Direct and Systemic Administration of a CNS-Permeant Tamoxifen Analog Reduces Amphetamine-Induced Dopamine Release and Reinforcing Effects

Colleen Carpenter[1,5], Alexander G Zestos[1,2,5], Rachel Altshuler[1], Roderick J Sorenson[3,4], Bipasha Guptaroy[1], Hollis D Showalter[3,4], Robert T Kennedy[1,2], Emily Jutkiewicz[1] and Margaret E Gnegy*,[1]

[1]Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA; [2]Department of Chemistry, University of Michigan, Ann Arbor, MI, USA; [3]Department of Medicinal Chemistry, University of Michigan, Ann Arbor, MI, USA; [4]Vahlteich Medicinal Chemistry Core, University of Michigan, Ann Arbor, MI, USA

Amphetamines (AMPHs) are globally abused. With no effective treatment for AMPH addiction to date, there is urgent need for the identification of druggable targets that mediate the reinforcing action of this stimulant class. AMPH-stimulated dopamine efflux is modulated by protein kinase C (PKC) activation. Inhibition of PKC reduces AMPH-stimulated dopamine efflux and locomotor activity. The only known CNS-permeant PKC inhibitor is the selective estrogen receptor modulator tamoxifen. In this study, we demonstrate that a tamoxifen analog, **6c**, which more potently inhibits PKC than tamoxifen but lacks affinity for the estrogen receptor, reduces AMPH-stimulated increases in extracellular dopamine and reinforcement-related behavior. In rat striatal synaptosomes, **6c** was almost fivefold more potent at inhibiting AMPH-stimulated dopamine efflux than [^{3}H]dopamine uptake through the dopamine transporter (DAT). The compound did not compete with [^{3}H]WIN 35,428 binding or affect surface DAT levels. Using microdialysis, direct accumbal administration of 1 μM **6c** reduced dopamine overflow in freely moving rats. Using LC-MS, we demonstrate that **6c** is CNS-permeant. Systemic treatment of rats with 6 mg/kg **6c** either simultaneously or 18 h prior to systemic AMPH administration reduced both AMPH-stimulated dopamine overflow and AMPH-induced locomotor effects. Finally, 18 h pretreatment of rats with 6 mg/kg **6c** s.c. reduces AMPH-self administration but not food self-administration. These results demonstrate the utility of tamoxifen analogs in reducing AMPH effects on dopamine and reinforcement-related behaviors and suggest a new avenue of development for therapeutics to reduce AMPH abuse.

INTRODUCTION

Amphetamine (AMPH) and its congeners are highly addictive stimulants and their abuse remains a significant health, social, and economic burden (Berman *et al*, 2008; Carvalho *et al*, 2012). Yet an effective treatment for AMPH abuse remains elusive. Similar to other drugs of abuse, the reinforcing effects of AMPH are attributed to its ability to significantly increase extracellular dopamine in the nucleus accumbens (Di Chiara and Imperato, 1988; Wise and Bozarth, 1985). AMPH achieves this effect through its action at the dopamine transporter (DAT). The primary role of DAT is to clear extracellular dopamine, thereby terminating presynaptic and postsynaptic dopamine signaling (Zhu and

Reith, 2008). AMPH, a substrate of DAT, disrupts this process by competitively blocking dopamine reuptake and also promoting reverse transport of dopamine via DAT (McMillen, 1983). Unlike stimulants such as cocaine, whose actions are more reliant on storage pools of monoamines, the release of newly synthesized dopamine also contributes to AMPH action (Chiueh and Moore, 1975; Parker and Cubeddu, 1986).

We found that protein kinase C (PKC) enhances AMPH-stimulated dopamine efflux. AMPH increases striatal particulate PKC activity (Giambalvo, 1992, 2004) and PKC stimulates the phosphorylation of N-terminal DAT residues (Foster *et al*, 2002). Phosphorylation of DAT is permissive for AMPH-stimulated dopamine release (Khoshbouei *et al*, 2004; Wang *et al*, 2016). Selective PKC inhibitors and genetic deletion of PKC significantly reduce AMPH-stimulated dopamine release from striatal synaptosomes and slices (Chen *et al*, 2009; Kantor and Gnegy, 1998). PKC inhibition, however, does not alter the normal uptake functioning of the transporter (Johnson *et al*, 2005; Kantor and Gnegy, 1998;

Zestos et al, 2016). Therefore, PKC represents a novel therapeutic target for the treatment of AMPH abuse.

The selective estrogen receptor (ER) modulator tamoxifen stands as the only commercially available central nervous system (CNS)-permeant PKC inhibitor (Zarate and Manji, 2009). Tamoxifen is commonly used to reduce ER-positive breast cancer recurrence and to prevent breast cancer in high-risk women (Fisher et al, 1998; Jordan, 2003). Early reports using purified PKC show that tamoxifen inhibits the calcium- and phospholipid-dependent activity of classical PKC isoforms, with IC_{50}s between 25 and 100 μM (Su et al, 1985). In cells, tamoxifen inhibits PKC at more pharmacologically relevant concentrations (1–5 μM) (Gundimeda et al, 1996; Horgan et al, 1986; Lien et al, 1991; O'Brian et al, 1985). There are findings that suggest PKC activity is elevated in patients suffering from bipolar mania, a disorder modeled by repeated AMPH administration in animals (Wang and Friedman, 1996). Interestingly, systemic tamoxifen reduces manic symptoms in patients with bipolar mania, and this effectiveness is believed to stem from the action of tamoxifen at PKC (Kulkarni et al, 2006; Zarate et al, 2007). These data point to the clinical relevance of tamoxifen as a CNS-permeant PKC inhibitor. Although tamoxifen is well tolerated overall, it can cause ER-mediated adverse effects, including increased risk of hot flashes, thromboembolisms, and endometrial cancers (Fisher et al, 1998; Gradishar, 2004). Therefore, a CNS-permeant tamoxifen analog lacking ER activity could be useful in the context of AMPH abuse treatment.

Extensive structure–activity relationship (SAR) studies have investigated tamoxifen substructures that contribute to its ability to bind to the ER and inhibit PKC (de Medina et al, 2004). We used this wealth of knowledge to synthesize a new generation of tamoxifen analogs with increased selectivity for PKC over ER (Carpenter et al, 2016). In this paper, we investigate the effect of our most promising novel compound, **6c** (Figure 1a), at DAT and also on the neurochemical, behavioral, and reinforcing actions of AMPH. Our key findings show that **6c** modulates DAT asymmetrically, in that it is more potent in reducing dopamine efflux than uptake. Importantly, intra-accumbal and systemic administration of the tamoxifen analog significantly reduces dopamine overflow and locomotion stimulated by AMPH. Finally, our self-administration studies demonstrate that **6c** effectively reduces AMPH self-administration but not food self-administration. This work supports the repurposing of the tamoxifen scaffold as a treatment for AMPH abuse and elucidates an effective route for blocking AMPH reinforcement.

MATERIALS AND METHODS

Compounds

6c (6c·2.5 HCl) was synthesized and provided by the Vahlteich Medicinal Chemistry Core at the University of Michigan (Carpenter et al, 2016). D-amphetamine hemi-sulfate, dopamine, nomifensine maleate salt, and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich. Ruboxistaurin and cocaine hydrochloride were provided by NIDA-NIH. [³H]Dopamine and [³H]WIN 35,428 ((−)-2-β-carbomethoxy-3-β-(4-fluorophenyl) tropane-1,5-napthalenedisulfonate) were purchased from Perkin Elmer. Heparin was purchased from Sagent Pharmaceuticals.

Animals

Animal use and procedures were approved by the Institutional Animal Care and Use Committee at the University of Michigan and followed the guidelines put forth by the National Institutes of Health. Male Sprague Dawley rats were purchased from Envigo and were maintained on a 12-h light cycle with lights on at 0700 hours and all experiments were performed during the light phase. For in vitro experiments, the rats were 7–12 weeks old. The age of rats for microdialysis and self-administration ranged from 7 weeks to 10 months. During self-administration, the rats were food restricted to 80–85% of their free-feeding body weight and given free access to water.

Synaptosome Preparation

Rat striata were dissected on ice and homogenized in 10 volumes of homogenization buffer comprised of 0.32 M sucrose, 1 mM EDTA, and cocktail of protease inhibitors (Complete Mini, Roche), pH 7.4. Homogenates were centrifuged at 3000 rpm for 10 min and the supernatants were saved. The supernatant fractions were then centrifuged at 14000 rpm for 15 min. For the PKC activity experiments, the supernatants were aspirated and the pellets resuspended in Krebs Ringer buffer (KRB) made of 145 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.4. For dopamine uptake and suprafusion experiments, the pellets were resuspended in KRB that included 10 mM glucose, 0.05 mM ascorbic acid, and 0.05 mM pargyline.

PKC Activity Assay

Synaptosomes were pretreated with **6c** for 1 h prior to activating PKC with PMA. Synaptosomes were lysed and probed for growth-associated protein-43 (GAP-43) and myristoylated alanine-rich C-kinase substrate (MARCKS) phosphorylation using western blotting. Details regarding the assay and western blotting are in Supplementary Methods.

[³H]Dopamine Uptake

Synaptosomes were treated with 0–3 μM **6c** at 37 °C for 1 h. [³H]Dopamine (PerkinElmer Life Sciences) was added for 3 min and [³H]dopamine uptake was terminated with 5 ml cold KRB. The samples were then rapidly filtered on Fisherbrand GF/C filters and washed with 5 ml of cold KRB twice. Non-specific uptake was determined using 100 μM cocaine. Once dried, the filters were counted in a Beckman LS 5801 liquid scintillation counter.

Biotinylation

The effect of **6c** on DAT surface levels in synaptosomes was investigated with sulfo-NHS-SS-biotin using a method previously described (Furman et al, 2009). See Supplementary Methods for more details.
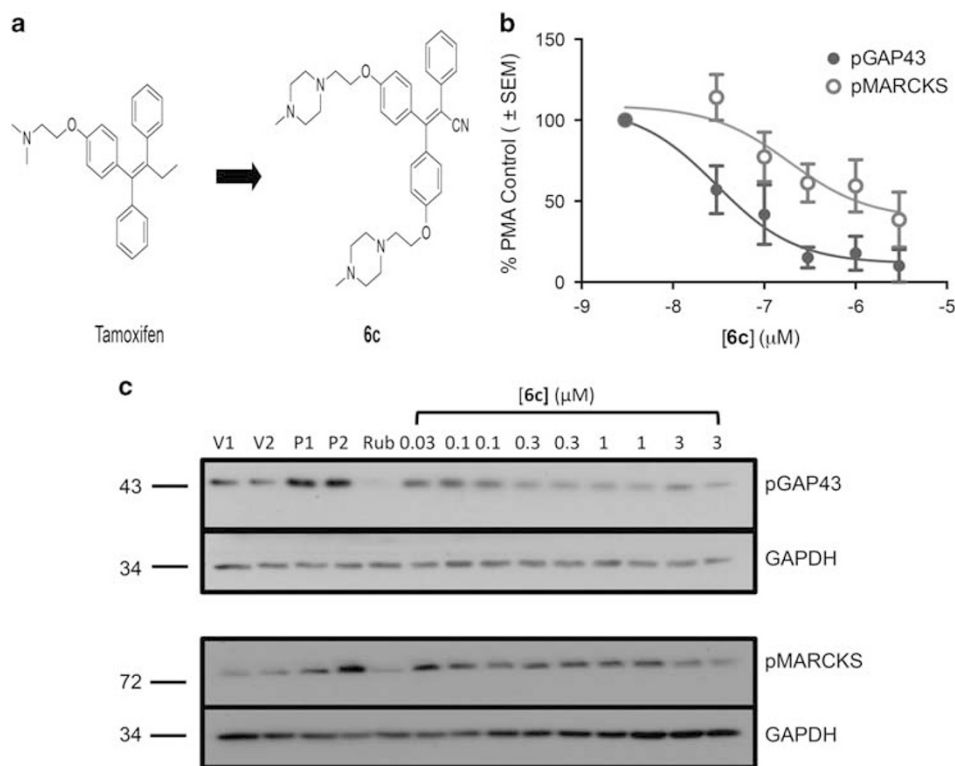
Figure 1 Structure of tamoxifen analog, **6c**, and its effect on PMA-induced PKC activity in synaptosomes. (a) Structures of tamoxifen and its analog, **6c**. (b, c). Rat striatal synaptosomes were incubated in the presence or absence of **6c** for 1 h at 37 °C. In all, 100 nM PMA was added for 15 min to stimulate PKC, and the samples were lysed and probed for phosphoser[41]-GAP-43 ($n = 4$) and phosphoser[152/156]-MARCKS ($n = 5$). GAPDH served as the loading control. (b) Data are represented as the percentage of vehicle optical density and each data set represents mean ± SEM. (c) Representative western blots. V1, V2: vehicle; P1, P2: PMA control; Rub: 500 nM ruboxistaurin, a control PKC inhibitor.

[³H]WIN 35,428 Binding

Radioligand saturation binding assays were performed by incubating synaptosomes with 3 µM **6c** at 37 °C for 1 h followed by 0–200 nM of the cocaine analog [³H]WIN 35,428 for 2 h at 4 °C. Five ml cold KRB was then added to samples followed by rapid filtration on Fisherbrand GF/C filters and washed twice with 5 ml of cold KRB. Non-specific binding was determined using 30 µM nomifensine.

Suprafusion

Synaptosomes were loaded onto filters in chambers of a Brandel perfusion apparatus (Brandel SF-12, Gaithersburg, MD) and perfused with 0–3 µM **6c** at 37 °C for 1 h at a rate of 400 µl/min. Following this wash, 16 fractions were collected in 2 min increments in vials containing internal standard solution (final concentration 50 mM perchloric acid, 25 µM EDTA, and 10 nM 2-aminophenol). Dopamine efflux was stimulated by the addition of 10 µM AMPH from fractions 6 to 11. The vehicle control or **6c** was also present during collection. Dopamine was quantified using high-performance liquid chromatography coupled to electrochemical detection (Thermo Scientific/ESA, Sunnyvale, CA).

Microdialysis and Locomotor Behavior

Probes were prepared and implanted as previously described (Zestos *et al*, 2016). The effect of direct accumbal administration or subcutaneous (s.c.) injection of **6c** on AMPH-induced neurochemical overflow and locomotion was investigated using a well-defined protocol (Zestos *et al*, 2016). The presence of **6c** and AMPH in the brain after s.c. administration were detected by LC/MS. See Supplementary Methods for more details.

Self-Administration

The effect of 6 mg/kg **6c** s.c. was evaluated on AMPH-self administration (0.032 mg/kg/infusion) and sucrose pellet self-administration under a fixed ratio (FR) 5 schedule of reinforcement. See Supplementary Methods for more details.

Statistics

The results were analyzed using the GraphPad Prism 6 software (San Diego, CA) and are plotted as mean ± SEM. Statistical significance was determined using two-way repeated measures (RM) ANOVA, one-way ANOVA or a two-tailed Student's *t*-test. When concentration–response curves were compared, comparison of fits in non-linear regression was used to determine whether curves differed from each other. The null hypothesis was that the best-fit parameters for the values did not differ. A conclusion of statistical significance represents a rejection of the null hypothesis and indicates a difference between designated values.

RESULTS

6c Inhibits PKC in Synaptosomes

Previously, we showed that **6c** inhibits PKC activity 250 times more potently than tamoxifen in the human neuroblastoma cell line SHSY5Y and displayed a $K_i > 10\,\mu M$ at the ERα (Carpenter *et al*, 2016). Here we examined the effect of **6c** on PKC activity in striatal synaptosomes, which contain dopaminergic terminals. To do this, we determined the effect of **6c** on the phosphorylation of the PKC substrates GAP-43 and MARCKS at the PKC-specific phosphorylation sites ser[41] and ser[152/156], respectively (Heemskerk *et al*, 1993; Nielander *et al*, 1990). **6c** decreased both GAP-43 and MARCKS phosphorylation in a concentration-dependent manner up to $3\,\mu M$ but showed greater potency in inhibition of GAP-43 phosphorylation as compared with MARCKS (Figure 1b). The IC_{50} of **6c** for inhibition of PMA-stimulated phosphoser[41]-GAP-43 was 30 nM (95% confidence interval (CI), 9–98 nM, $n = 4$) while that for inhibition of phosphoser[152/156]-MARCKS was 189 nM (95% CI, 25–1460 nM, $n = 5$). A comparison of fits in Prism 6 showed that the two IC_{50}s were significantly different ($F_{(2,45)} = 7.7$, $p < 0.01$).

6c Asymmetrically Blocks AMPH-Stimulated Dopamine Efflux and Uptake

We showed that selective PKC inhibitors reduce AMPH-stimulated dopamine release *in vitro* (Kantor and Gnegy, 1998). Using suprafusion of striatal synaptosomes, the effect of the PKC inhibitor **6c** on AMPH-stimulated dopamine efflux was tested. Synaptosomes were incubated with **6c** for 1 h before AMPH exposure. **6c** effectively and dose-dependently reduced dopamine efflux induced by $10\,\mu M$ AMPH (Figure 2a). No concentration of **6c** tested affected basal dopamine release (Supplementary Figure S1). Our group has shown that PKC inhibitors exhibit asymmetry in their effects on DAT activity, where the compounds preferentially block dopamine efflux without having significant effects on [3H]dopamine uptake (Johnson *et al*, 2005; Zestos *et al*, 2016). The effect of a 1 h pretreatment of 0.3–3 μM **6c** on [3H]dopamine uptake was determined in striatal synaptosomes. Only $3\,\mu M$ **6c** had a significant effect on dopamine uptake, reducing [3H]dopamine uptake by 60% compared with the vehicle treatment (Figure 2b). Calculation

of the dose-dependent percentage of inhibition of dopamine efflux and uptake demonstrates a 4.6-fold selectivity for efflux over influx (Figure 2c).

6c Does not Affect DAT Trafficking and Does Not Displace [3H]WIN 35,428 Binding

Two potential explanations for the effect of **6c** on dopamine uptake or efflux are that the compound is altering surface transporter levels or that it is directly binding to DAT. After incubating rat striatal synaptosomes with 3 μM **6c** for 1 h at 37 °C, our biotinylation studies showed that the compound did not cause a change in DAT surface levels as compared with vehicle (Figure 3). Many dopamine uptake blockers, such as cocaine, interact with a binding site close to the substrate site and hence reduce the normal uptake functioning at DAT (Beuming *et al*, 2008). To investigate whether **6c** is directly binding at this site, we tested the ability of the compound to alter binding of the cocaine analog [3H]WIN 35,428. We found that treating synaptosomes with 3 μM **6c** for 1 h at 37 °C caused no changes in the subsequent equilibrium binding of [3H]WIN 35,428. The K_d for [3H] WIN 35,428 in the absence or presence of **6c** in nM ± SEM was 77 ± 17 and 71 ± 19, respectively. The B_{max} for [3H]WIN 35,428 binding in the absence or presence of **6c** in pmol/mg protein ± SEM was 2.4 ± 0.4 and 2.2 ± 0.3, respectively, $n = 3$.
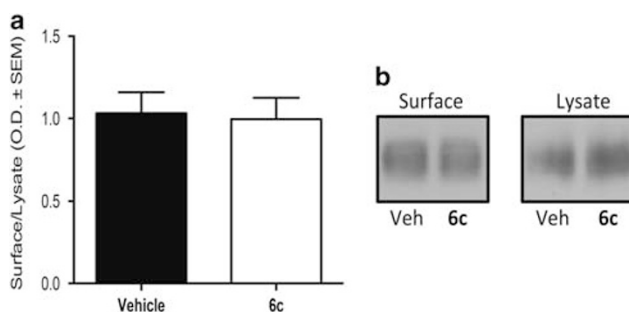

Figure 3 The action of **6c** on DAT surface expression. (a) Rat striatal synaptosomes were incubated in the presence or absence of 3 μM **6c** for 1 h at 37 °C, followed by the biotinylation of surface DAT as previously described in Methods section. (b) Representative western blots. Data shown as mean ± SEM ($n = 5$).
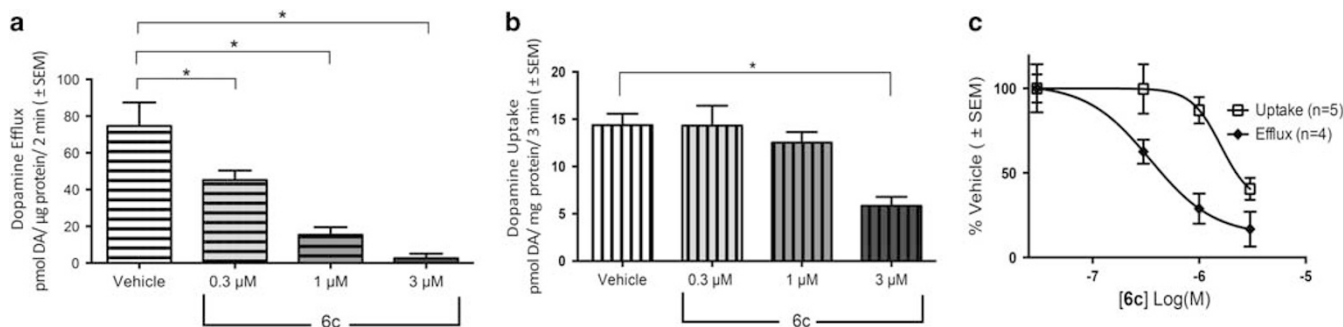

Figure 2 **6c** Modulation of DAT efflux and uptake processes. (a) Rat striatal synaptosomes were incubated in the presence or absence of **6c** for 1 h at 37 °C; efflux was stimulated with 10 μM AMPH ($n = 4$). *Post hoc* Dunnett multiple comparison test, *$p \leqslant 0.05$. (b) Synaptosomes were incubated with vehicle or **6c** for 1 h at 37 °C and [3H]dopamine uptake was quantified ($n = 5$). *Post hoc* Dunnett multiple comparison test, *$p < 0.01$. (c) Efflux and uptake results represented as the percentage of vehicle. All points are mean ± SEM.
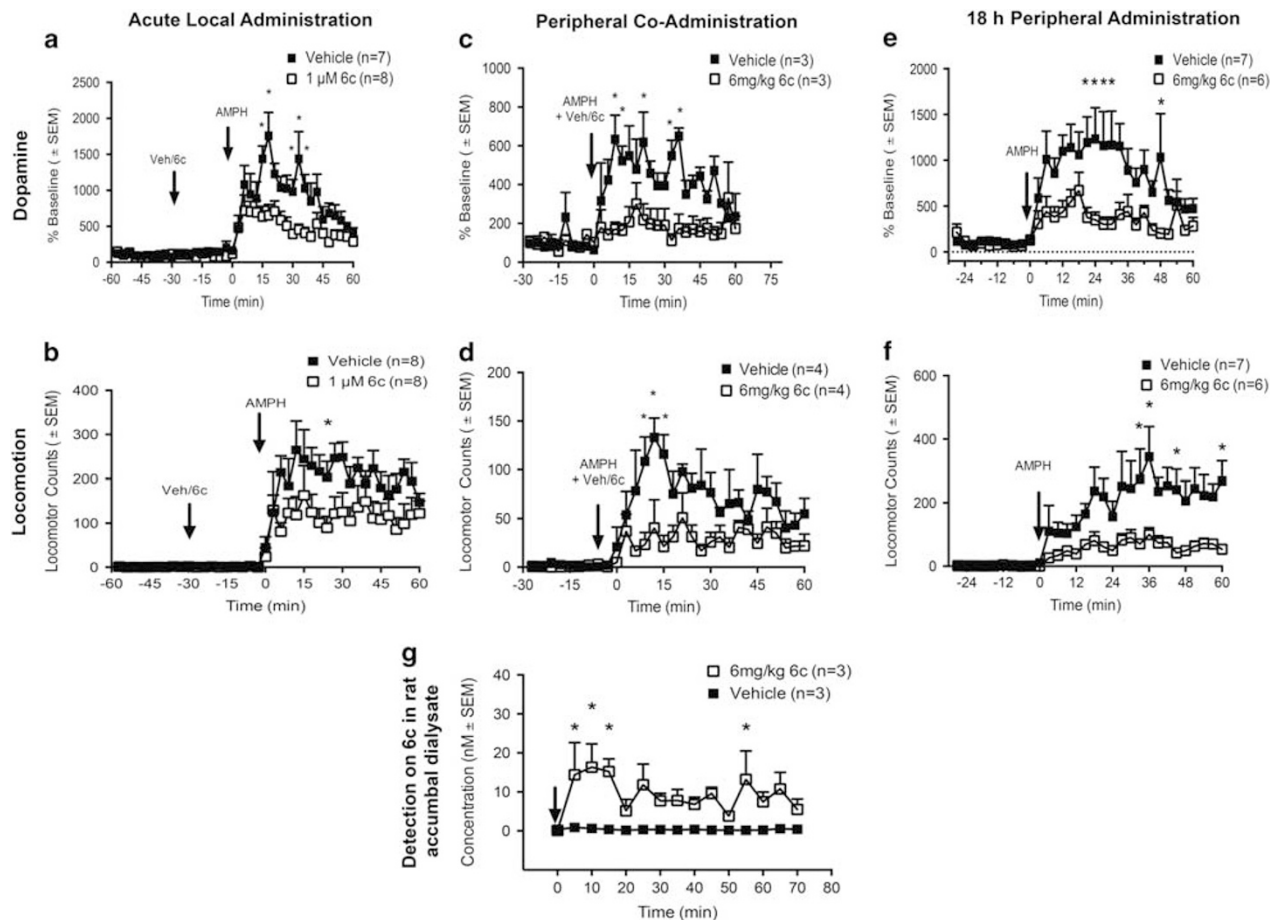
Figure 4 The effect of **6c** on *in vivo* AMPH-induced dopamine overflow and locomotion. (a, b) In all, 1 μM **6c** or vehicle was perfused into the nucleus accumbens using retrodialysis 30 min prior to the administration of 2 mg/kg i.p. AMPH. (a) Dopamine overflow; vehicle (*n* = 7) and **6c** (*n* = 8). *Post hoc* Sidak's multiple comparison test, *$p < 0.05$. (b) Locomotion; in *post hoc* Sidak's multiple comparison test, *$p < 0.05$; vehicle (*n* = 8), **6c** (*n* = 8). (c, d) In all, 6 mg/kg **6c** or vehicle were given to rats s.c. simultaneously with 1 mg/kg i.p. AMPH. (c) Dopamine overflow; *post hoc* Sidak's multiple comparison test, *$p < 0.05$; vehicle (*n* = 3), **6c** (*n* = 3). (d) Locomotor activity; *post hoc* Sidak's multiple comparison test, *$p < 0.01$; vehicle (*n* = 4), **6c** (*n* = 4). (e, f) In all, 6 mg/kg **6c** or vehicle was given 18 h prior to the administration of 2 mg/kg i.p. amphetamine (AMPH). (e) In *post hoc* Sidak's multiple comparison test, *$p < 0.05$ for dopamine overflow. (f) *Post hoc* Sidak's multiple comparison test, *$p < 0.05$ for locomotion. For dopamine and locomotor activity, vehicle (*n* = 7) and **6c** (*n* = 6). (g) In all, 6 mg/kg **6c** or vehicle (*n* = 3) was administered s.c., and dialysate collected from time 0 to 70 min. *Post hoc* Sidak's multiple comparison test, *$p < 0.05$. Levels of **6c** were quantified using LC-MS and corrected for recovery.

Direct Accumbal and Systemic Administration of *6c* Decreases AMPH-Stimulated Dopamine Overflow and Locomotion

To assess whether the effect of **6c** on reduction of AMPH-stimulated dopamine efflux in synaptosomes would occur *in vivo*, we conducted a series of microdialysis experiments in which dopamine overflow and locomotor activity were measured simultaneously. In the first experiment, we collected baseline samples for 30 min, and then 1 μM **6c** was perfused directly into the nucleus accumbens core 30 min prior to the peripheral administration of 2 mg/kg AMPH. As shown in Figure 4a, **6c** significantly reduced AMPH-stimulated dopamine overflow in freely moving rats at a concentration that did not reduce dopamine uptake (two-way RM ANOVA, $F_{(39, 507)} = 22.42$, $p < 0.0001$ for time; $F_{(1, 13)} = 7.23$, $p < 0.05$ for drug; and $F_{(39, 507)} = 4.04$, $p < 0.0001$ for time–drug interaction). Similarly, as depicted in Figure 4b, locomotor activity in response to the injection of AMPH was significantly reduced (two-way RM ANOVA,

$F_{(39, 546)} = 19.15$, $p < 0.0001$ for time; $F_{(1, 14)} = 4.26$, $p = 0.058$ for drug; and $F_{(39,546)} = 1.92$, $p < 0.01$ for interaction of drug and time).

Because **6c** was designed on the scaffold of the CNS-permeant drug, tamoxifen, there was some expectation that it might cross the blood–brain barrier. To test this possibility, we administered the compound systemically and checked for its presence in the nucleus accumbens using microdialysis coupled to LC-MS. In all, 6 mg/kg **6c** s.c. resulted in detectable levels of the compound in the nucleus accumbens (Figure 4g, two-way RM ANOVA, $F_{(1,4)} = 22.53$, $p < 0.01$ for drug). The data demonstrate that **6c** peaks in the brain within 10 min and remains relatively steady at concentrations between 10 and 20 nM over the next hour. Estimated percentage of recovery through the probe was 10–15% and was used to calculate the concentration of **6c** *in vivo*.

Based upon the rapid appearance of **6c** in the brain after peripheral injection, we examined if s.c. administration of **6c** with AMPH would reduce the effects of AMPH on dopamine
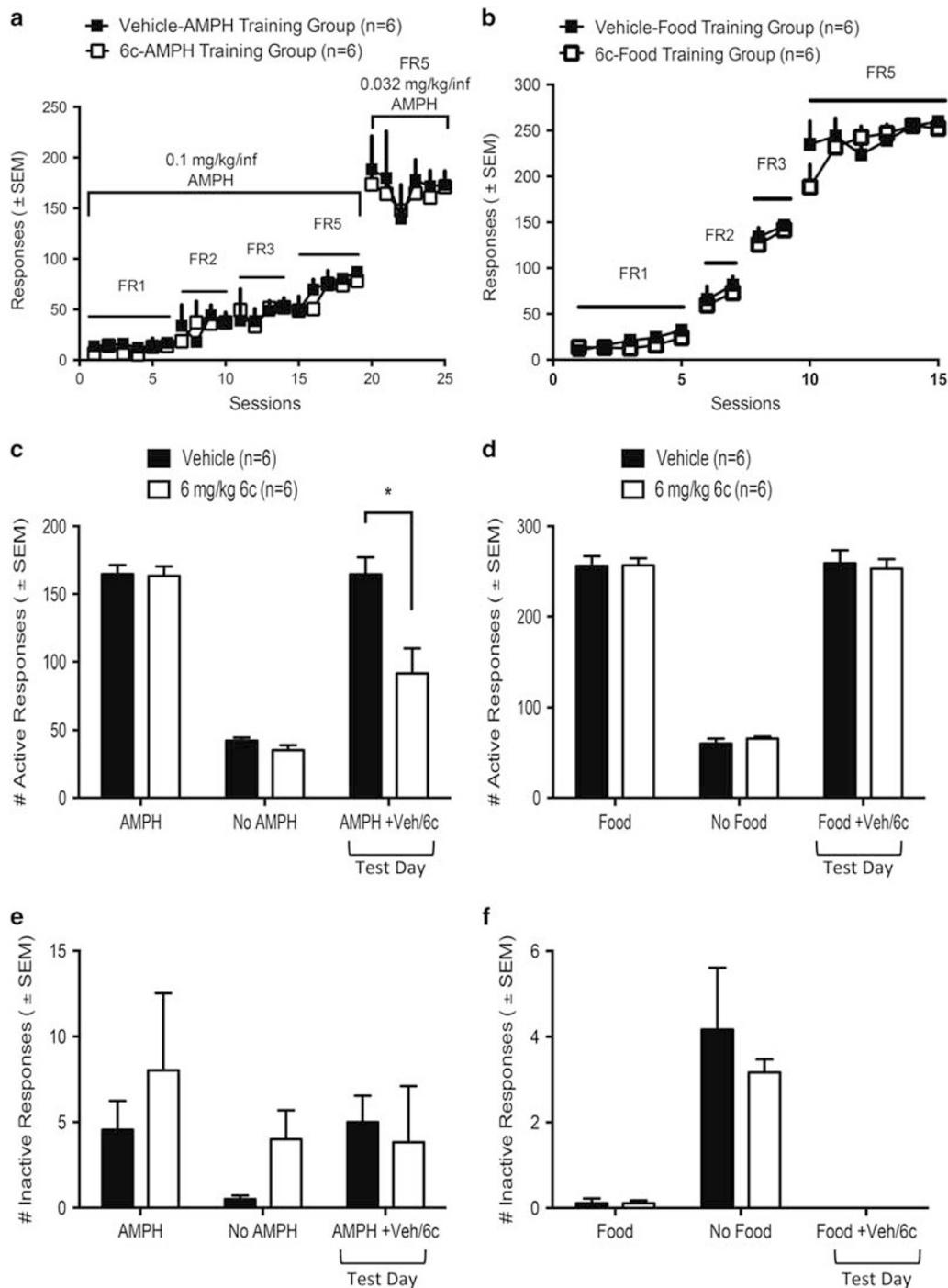
Figure 5 The effect of 6 mg/kg s.c. **6c** on AMPH and food self-administration. (a) Training data for rats used in AMPH self-administration experiments. Rats were escalated from a FR1 to a FR5 schedule with 0.1 mg/kg/infusion of AMPH. Finally rats were trained to stably administer on the FR5 schedule with 0.032 mg/kg/infusion of AMPH. (b) Training data for rats used in food self-administration. Rats escalated from a FR1 to a FR5 schedule for food pellets. (c) On test day, 6 mg/kg **6c** or vehicle was given 18 h prior to AMPH self-administration session. *Post hoc* Sidak's multiple comparison test, *$p < 0.0001$, a significant difference between vehicle- and **6c**-treated rats. (d) On test day, 6 mg/kg **6c** or vehicle was given 18 h prior to food self-administration session. (e, f) Inactive nose poke responses during AMPH and food self-administration sessions, respectively. All data sets are represented as mean ± SEM.

overflow and locomotion. As shown in Figure 4c, simultaneous s.c. injection of 6 mg/kg **6c** and 1 mg/kg AMPH resulted in a significant decrease in dopamine overflow as compared with AMPH alone (two-way RM ANOVA $F_{(29, 116)} = 5.17$, $p < 0.0001$ for time; $F_{(1, 4)} = 16.15$, $p < 0.05$ for drug; and $F_{(29, 116)} = 2.85$, $p < 0.0001$ for interaction of drug

and time). Figure 4d demonstrates that administration of **6c** elicited a corresponding significant decrease in AMPH-stimulated locomotor behavior compared with AMPH alone (two-way RM ANOVA, $F_{(29, 174)} = 6.45$, $p < 0.0001$ for time; $F_{(1, 6)} = 15.82$, $p < 0.01$ for drug; and $F_{(29, 174)} = 1.95$, $p < 0.01$ for drug and time interaction). To rule out whether **6c** was

simply reducing the amount of AMPH getting into the brain, we tested the concentrations of AMPH in the brain after **6c** was given s.c. with AMPH. Administration of s.c. **6c** with 1 mg/kg AMPH had no effect on the AMPH levels achieved in the nucleus accumbens when compared with rats treated with vehicle and 1 mg/kg AMPH (Supplementary Figure S2).

We next queried whether pretreatment with **6c** had extended effects. A s.c. injection of **6c** was given 18 h prior to AMPH and AMPH-stimulated dopamine overflow and locomotor behavior were measured. As shown in Figure 4e, pretreatment with 6 mg/kg **6c** s.c. 18 h prior to an injection of 2 mg/kg AMPH i.p. significantly decreased AMPH-stimulated dopamine overflow (in two-way RM ANOVA, $F_{(29, 319)} = 9.05$, $p < 0.0001$ for time; $F_{(1, 11)} = 5.05$, $p < 0.05$ for drug; and $F_{(29, 319)} = 2.87$, $p < 0.0001$ for drug and time interaction). Again, there was a corresponding decrease in the locomotor response to AMPH (Figure 4f) following **6c** pretreatment (in two-way RM ANOVA, $F_{(29, 319)} = 7.97$, $p < 0.0001$ for time; $F_{(1, 11)} = 9.87$, $p < 0.01$ for drug; and $F_{(29, 319)} = 2.56$, $p < 0.0001$ for interaction of time and drug). Although higher than null value, only low picomolar levels of **6c** were detected in accumbal dialysate 18 h after s.c. administration (data not shown).

Systemic 6c Reduces AMPH-Self Administration But Not Food Self-Administration

As **6c** effectively reduced AMPH neurochemical and behavioral effects *in vivo*, we next investigated whether the tamoxifen analog could effectively reduce the reinforcing effects of AMPH in a rat model of self-administration. Rats were trained to administer AMPH i.v. on a FR5 schedule of reinforcement and both groups of rats in the AMPH self-administration studies demonstrated similar patterns of responding for AMPH during training (Figure 5a). As shown in Figure 5c, 18 h following s.c. injection of **6c**, the number of infusions of AMPH earned over a 1 h session significantly decreased compared with the vehicle-treated animals (in two-way RM ANOVA, $F_{(2, 20)} = 76.56$, $p < 0.0001$ for responses; $F_{(1,10)} = 12.90$, $p < 0.01$ for drug; and $F_{(2, 20)} = 7.25$, $p < 0.01$ for interaction of infusion and drug). In a similar procedure, rats were trained to self-administer sucrose pellets on a FR5 schedule of reinforcement in 20 min daily sessions. Again, both groups of rats in the food self-administration studies demonstrated similar patterns of responding for food pellets during training (Figure 5b). We found that 18 h pretreatment with 6 mg/kg **6c** did not alter the number of active nose poke responses as compared with vehicle pretreatment (Figure 5d). There were no significant differences in the rats responding at the inactive nose poke for either the AMPH or food self-administration experiments (Figures 5e and f).

Discussion

In this paper, we report our findings on a novel tamoxifen analog and PKC inhibitor that modulates DAT functioning. It is well known that phosphorylation serves as a major regulator of DAT expression and activity (Foster *et al*, 2006). Enhanced PKC activity results in internalization of DAT, although a direct mechanism linking DAT phosphorylation and internalization has not been elucidated (Zahniser and

Doolen, 2001). DAT contains PKC consensus sites (Foster *et al*, 2002) in its N-terminal domain and truncation of this region attenuates DAT phosphorylation induced by the PKC activator PMA (Granas *et al*, 2003). Phosphorylation of DAT in the N-terminus has been reported to be permissive for AMPH-stimulated dopamine efflux with no effect on dopamine influx (Khoshbouei *et al*, 2004; Wang *et al*, 2016). Several reports have shown that PKC inhibitors reduce dopamine release (Kantor and Gnegy, 1998; Loweth *et al*, 2009; Zestos *et al*, 2016) while treatment of striatal synaptosomes with PKC activators stimulates dopamine efflux (Cowell *et al*, 2000; Opazo *et al*, 2010). We previously demonstrated that specific inhibitors of PKCβ inhibit AMPH-stimulated dopamine overflow and locomotor activity when administered directly into the core of the nucleus accumbens (Zestos *et al*, 2016).

In Vitro Studies

AMPH-induced dopamine efflux is central to the abuse liability of the stimulant. Therefore, we hypothesize that a CNS-permeant PKC inhibitor may be therapeutically beneficial in the treatment of AMPH abuse. As the only CNS-permeant PKC inhibitor, tamoxifen, causes undesirable ER-mediated side effects, we used previously available SAR information to create a CNS-permeant PKC inhibitor lacking ER affinity (Carpenter *et al*, 2016). As shown in Figures 1b and c, **6c** reduced PMA-stimulated PKC activity in striatal synaptosomes. Moreover, the compound demonstrated selectivity for PKC substrates, in that its inhibitory activity was more potent for phospho-GAP-43 than for phospho-MARCKS. Tamoxifen acts as an allosteric inhibitor of PKC by interacting with its regulatory domain and competing with phospholipids (O'Brian *et al*, 1985). It is possible that the mechanism of action of **6c** at PKC is also at the regulatory site but further studies must be undertaken to confirm this. An action at a regulatory subunit of PKC, similar to that of tamoxifen, could explain the differential effect on substrates.

There was also a differential potency of **6c** on the direction of dopamine transport in that **6c** more potently inhibited reverse transport of dopamine than inward transport. This observation is comparable to, but not identical to our previous studies examining the effects of PKCβ inhibitors on DAT. We and others have reported that PKC inhibitors, including specific PKCβ inhibitors, reduce AMPH-stimulated dopamine efflux but have no effect on dopamine uptake (Browman *et al*, 1998; Loweth *et al*, 2009; Zestos *et al*, 2016). The asymmetric regulation of DAT is possible because forward and reverse transport appear to be mediated by different factors (Sitte and Freissmuth, 2015). For example, the DAT ligand SoRI-20041 inhibited substrate uptake with no significant effects on efflux (Rothman *et al*, 2009; Schmitt *et al*, 2013). **6c** has different effects on DAT from the standard PKC inhibitors, however, because at higher concentrations it will inhibit dopamine uptake. This suggests that the compound might interact directly with DAT, although not at the cocaine-binding site as up to 3 μM **6c** had no effect on the binding of [³H]WIN 35,428. Although the cocaine- and dopamine-binding sites on DAT overlap, they are not the same (Beuming *et al*, 2008). Therefore, it is possible, albeit unlikely, that **6c** can directly bind to DAT and affect substrate but not cocaine binding.

The biotinylation experiments showed that the inhibition of dopamine uptake could not be attributed to a reduction in cell-surface DAT. It is possible that the diphenyl structural motif in **6c** could partially account for its blockade of dopamine uptake because this motif also exists in atypical DAT blockers such as modafinil and benztropine, which appear to bind preferentially to the inward-facing conformation of DAT (Reith *et al*, 2015; Schmitt *et al*, 2013). In addition to the well-characterized substrate site (S1), crystal structures of the bacterial leucine transporter (Zhen and Reith, 2016), the drosophila DAT, and the serotonin transporter show the presence of secondary allosteric sites (S2 and S3), where compounds can bind and modify the conformation and function of DAT (Reith *et al*, 2015; Schmitt *et al*, 2013). It is possible that **6c** could occupy one of these allosteric sites. Alternatively, the effect on uptake could be through an as yet unidentified mediator.

In Vivo Studies

We previously demonstrated that direct administration of specific inhibitors of PKCβ via retrodialysis into the nucleus accumbens inhibits AMPH-stimulated dopamine overflow and locomotor activity (Zestos *et al*, 2016). This result was recapitulated with the structurally unrelated **6c**, using a concentration (1 μM) that did not disrupt inward transport in our *in vitro* studies. A notable advantage of **6c** over the other inhibitors, however, is that it can cross the blood–brain barrier, like its parent compound tamoxifen. We found that giving 6 mg/kg **6c** s.c. led to detectable levels of the compound in the brain within the first 5 min following administration. The maximally measured concentration of **6c** in the brain was approximately 15 nM, but it appeared biologically active even at that concentration. Simultaneous s.c. injection of 6 mg/kg **6c** with 1 mg/kg AMPH resulted in a rapid reduction in both dopamine overflow and locomotor behavior. Therefore, despite the seemingly low level, the compound was active. Also, the low concentration of **6c** in the nucleus accumbens was comparable to the IC$_{50}$ of **6c** for inhibition of GAP-43 phosphorylation in synaptosomes (Figure 1b).

Our ultimate goal is to determine whether **6c** would alter the reinforcing properties of AMPH. Therefore, we measured the effect of s.c. administration of the compound on AMPH self-administration. As previously mentioned, giving **6c** 18 h prior to AMPH significantly decreased AMPH-induced dopamine overflow and locomotion. In agreement with this, we found that administering 6 mg/kg **6c** 18 h prior to a AMPH self-administration session significantly reduced responding on the active nose poke manipulandum as compared with vehicle pretreatment (Figure 5c). This finding serves as direct evidence that **6c** decreases on-going AMPH self-administration. Ultimately, we are interested in investigating how **6c** affects the inverted U-shaped dose–response curve for AMPH self-administration and whether the results obtained in this current study translate to other schedules of reinforcement, such as a progressive ratio schedule. The lack of effect of **6c** on sucrose self-administration demonstrates that the drug did not alter responding for all reinforcers and did not suppress all operant responding.

We are in the process of performing time-course studies documenting the onset and duration of the ability of **6c** to reduce the neurochemical and behavioral effects of AMPH *in vivo*. Nonetheless, it is highly interesting that, 18 h after s. c. administration, **6c** can still produce a reduction in AMPH-stimulated dopamine overflow and locomotor activity. Our measurement of **6c** in the nucleus accumbens showed that it is still present, albeit at extremely low levels. Nothing is known of the metabolism of this compound, but it is possible that there is a long-lasting metabolite that remains active. Another explanation is that the initial action of the compound, perhaps PKC inhibition, elicits a long-term change in downstream signaling that maintains inhibition of AMPH action. A limitation to our methodology for measuring **6c** levels in the brain is that it only reflects extracellular concentrations. It is therefore also conceivable that the long-term effects of **6c** could be due to intracellular accumulation of the compound, which we did not sample.

PKC is widely expressed in the body and is involved in a diverse collection of GPCR and growth factor-dependent cellular pathways. Therefore, there are concerns that targeting the enzyme could lead to significant, unwanted side effects. However, clinical work with PKC inhibitors proves otherwise. For instance, chronic tamoxifen administration is generally well tolerated by women and selective PKC inhibitors have not caused severe toxicities in clinical studies (Mochly-Rosen *et al*, 2012). Recent findings suggest that PKC inhibitors would be useful in other CNS disorders, such as Alzheimer's disease where activating forms of PKCα support Aβ activity (Alfonso *et al*, 2016). A total block of PKC activity is likely not required for adequate treatment; if increases in PKC activity are involved in a particular pathology, reduction to normal levels could be effective. Repeated treatment with AMPH, for example, leads to an increase in striatal PKC activity in rats (Iwata *et al*, 1997). Development of inhibitors selective for specific isozymes or substrate-specific inhibitors would increase the utility of PKC inhibitors. Our data showing greater potency of **6c** for inhibition of phosphorylation of GAP-43 over that of MARCKS demonstrates that substrate selectivity is possible, especially if the drug does not act directly at the ATP-substrate-binding site on the PKC isozyme. We are hopeful in the possibility of creating even more selective CNS-permeant tamoxifen analogs and also implementing tools that will allow site-specific targeting of these compounds.

In summary, this study presents a new CNS-permeant PKC inhibitor, **6c**, which attenuated *in vitro* and *in vivo* neurochemical and behavioral effects of AMPH. Furthermore, systemic administration of **6c** significantly reduced AMPH self-administration without generally suppressing behavior. These findings further support the targeting of PKC for the treatment of AMPH abuse and also illuminate the use of tamoxifen as a scaffold to create a new generation of CNS-permeant PKC inhibitors. Additionally, **6c** may serve as a useful tool for investigating other PKC-related brain disorders, such as bipolar mania (Mochly-Rosen *et al*, 2012).

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