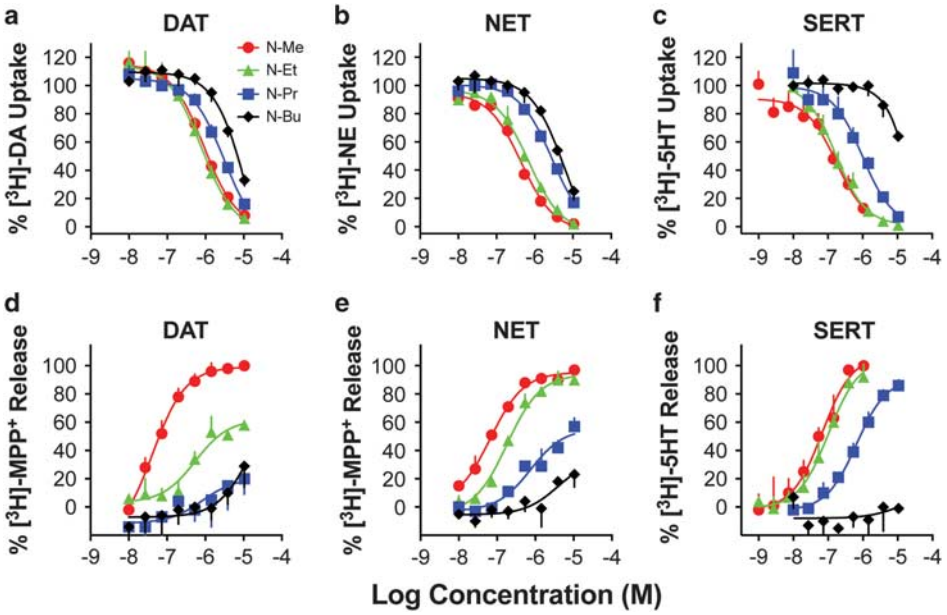

Figure 1 Effects of *N*-substituted 4-MA analogs on monoamine uptake and release in rat brain synaptosomes. Top panels (a–c) show drug effects on uptake inhibition. Synaptosomes were incubated with different concentrations of the *N*-substituted 4-MA analogs in the presence of 5 nM [^{3}H]DA (a, for DAT), 10 nM [^{3}H]NE (b, for NET), or 5 nM [^{3}H]5HT (c, for SERT) and the effect on uptake was plotted as a percent of maximal uptake. Bottom panels (d–f) show drug effects on monoamine release. Synaptosomes were preloaded with 9 nM [^{3}H]MPP$^+$ for DAT (d) and NET (e) or 5 nM [^{3}H]5HT for SERT (f), followed by incubation with different concentrations of the *N*-substituted 4-MA analogs to stimulate release that was plotted as a percent of maximal release elicited by tyramine. EC_{50} and IC_{50} values determined from these curves are reported in Table 1. All points show mean ± SEM of three experiments.

additional summary measure of drug effects on ICSS, the total number of stimulations delivered across all frequencies was also calculated and normalized to baseline data for each drug dose in each rat, and then averaged across rats in each experimental condition.

RESULTS

Uptake and Release Assays in Rat Brain Synaptosomes

Figure 1 depicts the dose–response effects of *N*-substituted 4-MA analogs on uptake inhibition and release at DAT, NET, and SERT in rat brain synaptosomes. Table 1 summarizes drug potencies for uptake inhibition (IC_{50} values) and release (EC_{50} values). In general, the potency for *N*-substituted 4-MA analogs to inhibit uptake at DAT, NET, and SERT decreased in a stepwise manner as *N*-alkyl chain length increased beyond a methyl group (Figures 1a–c). The potency for *N*-butyl 4-MA at SERT was so weak that an IC_{50} value could not be determined. The potency for 4-MA analogs to promote release also decreased as the *N*-substitution was elongated. However, the most dramatic effect of increasing *N*-alkyl chain length on releasing activity was reduction in efficacy (ie, flattening of the dose–response curves), especially at DAT and NET (Figures 1d–f). Although *N*-methyl 4-MA elicited maximal release efficacy at DAT, the release efficacies of *N*-ethyl, *N*-propyl, and *N*-butyl analogs at DAT were 58%, 20%, and 25% of maximal release, respectively. In a similar manner, *N*-methyl and *N*-ethyl 4-MA produced maximal release at NET, but *N*-propyl and *N*-butyl displayed reduced efficacies of 57% and 23% of maximal, respectively. Finally, all of the analogs produced fully efficacious release at SERT, with the exception of *N*-butyl 4-MA that produced no observable effects, perhaps because of its low potency at SERT. The data from synaptosomes provided evidence that the *N*-ethyl and *N*-propyl analogs of 4-MA might display hybrid transporter activity, acting as inhibitors at DAT but substrates at SERT. Definitive determination of inhibitor *vs* substrate activity of 4-MA analogs required additional approaches examining transporters in cells.

Transporter-Mediated Ionic Currents in Transporter-Expressing Oocytes

We evaluated the electrophysiological effect of the test compounds at hDAT and hSERT in *X. laevis* oocytes expressing these transporters and voltage-clamped to −60 mV. As noted previously, transporter substrates induce inward depolarizing currents whereas inhibitors induce outward currents (Solis, 2017; Solis *et al*, 2012). Figure 2 shows the effects of 4-MA analogs on transporter-mediated currents. When examining effects at hDAT, *N*-methyl 4-MA induced an inward depolarizing current indicative of substrate activity, whereas *N*-ethyl, *N*-propyl, and *N*-butyl analogs induced outward currents consistent with nontransported inhibitor activity (Figures 2a–d). All analogs of 4-MA induced inward currents at SERT, but the magnitude of response varied (Figures 2e–h). SERT-mediated currents for *N*-methyl 4-MA and *N*-ethyl 4-MA were much larger than the effect of the endogenous substrate 5-HT, whereas *N*-propyl 4-MA induced a current similar in magnitude to the 5-HT-induced current. *N*-butyl 4-MA induced a small but detectable inward current at SERT, characteristic of a substrate. These results show that elongating the *N*-alkyl chain of 4-MA changes drug mechanism at DAT from

Table 1 IC_{50} and EC_{50} Values (nM) for *N*-Substituted 4-MA Analogs to Inhibit Uptake or Stimulate Release at Monoamine Transporters in Rat Brain Synaptosomes or to Block Monoamine-Induced Ca^{2+} Flux or Stimulate Ca^{2+} Flux in Flp-In T-REx 293 Cells

	***N*-methyl 4-MA**	***N*-ethyl 4-MA**	***N*-propyl 4-MA**	***N*-butyl 4-MA**
Rat brain synaptosomes				
DAT uptake, IC_{50}	910 ± 38	795 ± 71	3064 ± 208	13 660 ± 2050
NET uptake, IC_{50}	438 ± 24	685 ± 45	2916 ± 183	6009 ± 701
SERT uptake, IC_{50}	171 ± 23	166 ± 17	984 ± 124	*
DAT release, EC_{50} (%)	41 ± 3.1 (100)	550 ± 75 (58)	– (20)	– (29)
NET release, EC_{50} (%)	67 ± 4.8 (97)	182 ± 11 (90)	742 ± 98 (57)	– (23)
SERT release, EC_{50} (%)	67 ± 7.8 (100)	102 ± 9.0 (92)	650 ± 51 (86)	– (0)
Cells coexpressing transporters/channels				
DAT blockade, IC_{50}		4353 ± 612	18 260 ± 3600	60 960 ± 12 640
NET blockade, IC_{50}			14 350 ± 3560	30 280 ± 11 710
SERT blockade, IC_{50}				*
DAT influx, EC_{50}	208 ± 21.0			
NET influx, EC_{50}	252 ± 39.6	728 ± 184		
SERT influx, EC_{50}	479 ± 104	683 ± 90	2444 ± 388	

All IC_{50} and EC_{50} values were derived from data shown in Figures 1 and 3. *Indicates an IC_{50} that could not be calculated. The lower limit of release efficacy is considered 30% of maximal, and no EC_{50} values were obtained for efficacies below this level (marked as '–'). For the transporter/Ca^{2+} channel assay, either EC_{50} or IC_{50} values were calculated for each compound depending on their action on the transporter.

substrate to inhibitor, whereas all analogs behave as SERT substrates regardless of *N*-alkyl chain length.

Transporter-Mediated Depolarization in Cells Coexpressing Transporters and Ca^{2+} Channels

To further explore the nature of the interactions of 4-MA analogs with monoamine transporters, we employed HEK cells expressing hDAT, hNET, or hSERT in combination with the voltage-gated Ca^{2+} channel $Ca_v1.2$ (Cameron *et al*, 2015; Ruchala *et al*, 2014). In this assay, transporter substrates elicit transporter-mediated depolarizing currents that activate Ca^{2+} channels, allowing Ca^{2+} influx and generation of a traceable Ca^{2+} signal. As transporter inhibitors lack transporter-mediated depolarizing currents, Ca^{2+} channels will not be activated and no Ca^{2+} signal will be produced by inhibitors. Figure 3a depicts representative raw data showing *N*-methyl 4-MA elicits a dose-dependent Ca^{2+} signal similar to the effects of dopamine that confirms its activity as a DAT substrate. Figure 3b shows that *N*-ethyl 4-MA is unable to produce a Ca^{2+} signal at hDAT, indicating its lack of activity as a substrate, agreeing with the electrophysiological results showing this drug is a nontransported inhibitor at hDAT. Importantly, Figure 3b also demonstrates that *N*-ethyl 4-MA is able to inhibit the Ca^{2+} signal produced by dopamine in a dose-dependent manner. Using this assay, we determined whether the *N*-substituted 4-MA analogs behaved as substrates or inhibitors at the three monoamine transporters. As summarized in Figures 3c–e, transporter substrates produced dose-dependent depolarization-induced Ca^{2+} signals that are depicted as upward curves, whereas inhibitors produced dose-dependent reductions in Ca^{2+} signals elicited by endogenous transmitters, and are depicted as downward curves (raw data are shown in Supplementary Figure 2 and potencies are shown in Table 1). For hDAT experiments, elongating the *N*-alkyl chain greater than an *N*-methyl group converted the drug mechanism from substrate to inhibitor. Similarly, for hNET, elongating the *N*-alkyl chain greater than an *N*-ethyl flipped the drug mechanism from substrate to inhibitor. Importantly, *N*-alkyl chain length did not alter substrate activity of compounds at SERT. Similar to the hSERT currents observed in oocytes, lengthening the *N*-alkyl chain of 4-MA only diminished the magnitude of the Ca^{2+} response. *N*-butyl 4-MA acted as a substrate but was so weak that a potency measure could not be acquired (Supplementary Figure 2l). It is noteworthy that 4-MA analogs that acted as low-efficacy partial releasers at DAT and NET in rat brain synaptosomes acted as inhibitors in the Ca^{2+} assays shown here.

We next wished to correlate the findings from Ca^{2+} assays in cells with the data obtained from rat brain synaptosomes. To this end, we first classified 4-MA analogs as either transporter inhibitors or substrates based on the findings from the Ca^{2+} flux assays; it is noteworthy that using this classification scheme, low-efficacy partial releasers in the synaptosome assays (eg, *N*-ethyl 4-MA at DAT) are considered transporter inhibitors. For those compounds that behaved as inhibitors or low-efficacy releasers in the rat synaptosome assays and inhibitors in the Ca^{2+} assays, we found a significant correlation between the IC_{50} values obtained from both methods (Figure 4a). Similarly, for the compounds that behaved as fully efficacious substrates in the rat synaptosome and Ca^{2+} assays, we found a significant correlation between the EC_{50} values obtained from both methods (Figure 4b).

Intracranial Self-Stimulation in Rats

As a means to assess the abuse-related effects of 4-MA analogs, we tested these agents in a rat ICSS procedure.

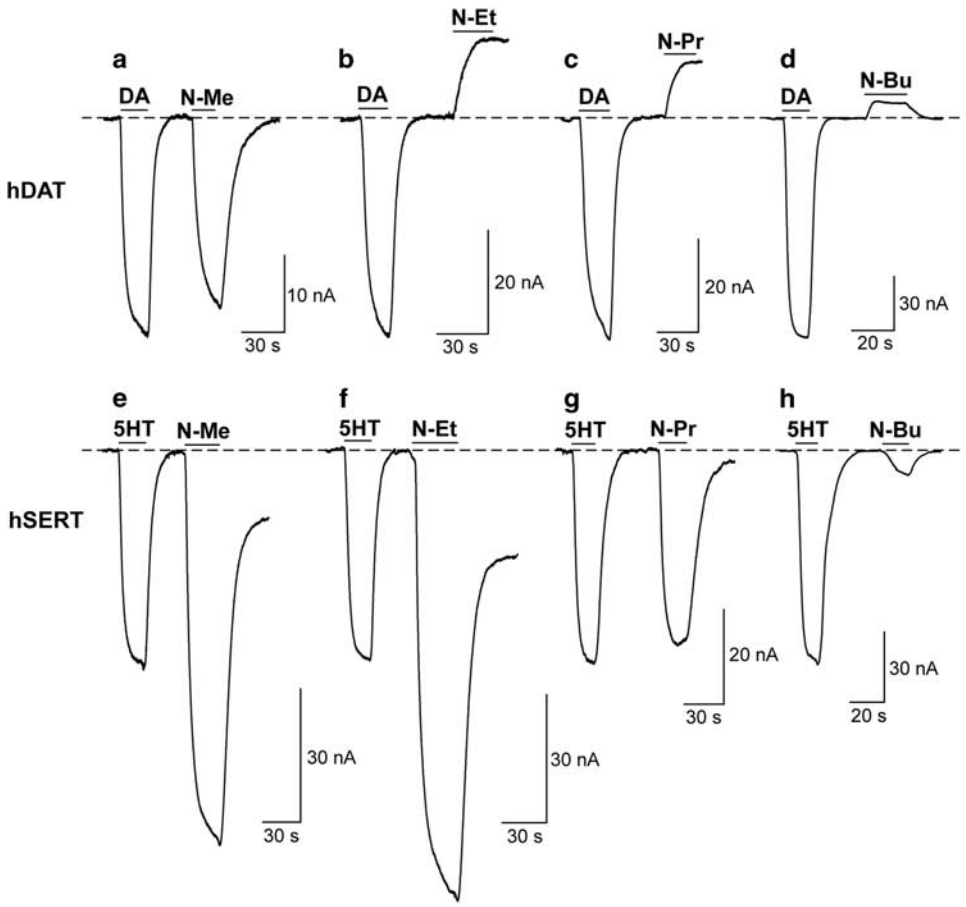

Figure 2 Effect of *N*-substituted 4-MA analogs on membrane currents in oocytes transfected with hDAT or hSERT. In voltage-clamped ($V_{com} = -60$ mV) hDAT-expressing oocytes 10 μM *N*-methyl 4-MA produces an inward current (a), and 10 μM *N*-ethyl 4-MA (b), *N*-propyl 4-MA (c), and *N*-butyl 4-MA (d) display a block of the endogenous hDAT leak current. For comparison, individual traces are adjusted to match the inward current in response to hDAT endogenous substrate DA (5 μM, shown before application of the *N*-substituted 4-MA analogs) (a–d). In voltage-clamped ($V_{com} = -60$ mV) hSERT-expressing oocytes, all *N*-substituted 4-MA analogs produce inward currents (e–h); however, 10 μM *N*-methyl 4-MA (e) and *N*-ethyl 4-MA (f) display large responses, *N*-propyl 4-MA displays a moderate response (g), and *N*-butyl 4-MA (h) produces a small response. For comparison, individual traces are adjusted to match an inward current in response to hSERT endogenous substrate 5HT (5 μM, shown before application of the *N*-substituted 4-MA analogs) (e–h). Horizontal dotted lines along the baseline (ie, holding current) for hDAT and hSERT recordings are added to compare compound responses.

Figure 5 shows effects of *N*-substituted 4-MA analogs on ICSS in rats. The average ± SEM baseline MCR was 62.53 ± 2.48 stimulations per trial, and the mean ± SEM number of total baseline stimulations was 296.90 ± 18.40 stimulations per component. After vehicle administration, electrical brain stimulation maintained a frequency-dependent increase in ICSS rates (Figures 5a-d, dotted lines), and frequency-rate curves after vehicle treatment were not different from baseline curves determined before vehicle (data not shown). All four *N*-alkylated 4-MA analogs produced dose-dependent changes in ICSS, but both the potency and the extent of abuse-related and presumably dopamine-mediated facilitation of ICSS rates tended to decrease as *N*-alkyl chain length increased. Figures 5a and e show that *N*-methyl 4-MA produced exclusive facilitation of ICSS rates (1.9–1.95 log Hz) at 0.32 mg/kg, whereas a higher dose of 1.0 mg/kg produced mixed effects consisting of facilitation of ICSS rates at low stimulation frequencies (1.8–1.95 log Hz) and depression of ICSS rates at high frequencies (2.15–2.2 log Hz). The highest dose of 3.2 mg/kg *N*-methyl 4-MA produced only depression of ICSS rates. Figures 5b and f show that *N*-ethyl 4-MA produced mixed ICSS effects at 1.0 mg/kg (facilitation of ICSS rates at 1.9–1.95 log Hz, depression of ICSS rates at 2.2 log Hz) but only depression of ICSS rates at 3.2 mg/kg (2.0–2.2 log Hz). Both *N*-propyl (3.2–32 mg/kg; Figures 5c and g) and *N*-butyl (0.32–10 mg/kg; Figures 5d and h) analogs produced only dose-dependent depression of ICSS rates across a range of intermediate to high brain stimulation frequencies.

DISCUSSION

The main purpose of the present investigation was to examine the effects of lengthening the amine substituent of 4-MA on drug interactions at monoamine transporters and the resulting behavioral consequences. We observed that increasing the *N*-alkyl chain length of 4-MA from *N*-methyl to *N*-butyl decreased the potency to inhibit uptake at DAT, NET, and SERT in rat brain synaptosomes. Similarly, the potency of substrate activity at monoamine transporters decreased as the *N*-alkyl group was elongated, but the efficacy to promote release in synaptosomes differed

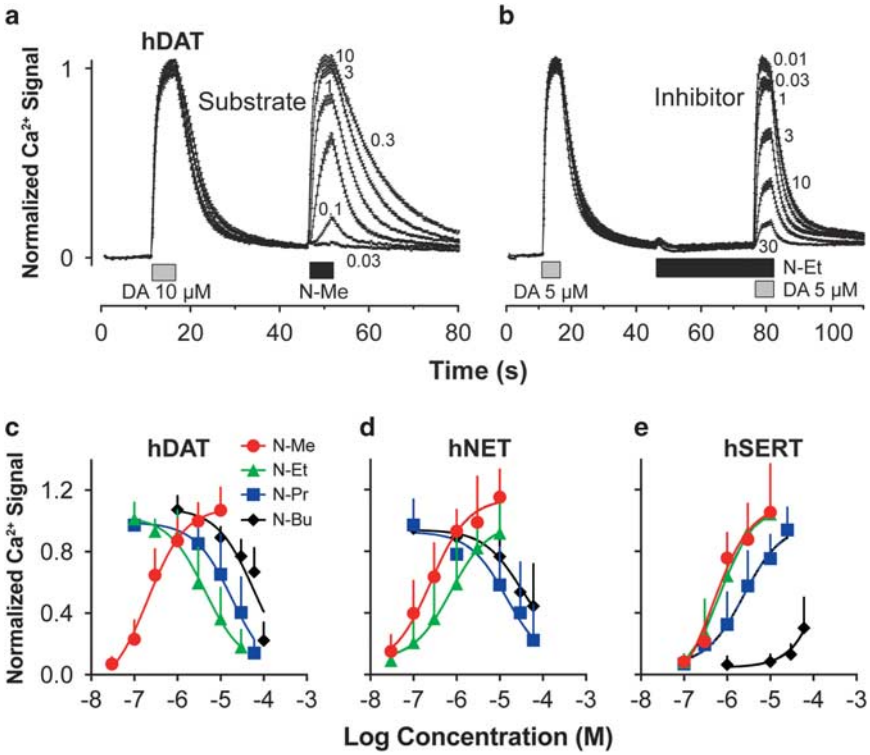

Figure 3 Effects of *N*-substituted 4-MA analogs on hDAT-, hNET-, and hSERT-mediated L-type Ca^{2+} signals in Flp-In T-REx 293 cells. Intracellular Ca^{2+} levels were determined by fluorescence microscopy using the Ca^{2+}-sensitive dye Fura2AM in Flp-hDAT, Flp-hNET, or Flp-hSERT cells that were cotransfected with $Ca_v1.2$ and the β_3, $\alpha_2\delta$, and EGFP plasmids 3 days before each experiment. (a) In cells coexpressing hDAT and $Ca_v1.2$ channels, a Ca^{2+} signal is produced in response to cell depolarization induced by a depolarizing hDAT-mediated current (produced with 10 μM DA). *N*-methyl 4-MA (0.03–10 μM) elicits Ca^{2+} signals of increasing magnitude. (b) In cells coexpressing hDAT and $Ca_v1.2$ channels, *N*-ethyl 4-MA does not produce a Ca^{2+} signal regardless of concentration, but *N*-ethyl 4-MA (0.01–30 μM) is able to inhibit DA-induced Ca^{2+} signals in a dose-dependent manner. (c–e) For *N*-substituted 4-MA analogs that behaved as substrates, the dose responses to produce Ca^{2+} signals were obtained (*N*-methyl 4-MA at hDAT; *N*-methyl and *N*-ethyl 4-MA at hNET; all 4 analogs at hSERT); for analogs that behaved as inhibitors (*N*-methyl, *N*-ethyl, and *N*-propyl 4-MA at hDAT; *N*-methyl and *N*-ethyl 4-MA at hNET), the dose responses to inhibit Ca^{2+} signals induced by the endogenous transmitter were obtained. EC_{50} values were determined from a two-pulse protocol in which exposure to a fixed concentration of the endogenous transmitter for each transporter (DA, NE, and 5HT for hDAT, hNET, and hSERT, respectively) was followed by a brief exposure to increasing concentrations of an *N*-substituted 4-MA analog. IC_{50} values were determined from a two-pulse protocol in which brief exposure of a fixed concentration of the endogenous transmitter for each transporter was followed by exposure to increasing concentrations of the *N*-substituted 4-MA analogs for 30 s (yielding no response) and coapplication of the endogenous transmitter. Dose–response curves were obtained for both depolarizing and inhibition induced by the *N*-substituted 4-MA analogs at hDAT, hNET, and hSERT by fitting the responses to the equation described in methods. EC_{50} and IC_{50} values for all drug–transporter interactions are summarized in Table 1. The data shown in (a) and (b) are mean ± SEM, and in (c–e) are mean ± SD.

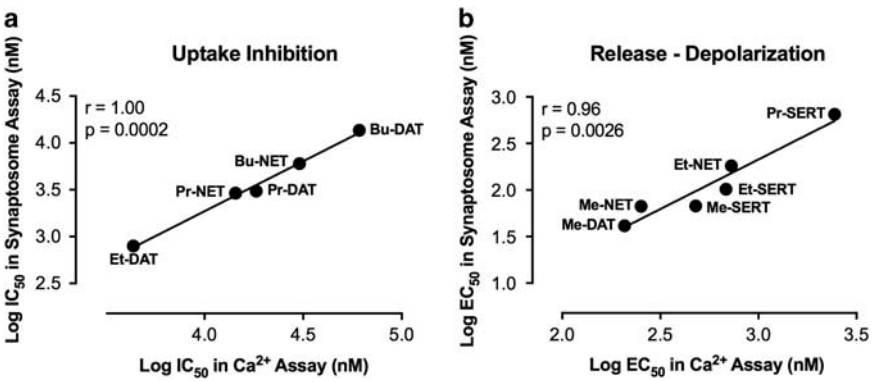

Figure 4 Correlations of rat synaptosome and fluorometric Ca^{2+} channel assays. Data used for these correlations were obtained from experiments performed in the rat synaptosome and the fluorometric transporter-Ca^{2+} channel assays testing the effects of *N*-substituted 4-MA analogs on transporters (Figures 1 and 3 and Table 1). (a) Log IC_{50} values from uptake inhibition in the synaptosome assay are correlated to log IC_{50} values from the Ca^{2+} assay. Only values from compound–transporter combinations that elicited clear inhibitor activity in the Ca^{2+} assay were used. (b) Log EC_{50} values obtained from the synaptosome release assay are correlated to log EC_{50} values from the transporter-Ca^{2+} channel assay. Values used were from compound–transporter combinations that promoted maximal or near-maximal release in the synaptosome assay and evoked prominent Ca^{2+} signals in response to transporter-mediated depolarization.

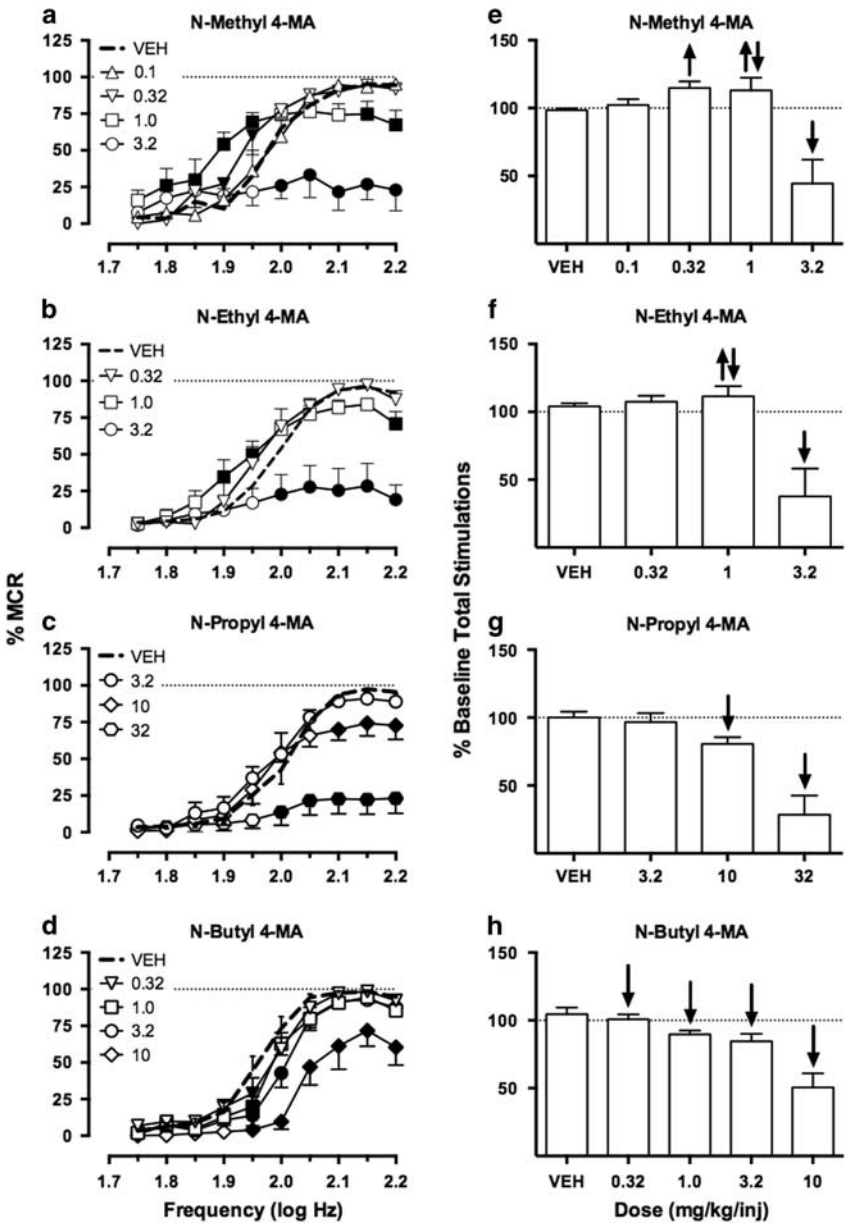

Figure 5 Effects of *N*-substituted 4-MA analogs on ICSS in rats. Left panels (a–d) show full ICSS frequency-rate curves. Abscissae indicate brain stimulation frequency in log Hz. Ordinates indicate percent maximum control response rate (%MCR). Filled points show drug effects significantly different from VEH treatment as determined by two-way ANOVA followed by Holm–Sidak *post hoc* test ($p < 0.05$). For all panels, there were significant main effects of frequency ($p < 0.001$) and dose ($p < 0.001$) (data not shown). Frequency–dose interactions were also significant (all $p < 0.001$), and ANOVA results are as follows: *N*-methyl 4-MA, $F(36, 180) = 9.91$, *N*-ethyl 4-MA, $F(36, 180) = 6.38$, *N*-propyl 4-MA, $F(36, 180) = 7.01$, and *N*-butyl 4-MA, $F(36, 180) = 2.93$. Right panels (e–h) show summary data for the total number of stimulations delivered across all brain-stimulation frequencies. Abscissae indicate drug dose in mg/kg. Ordinates indicate total number of stimulations per component expressed as percent baseline (% baseline total stimulations). Upward/downward arrows indicate that ICSS rates were significantly increased/decreased for at least one brain-stimulation frequency as determined by analysis of full frequency-rate curves in the left panels. All points and bars show mean ± SEM in six rats.

markedly across the three transporters. In general, increasing the *N*-alkyl chain tended to convert DAT and NET substrates to inhibitors. For instance, when the amine substituent was lengthened from *N*-methyl to *N*-ethyl, release efficacy at DAT dropped from ~100% to ~60% of maximal, respectively. *N*-propyl and *N*-butyl analogs displayed <30% releasing efficacy at DAT consistent with the effects of DAT inhibitors. In contrast, *N*-methyl, *N*-ethyl, and *N*-propyl 4-MA displayed maximal or near-maximal release efficacy (80–100% of maximal) at SERT consistent with the profile of substrates. *N*-butyl 4-MA appeared to display no releasing activity in synaptosomes, but this lack of effect was most likely because of its extremely low potency. The structure–activity findings shown here are consistent with prior data demonstrating that increasing the steric bulk of amine substituents on amphetamine analogs converts substrates at DAT and NET to nontransported inhibitors (Rothman *et al*, 2012; Sandtner *et al*, 2016).

Previous investigations in synaptosomes and transporter-expressing cells have shown that potent uptake inhibitors can induce low-efficacy efflux of preloaded [^{3}H]MPP$^+$ in release assays, in the range of 20–30% of maximal releasing response (Baumann *et al*, 2013b; Scholze *et al*, 2000). Low-efficacy efflux of this magnitude, or 'pseudoefflux', is due to the diffusion of [^{3}H]MPP$^+$ out of synaptosomes that is unmasked in the presence of transporter blockade. Under these circumstances, the effects of transporter inhibitors in release assays could be erroneously interpreted as *bona fide* transporter-mediated reverse transport. Other studies in rat brain synaptosomes have identified transporter ligands exhibiting more substantial 'partial releasing' effects, in the range of 50–75% of maximal releasing response, similar to the effects of *N*-ethyl 4-MA at DAT observed here (Rothman *et al*, 2012; Sandtner *et al*, 2016). As a specific example, we demonstrated previously that 3,4-methylenedioxyethamphetamine (MDEA), the *N*-ethyl analog of 3,4-methylenedioxyamphetamine (MDA), induces partial release at DAT with ~60% efficacy (Rothman *et al*, 2012). The precise molecular underpinnings of transporter-mediated partial release are not fully understood, but our prior data provide compelling evidence that the blunted releasing efficacy of MDEA at DAT is because of a much slower rate of [^{3}H]MPP$^+$ efflux (ie, a slower rate of reverse transport) when compared with full-efficacy releasers like MDA. The present findings with *N*-ethyl 4-MA reveal an important caveat of the synaptosome assays: methods using rat brain tissue cannot definitively discriminate transporter inhibitors from substrates when drugs display partial releasing actions. To further explore the mechanism of action of 4-MA analogs at transporters, we employed additional methods that measure different aspects of transporter activity.

By using the TEVC technique in *X. laevis* oocytes overexpressing monoamine transporters, we previously showed that transportable substrates produce large Na^+-dependent inward currents, whereas nontransported inhibitors do not. In fact, transporter inhibitors evoke an apparent outward current because of blockade of the endogenous transporter 'leak' current (Cameron *et al*, 2013; Rodriguez-Menchaca *et al*, 2012; Solis, 2017; Solis *et al*, 2012). As expected, compounds eliciting maximal release efficacy in synaptosomes (eg, *N*-methyl 4-MA at DAT) also produced large inward currents through hDAT. In contrast, *N*-ethyl 4-MA displayed outward currents at DAT consistent with a nontransported inhibitor, and elongating to *N*-propyl or *N*-butyl 4-MA induced sequentially smaller inhibitor-like currents at DAT. The inhibitor-like outward currents produced by *N*-ethyl 4-MA in DAT-expressing oocytes might seem at odds with the partial substrate actions of this compound at DAT in synaptosomes. As noted above, partial releasing properties of *N*-ethyl analogs like MDEA in synaptosomes are characterized by a substantially slower rate of DAT-mediated efflux when compared with fully efficacious releasers. Thus, one possible explanation for the discordant findings with *N*-ethyl 4-MA in oocytes *vs* synaptosomes could be related to the different time courses for each assay. More specifically, the oocyte assays measure transporter-mediated currents on the timescale of seconds, whereas the synaptosome assays measure [^{3}H]substrate efflux on the timescale of minutes. Because of the abbreviated time window of electrophysiological measurements, it seems feasible that only the inhibitor activity of 4-ethyl 4-MA at DAT is observed under the oocyte assay conditions. Other explanations for discrepancies in the data between oocytes and synaptosomes could be the existence of species differences in responsiveness to specific transporter ligands, or intermediate conformational states in transporter proteins that allow some reverse transport in the absence of an inward current. Further investigations are required to address these intriguing possibilities.

In contrast to the divergent effects of 4-MA analogs on DAT-mediated currents, all of the 4-MA analogs produced inward currents consistent with transporter substrates in hSERT-expressing oocytes. Although *N*-butyl 4-MA displayed no discernable activity at SERT in the synaptosome release assay, it elicited a small inward current through hSERT in oocytes indicative of substrate activity. Although the TEVC technique provides detailed mechanistic information about the action of compounds on transporters, there are significant drawbacks. For instance, hSERT is efficiently expressed in oocytes, whereas hDAT shows significant expression variability among oocyte batches. Moreover, hNET activity is undetectable in oocytes because it does not properly incorporate into oocyte plasma membranes. Thus, the oocyte TEVC system cannot be used for comparison of drug effects across DAT, NET, and SERT.

To overcome limitations of the synaptosome assays and TEVC methods, we employed a fluorometric assay that takes advantage of the electrogenic nature of transporter proteins (Cameron *et al*, 2015; Ruchala *et al*, 2014). This assay consists of HEK cell lines that coexpress either hDAT, hNET, or hSERT along with voltage-gated Ca^{2+} channels (VGCC) and uses the Fura2 dye to measure changes in Ca^{2+} influx into individual cells. In recent work, we demonstrated that transporter-mediated currents induced by substrates are strong enough to evoke opening of L-type Ca^{2+} channels in expression systems where transporters and Ca^{2+} channels are coexpressed (Cameron *et al*, 2015; Ruchala *et al*, 2014). Because monoamine transporter currents are electrically coupled to the opening of voltage-gated Ca^{2+} channels, the magnitude of Ca^{2+} influx is proportional to the concentration of substrate drug applied, allowing for the generation of reliable dose–response curves and potencies of different drugs to be determined (Cameron *et al*, 2015). Transporter inhibitors do not induce depolarizing currents and do not produce a Ca^{2+} signal, but they are able to block Ca^{2+} signals induced by substrates (Cameron *et al*, 2015; Ruchala *et al*, 2014). Using this assay, we first characterized the molecular mechanism of action for *N*-substituted 4-MA analogs at monoamine transporters, and used these results to classify the compounds as either transporter substrates or inhibitors. We then performed dose–response experiments in which we determined EC_{50} values for substrate-type drugs and IC_{50} values for inhibitor-type drugs. Importantly, the compounds that elicited maximal releasing efficacy in synaptosomes and inward currents in oocytes also elicited depolarization-induced Ca^{2+} responses in the fluorescence assay compounds. In contrast, the compounds that behaved as transporter inhibitors or partial releasers in synaptosomes, and behaved as blockers in the electrophysiological assay, did not elicit Ca^{2+} signals in the fluorescence assay. *N*-butyl 4-MA, which lacked measurable potency as a SERT substrate in synaptosomes, induced an inward current in oocytes and a

positive response in the fluorometric Ca^{2+} assay. Importantly, the small outward hDAT current induced by *N*-butyl 4-MA in the TEVC experiments was substantiated by inhibition of dopamine-induced Ca^{2+} increases. These results highlight the sensitivity of the Ca^{2+} fluorescence assay to determine the molecular mechanism of action for compounds at monoamine transporters, even those with weak potency that cannot be effectively characterized in the synaptosome-based assays. It is worth mentioning that the differences in potency across the rat synaptosome and the transporter/Ca^{2+} channel fluorescence assays are likely because of technical differences in methods employed. For example, the IC_{50} value for a compound blocking a Ca^{2+} response elicited by a transmitter depends on the concentration of the transmitter being inhibited (ie, if a lower concentration of transmitter is competed off by a compound, the IC_{50} value would be more potent).

When we used the results from the transporter/Ca^{2+} channel assays to classify compounds as either transporter inhibitors or substrates at DAT, NET, and SERT, we observed remarkable correlations between the potencies obtained from the Ca^{2+} assays and rat synaptosome methods. More specifically, the IC_{50} values for transporter inhibitors in the Ca^{2+} fluorescence assays were positively correlated with the IC_{50} values for transporter inhibitors and partial substrates in the synaptosome uptake inhibition assays. Similarly, the EC_{50} values for transporter substrates in the Ca^{2+} fluorescence assays were positively correlated with fully efficacious substrates in the synaptosome assays. Such correlations validate the Ca^{2+} fluorescence assay as a suitable and complementary technique to study monoamine transporter pharmacology as compared with the established [^{3}H] transmitter uptake inhibition and release assays. In addition, the fluorometric transporter-Ca^{2+} channel assay has the added advantage to accurately discern the mechanism of action of a compound, possesses high signal-to-noise ratio, and is amenable to studying dose–response effects for many compounds. From the correlation results, we can make the case that monoamine transporters from distinct mammalian species (rats *vs* humans) interact similarly with substrates and blockers that differs from drug interactions with nonmammalian monoamine transporters. For example, cocaine has different actions at drosophila DAT and hDAT (Porzgen *et al*, 2001), and chicken SERT is much less sensitive to reuptake inhibitors than hSERT (Larsen *et al*, 2004).

Previous studies have identified DAT *vs* SERT selectivity as one important determinant of abuse-related effects of monoamine transporter ligands in ICSS procedures (Bauer *et al*, 2013; Bonano *et al*, 2014, 2015; Hutsell *et al*, 2016; Miller *et al*, 2015; Rosenberg *et al*, 2013), as well as in other behavioral procedures, such as drug self-administration (Czoty *et al*, 2002; Negus *et al*, 2007; Rothman *et al*, 2008; Wang and Woolverton, 2007). In general, transporter ligands with greater DAT selectivity are associated with abuse-related effects, whereas those with greater SERT selectivity tend to produce abuse-limiting effects. The behavioral effects of *N*-substituted 4-MA analogs in the ICSS experiments were generally consistent with their function at DAT and SERT, and selectivity for DAT *vs* SERT. In particular, *N*-methyl 4-MA produced the most potent and robust abuse-related facilitation of ICSS rates, whereas higher doses engendered rate-decreasing effects. This behavioral profile is consistent with the function of *N*-methyl 4-MA as a nonselective transporter substrate with somewhat greater potency at DAT. The expression of abuse-related effects and overall drug potency declined with increasing *N*-alkyl chain length that was associated with: (1) loss of selectivity for DAT *vs* SERT, (2) overall loss of potency at all transporters, and (3) change in function from DAT substrate to inhibitor. Thus, in comparison with *N*-methyl 4-MA, *N*-ethyl 4-MA produced weaker and less potent facilitation of ICSS rates, but displayed similar potency for depression of ICSS rates. This profile is consistent with the function of *N*-ethyl 4-MA as a DAT blocker with lower DAT potency than *N*-methyl 4-MA and its inverted selectivity for DAT $<$ SERT. *N*-propyl 4-MA produced only low-potency depression of ICSS rates, agreeing with its low DAT potency and inverted DAT $<$ SERT selectivity. Finally, *N*-butyl 4-MA also produced only depression of ICSS rates, consistent with its inactivity at DAT; however, *N*-butyl 4-MA also displayed very low potency at SERT. The finding that *N*-butyl 4-MA was more potent at depressing ICSS than *N*-propyl 4-MA, despite having weaker SERT potency, suggests that *N*-butyl 4-MA induced depression of ICSS rates independent of SERT.

In summary, the converging lines of evidence presented here demonstrate that lengthening the *N*-alkyl chain of 4-MA decreases potency to inhibit monoamine transporters. Perhaps more importantly, elongating the *N*-alkyl chain greater than a methyl group converts DAT substrates to inhibitors, whereas elongating the chain greater than an ethyl group converts NET substrates to inhibitors. Thus, the *N*-ethyl, *N*-propyl, and *N*-butyl analogs of 4-MA display hybrid transporter activity characterized by differential mechanisms at DAT, NET, and SERT for the same drug molecule (Blough *et al*, 2014; Saha *et al*, 2015). It is important to note that elucidating the complex pharmacology of 4-MA compounds could not have been accomplished without the multipronged approach utilized in our study. Although synaptosome assays provide physiologically relevant data from native tissue, and can discriminate fully efficacious transporter releasers from transporter inhibitors, these methods cannot discern the precise mechanism of action for drugs acting as partial releasers. The electrophysiological and Ca^{2+} fluorescence assays employed here demonstrate that partial releasers in the synaptosome assays can function as pure transporter inhibitors in cells expressing human transporters. Determining the molecular mechanisms responsible for the apparent disparities between the effects of partial releasers in native tissue preparations *vs* their effects in cell-based systems warrants further study. The *in vivo* ICSS data corresponded well with the *in vitro* data as increasing alkyl chain length reduced abuse-related effects of drugs in parallel with decreased DAT potency. Overall, our findings indicate that the *N*-alkylated analogs of 4-MA display less abuse liability than the parent compound. Given the continued appearance of stimulant-like NPS in the street drug marketplace, future investigations are warranted to evaluate the pharmacology and toxicology of these substances using an experimental approach that employs complementary *in vitro* and *in vivo* methods.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)