

Investigating the Role of Protein Kinase C Inhibitors in Amphetamine-Mediated Behaviors

by

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Dedication

To my family, who was with me every step of this journey

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List of Abbreviations

- aCSF: Artificial cerebral spinal fluid
- ADHD: Attention deficit hyperactivity disorder
- ALDH: Aldehyde dehydrogenase
- AMPH: Amphetamine
- AUC: Area under curve
- COMT: Catechol-O-methyl transferase
- CPP: Conditioned place preference
- DAT: Dopamine transporter
- DSM-5: The Diagnostic and Statistical Manual of Mental Disorders 5
- FR: Fixed-ratio
- GAP-43: Growth associated protein-43
- GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
- GPCR: G-protein coupled receptor
- ICSS: Intracranial self-stimulation
- i.c.v.*: Intracerebroventricular
- L-DOPA: 3,4-dihydroxyl-L-phenylalanine
- MAO: Monoamine oxidase
- NAc: Nucleus accumbens
- PKC: Protein kinase C
- PMA: Phorbol 12-myristate 13-acetate
- PR: Progressive-ratio
- TAAR: Trace amine-associated receptor

VMAT: Vesicular monoamine transporter

VTA: Ventral tegmental area

Abstract

Amphetamines (AMPH) are among the most widely misused drugs in the United States and worldwide. Currently, there are no pharmacological interventions for amphetamine-use disorder. A close examination of mechanism of action of AMPH identified protein kinase C (PKC β) as a potential target for a new therapeutic intervention. The reinforcing effects of AMPH are elicited through an increase in extracellular dopamine levels in the brain. PKC β inhibition attenuates AMPH-stimulated increases in dopamine levels *in vivo* and *ex vivo*, leading to the possibility that PKC β inhibition decreases the reinforcing effects of AMPH. These findings highlight the potential for developing PKC β inhibitors as a pharmacological intervention for AMPH-use disorder.

To assess the feasibility of utilizing PKC β inhibitors as a treatment for AMPH-use disorders, I examined whether pharmacological inhibition of PKC β attenuated AMPH-stimulated behaviors. I used the PKC β inhibitors in proof-of-concept studies demonstrating that PKC β inhibitors decrease AMPH-stimulated locomotor activity and AMPH self-administration. Using a self-administration model to investigate the reinforcing properties of AMPH, I showed that an intracerebroventricular injection of a PKC β inhibitor 18 hr prior to a self-administration session decreased responding for AMPH under a fixed-ratio 5 schedule of reinforcement and shifted the ascending limb of the AMPH dose-effect curve for self-administration to the right. Furthermore,

PKC β inhibitors decreased responding for AMPH under a progressive-ratio schedule of reinforcement, demonstrating that PKC β inhibition decreased the reinforcing strength of AMPH and the motivation to work for AMPH. PKC β inhibitors did not decrease responding for sucrose under a fixed-ratio or progressive ratio schedule, indicating that the doses tested did not inhibit general behavior. These properties are desirable for a pharmacological intervention for substance-use disorders and support the development of PKC β inhibitors as new therapeutics for the treatment of AMPH-use disorder.

Interestingly, my findings suggested that PKC β inhibitors acted acutely to decrease AMPH-stimulated behaviors but also decreased AMPH-stimulated behaviors through a secondary mechanism. To probe these mechanisms, I compared locomotor activity and protein expression at different time points following administration of a PKC β inhibitor into the nucleus accumbens (NAc) or ventral tegmental area (VTA). I found that PKC β inhibition in the NAc acutely decreased AMPH-stimulated locomotor activity whereas PKC β administration into the VTA required an extended period of time before attenuating locomotor activity. Furthermore, PKC β levels were decreased in the VTA 18 hr following administration of a PKC β inhibitor. These findings support the hypothesis that PKC β inhibitors act through a secondary mechanism, possibly the downregulation of PKC β , to decrease AMPH-mediated behaviors. Altogether, these findings support the use of PKC β inhibitors as a new therapeutic for AMPH-use disorder and further our understanding of the mechanism by which the inhibitors act to decrease AMPH-stimulated behaviors.

Chapter 1 Introduction

Substance-use disorders

Substance-use disorders are a serious public health concern worldwide. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) characterizes the criteria for substance-use disorder as a pattern of use of drugs (including stimulants, sedatives, inhalants, and hallucinogens) that leads to physical or psychological dependence, as well as recurrent and increased use despite consequences, tolerance and withdrawal, and cravings (American Psychiatric Association 2013). Many factors including genetics, mental health, the environment, and exposure to drugs are implicated in the development of substance-use disorders (NIDA 2018a). A 2016 study estimated that nearly 300 million people worldwide used controlled substances during the previous year and 11% of people who use drugs can be diagnosed with a substance-use disorder (UNODC 2018). Within the United States of America, more than 63,000 people died of drug misuse-related causes in 2015, with opioids, cocaine, and amphetamine-type stimulants representing the top drugs related to the deaths (UNODC 2016a). Substance-use disorders have cost the United States an estimated \$700 billion in costs for criminal justice, health care, and loss of productivity (McCollister et al. 2017; NIDA 2017). With the substantial costs of substance-use disorders on individuals and communities, there is a strong need for more effective treatments for substance-use disorder.

Currently, the most common treatments for substance-use disorders are behavioral therapies and community interventions (Carroll and Onken 2005; NIDA 2018b) . Behavioral therapies may be implemented in different forms, such as providing incentives for a decrease in drug use or training patients to recognize risks and situations associated with drug use (Carroll and Onken 2005). One of the limitations to the effectiveness of these treatments are the vast array of factors that play into the maintenance of substance-use disorders, often making it necessary to combine different types of interventions (UNODC 2016b). Pharmacological interventions can be effective for certain classes of drugs such as opioids and nicotine; however, there are no accepted medications to treat addiction to other classes of drugs, leaving behavioral therapy as the primary source for treatments (UNODC 2016b). Thus, research developing novel pharmacological interventions to supplement behavioral therapies is necessary to expand the effectiveness of substance-use therapeutic strategies.

Investigating novel treatments for drug abuse

Behavioral pharmacology has long been a field vital to the understanding of drugs of abuse and the development of pharmacological therapeutics. In humans and animals, drugs of abuse can produce physiological effects and changes in observable behaviors such as hyperactivity or dependence. However, it is often called into question whether these measures contribute to or influence the abuse liability of psychoactive drugs. Abuse liability can be better assessed by exploring behaviors controlled by reinforcement, motivation, and learning. Some of the early studies within the field of behavioral pharmacology relied on techniques developed by prominent psychologists B.F. Skinner and Ivan Pavlov to show that psychoactive drugs can selectively alter or control different behaviors (Dews 1955; Pickens 1977). These studies began to challenge stereotypes regarding patients with substance-use disorders (Balster et al. 2009).

Animal behavior can be used to study the mechanisms of drug action, to evaluate abuse liability of drugs, and to assess therapeutics for the treatment of substance-use disorders (Griffiths et al. 1979; Henningfield et al. 2016). Methods such as conditioned-place preference, intracranial self-stimulation, drug discrimination, and drug self-administration are some of the most commonly used techniques to address these types of studies. These techniques rely on the concepts of reward and reinforcement; reward is an event with a positive value (i.e. food or drug) and positive reinforcement is an increase in the behavior paired with a reward. Well-designed animal studies can model different aspects of substance-use disorders such as reward and reinforcement, drug-taking and drug-seeking, environmental variables, and relapse.

Conditioned-place preference (CPP) is a technique that examines the rewarding properties of drugs of abuse. In this assay, rodents are exposed to different environments (different floor patterns or coloring) and are conditioned to associate one environment with an injection of drug and the other environment with an injection of vehicle. Following a conditioning period, the animals are placed in the chambers and allowed to roam freely. The time spent in the drug-paired side of the box as opposed to the vehicle-paired side is recorded. An increase in time spent on the drug-paired side indicates a preference for the drug, whereas a decrease indicates if the drug is aversive (Prus et al. 2009). Psychostimulants have been well characterized in place-preference assays and are known to produce CPP (Itzhak and Martin 2002; Spyraki et al. 1982). Pharmacological and genetic studies with CPP are used to study mechanisms behind drug reward, to model relapse, and to screen therapeutics for the treatment of substance-use disorders (Bardo and Bevins 2000; Napier et al. 2013). This technique can additionally be used to probe questions on cue-related memory and learning (McIntyre et al. 2002). While CPP is a good model for assessing the rewarding effects of drugs, this model does

not address other behaviors associated with drugs of abuse, such as drug-taking behaviors, making it necessary to include additional techniques in the studies of drugs of abuse (Nader 2016).

Intracranial self-stimulation (ICSS) is another method used to model the effects of drugs of abuse. In these studies, animals are implanted with intracranial electrodes that deliver electrical stimulation to the regions of the brain associated with motivation and reward such as the ventral tegmental area (VTA) (Wise 1996). Animals make responses on a manipulandum in order to receive electrical stimulation. Early studies with this technique demonstrated that stimulation in the VTA or hypothalamus are reinforcing; an animal will increase responding that has been paired with the electrical stimulation, or the reward (Olds and Milner 1954). By varying the frequency of the stimulations, it is possible to generate rate-frequency curves comparing the frequency of the stimulation to the number or rate of responses. Animals display increased levels of responding for higher frequencies, which are more rewarding (Wise 1996). Drugs of abuse generally shift the rate-frequency curves to the left; the stimulation threshold is lower, and animals respond more for lower frequencies in the presence of certain drugs. The results from ICSS studies are used as an indicator of a drug's abuse liability (Negus and Miller 2014). ICSS is also used to identify regions in the brain important for reward circuitry (Koob 2009). ICSS is an indirect method to study drugs of abuse because it does not measure drug-taking or drug-seeking behaviors, so it is often used in conjunction with other behavioral assays.

Drug discrimination is a technique that is used to measure the subjective effects of a drugs of abuse and provide insight into abuse liability of the drugs tested, their underlying mechanisms, and potential therapeutics (Holtzman 1990; Porter et al. 2018; Rocha et al. 2008). In discrimination studies, animals are trained using operant procedures to respond for a reward,

often a food pellet. The animals are trained to make one response if they have been treated with a CNS active drug and another response if they receive an injection of saline. During training, animal only earn a food reward by making the injection-appropriate response. Once a drug has been established as a discriminative stimulus, the interoceptive (or subjective) properties of a novel drugs can be evaluated. Drug discrimination studies are used by government agencies in order to determine if a novel drug should be regulated or controlled; if a new compound generalizes to a controlled substance, such as morphine or amphetamine, then the new compound likely has the potential to be abused (Nader 2016; Rocha et al. 2008). Some limitations to the use of drug discrimination assays are that there are many variables that can affect the results of the studies, such as the training drug used and dose (Grant et al. 2000; Nader 2016). As with ICSS and CPP, this technique is a useful, but indirect, tool for studying drugs of abuse since it doesn't directly model behaviors associated with substance use disorders such as drug-taking or drug-seeking behaviors. Therefore, drug discrimination studies are often combined with more direct assays such as drug self-administration.

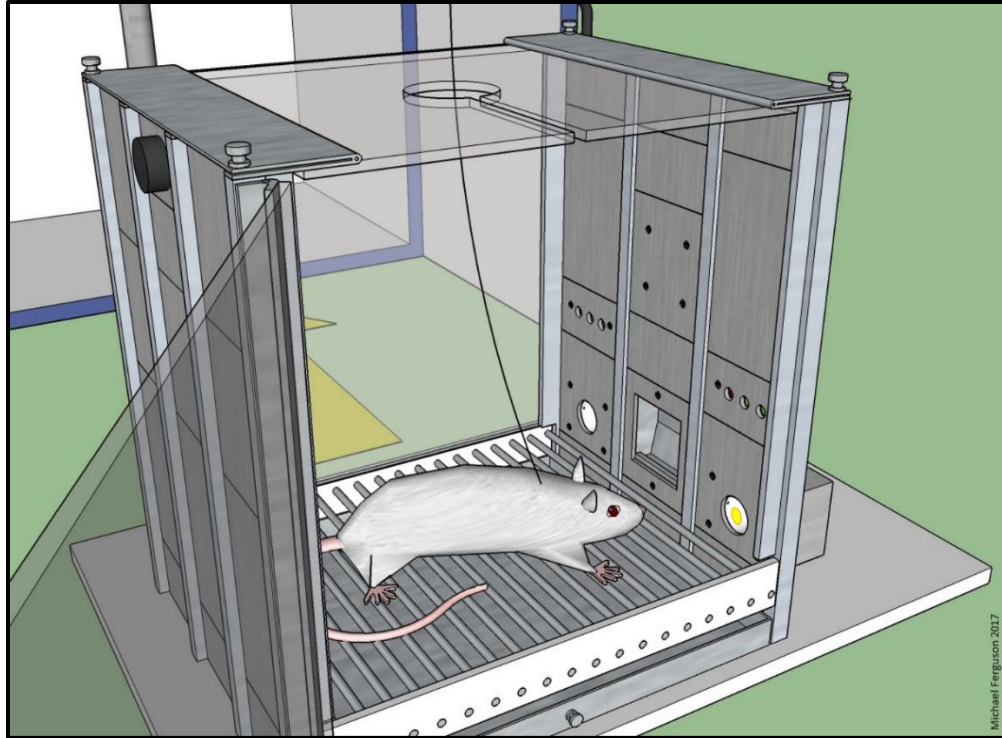


Figure 1.1 Operant boxes for rodent self-administration

Image displays a rat in an operant chamber with two nose pokes and a pellet dispenser. The “active” nose poke is highlighted in yellow and indicates the active nose poke in which a response results in drug delivery (also known as the discriminative stimulus). Artwork by Michael Ferguson, University of Michigan, 2017.

The gold standard technique used to study drugs of abuse and abuse liability is drug self-administration. Animals readily self-administer certain drugs commonly abused by humans such as cocaine, amphetamine, and opioids (Griffiths et al. 1979; O'Connor et al. 2011), thus this technique is often used to examine the reinforcing properties of drugs of abuse and determine the abuse liability of novel drugs. Self-administration procedures also model different aspects of substance-use disorders such as drug-taking behaviors, drug-seeking behaviors, and relapse, making them a useful, direct tool for studying drugs of abuse. Governmental regulatory agencies have previously taken self-administration data into consideration when scheduling drugs

(O'Connor et al. 2011). These studies allow animals to self-administer drugs of abuse orally, intravenously, or through inhalation. In standard self-administration studies, animals are placed in an operant box (Figure 1.1) where they can respond on levers or other manipulanda for a reward. When the animal responds under a specific schedule, it receives a reward such as food or drug. A reward is considered a reinforcer if it increases the likelihood of a behavior (i.e. lever pressing) occurring. A discriminative stimulus (such as a light in the nose poke) may signal to the animal the availability of a reinforcer. A conditioned stimulus is often paired with delivery of the reinforcer; for example, a tone or light occurring every time the animal receives a reinforcer. Conditioned stimuli often carry their own salience, likely due to their association with a reward (Flagel et al. 2011). One of the most common schedules used is the fixed-ratio (FR) schedule of reinforcement, where a set number of responses always results in the delivery of a reinforcer. FR schedules are among the simplest and most common procedures to use when studying drug-taking behavior (Panlilio and Goldberg 2007; Spealman and Goldberg 1978). Utilizing self-administration as a model for substance-use disorders is a highly useful tool but inevitably contains some limitations, mainly that self-administration models do not accurately model all the social and environmental factors associated with drug use. Despite these limitations, self-administration techniques remain one of the best tools to study drugs of abuse.

More intricate schedules of reinforcement can model drug-seeking behavior and examine the reinforcing efficacy of different drugs. One example of these more complex schedules is the progressive-ratio (PR) schedule of reinforcement. Under PR schedules of reinforcement, the work requirement (number of responses) for the delivery of a reward increases within or between sessions, until the animal no longer completes the necessary work requirement. The final ratio completed is referred to as the breakpoint. PR studies are thought to model drug craving or

motivation (Hodos and Kalman 1963; Markou et al. 1993). Furthermore, comparing the breakpoint across different drug reinforcers allows experimenters to draw conclusions about the reinforcing efficacy of the drugs (Brady 1991; Hodos 1961; Richardson and Roberts 1996). Richardson and Roberts have argued that while responding under an FR schedule of reinforcement provides data on whether or not a drug can act as a reinforcing stimulus, a benefit of a PR schedule is that it provides quantitative data on the reinforcing or motivational strength of drugs (Richardson and Roberts 1996; Roberts and Richardson 1992).

All of the assays above can be used to identify and assess novel therapeutics for the treatment of substance-use disorders (Haney and Spealman 2008; Mello and Negus 1996; Nader 2016). An ideal therapeutic would decrease responding for the drug or alter its reinforcing efficacy in self-administration models without altering all operant responding. This can be assessed by examining if the drug in question alters responding for a natural reinforcer such as food. Multiple doses of drugs need to be studied to determine the extent of the drugs' actions; an ideal treatment for drug abuse would not simply shift the dose-effect curve for drug self-administration to the right but dampen responding for a drug (Mello and Negus 1996). Strategies such as CPP, ICSS, drug discrimination, and self-administration require considerable time and training in order to collect data, therefore behaviors that do not require training, such as locomotor activity, are used as a quick and early screening process when identifying new medications (Nader 2016). Altogether, these techniques have been used during the development of medications (i.e. buprenorphine, varenicline) that successfully treat substance-use disorders (Biala et al. 2010; Wiebelhaus et al. 2016; Winger et al. 1992)

Dopamine and the reward pathway

Drugs that are commonly abused are divided into many different categories. One of the categories of drugs most commonly abused are psychostimulants. Psychostimulants, including caffeine, nicotine, cocaine, and amphetamine, are named due to their ability to stimulate the central nervous system (Favrod-Coune and Broers 2010) and can result in arousal and alertness. Within the larger class of psychostimulants are indirect sympathomimetics, which increase norepinephrine (Burn and Rand 1958; Koob et al. 2014) and dopamine levels in the brain; (McCreary et al. 2015; Sulzer 2011); this class includes amphetamine and cocaine. Additional classes of abused drugs include depressants such as alcohol, opioids (e.g. heroin), and cannabis. One thing these drugs all have in common is that they increase dopamine levels in the brain (Sulzer 2011).

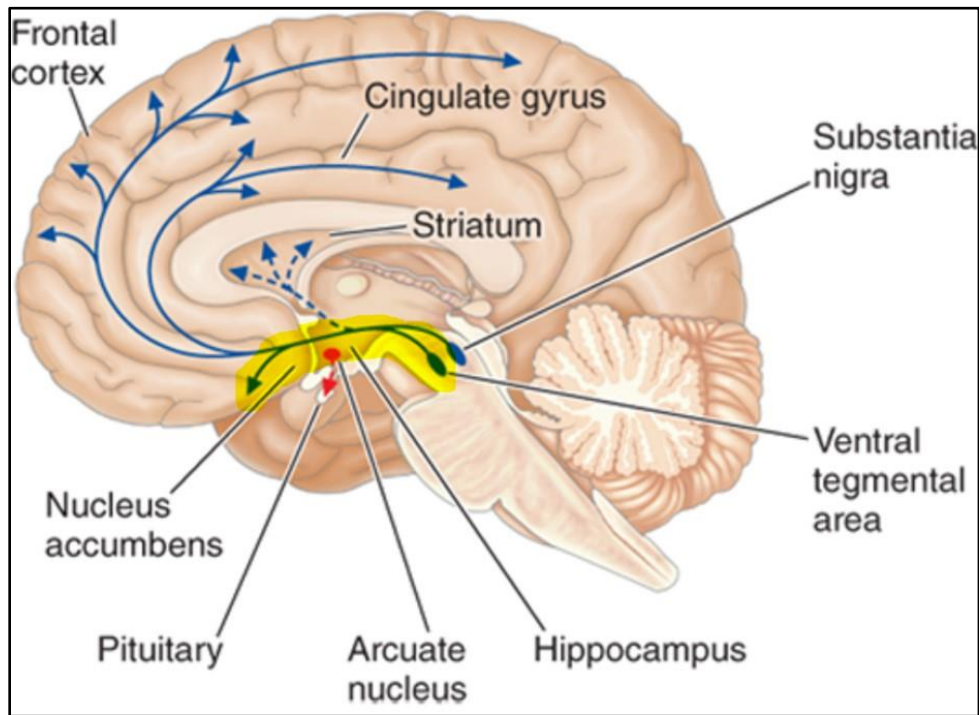


Figure 1.2 Main dopaminergic pathways in the brain

Adapted from Goodman and Gilman's: The Pharmacological Basis of Therapeutics, 13 edition, this figure shows the mesolimbic, mesocortical, and nigrostriatal pathways in blue and

the tuberoinfundibular pathway in red. The mesolimbic pathway is highlighted in yellow (Sibley et al. 2017).

Dopamine is a catecholamine that is important for the body and brain. Dopamine within the body plays a role in cardiovascular and renal functions (Missale et al. 1998). Within the brain, dopamine is important for functions including learning, movement, attention, and memory (Sibley 1999). Dysregulation of dopamine has been implicated in diseases such as Parkinson's disease, schizophrenia, and substance-use disorders (Abi-Dargham 2014; Ehringer and Hornykiewicz 1960; Volkow et al. 2004). The main dopaminergic pathways in the brain are the mesolimbic pathway, the mesocortical pathway, the nigrostriatal pathway, and the tuberoinfundibular pathway (Figure 1.2). The mesolimbic pathway has been identified as an important pathway that regulates reward, including natural rewards such as food and sex as well as drugs of abuse. This pathway begins with dopaminergic cell bodies in the ventral tegmental area (VTA) that extend to the nucleus accumbens (NAc). Drugs of abuse increase activity of dopaminergic neurons along this pathway, and dopamine increase in the NAc is important for the salience of rewards (Di Chiara and Imperato 1988; Volkow et al. 2004).

Dopamine is synthesized when tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) through the addition of a hydroxyl group to tyrosine by tyrosine hydroxylase. L-DOPA is then converted to dopamine through the removal of a carboxyl group by aromatic amino acid decarboxylase. The tyrosine hydroxylase conversion of tyrosine to L-DOPA is considered the rate-limiting step in dopamine synthesis and is important for negative feedback to regulate dopamine levels (Meyer and Quenzer 2005). Dopamine can be further metabolized to norepinephrine by dopamine β -hydroxylase and degraded by monoamine oxidase (MAO), aldehyde dehydrogenase (ALDH), and catechol-O-methyl transferase (COMT). As MAO is

active within the presynaptic neuron, storage in synaptic vesicles prevents MAO from degrading dopamine. Dopamine is transported into vesicles through the vesicular monoamine transporter (VMAT). When an action potential depolarizes the neuron, dopamine is released into the extracellular space via exocytosis. Once released, dopamine can bind to presynaptic and postsynaptic dopamine receptors. Postsynaptic dopamine receptors are G-protein coupled receptors (GPCR) that are classified as D₁-like receptors (coupled to stimulatory G_s proteins) and D₂-like receptors (coupled to the inhibitory G_{i/o} proteins). Activation of these receptors modulate functions such as motor activity, attention, and sleep (Beaulieu and Gainetdinov 2011). D₂-like receptors are also found on postsynaptic neurons as well as presynaptic neurons and are involved in dopamine regulation (further discussed in the next session).

Dopamine regulation

Fine-tuning dopaminergic signaling is important for proper physical and mental health. In order to terminate dopaminergic signaling, dopamine is cleared from the extracellular space by degradation, diffusion or reuptake into neurons. The machinery necessary for reuptake is the dopamine transporter (DAT). DAT is a transmembrane protein in the presynaptic neuron that removes dopamine from the extracellular space into the neuronal terminal where it is repackaged into vesicles or recycled. DAT has 12 transmembrane domains and intracellular-facing N-terminal and C-terminal domains. These domains contain multiple sites for posttranslational modifications such as phosphorylation, palmitoylation, and ubiquitination that help modulate DAT function, degradation, and/or trafficking (Vaughan and Foster 2013). A crystal structure from a homologous Na⁺, Cl⁻ dependent transporter, LeuT, suggests that DAT exists in both inward (intracellular)-facing and outward (extracellular)-facing conformations; movement between the two conformations is important for the transport of substrates into or out of the

neuron. Along with two Na⁺ and one Cl⁻, dopamine binds to DAT when it is in its outward-facing conformation. When DAT shifts to its inward-facing conformation, dopamine and the ions dissociate from DAT and are released into the cytoplasm. The Na⁺ and Cl⁻ gradients drive the transport (Pramod et al. 2013; Shan et al. 2011; Yamashita et al. 2005).

An additional mechanism of dopamine regulation is through the dopamine receptors. Several GPCRs in the D₂-like family, D₂, D₃, and D₄ receptors, are found presynaptically and are referred to as autoreceptors. Activation of these receptors can regulate dopamine through multiple mechanisms: by decreasing exocytotic release of dopamine, increasing dopamine uptake and altering rates of dopamine synthesis (Beaulieu and Gainetdinov 2011; Joseph et al. 2002). Multiple studies have suggested that D₂ or D₃ activation can decrease the rate of firing in dopaminergic neurons, likely through activation of potassium channels or inactivation of calcium channels, which ultimately decreases extracellular dopamine release (Kuzhikandathil et al. 1998; Neve et al. 2004). Studies have also indicated that autoreceptor activation increases DAT activity, resulting in an increase in dopamine clearance from the synapse (Wu et al. 2002). Further studies have suggested that D₂ and D₃ receptor activation affects DAT trafficking, with short exposures to D₃ agonists increasing surface DAT and long exposures increasing DAT internalization (Bolan et al. 2007; Zapata et al. 2007). Additionally, autoreceptor activation inhibits tyrosine hydroxylase, thus decreasing dopamine levels within the neuron (O'Hara et al. 1996).

Amphetamines

History of amphetamine

One of the most commonly misused class of drugs worldwide is amphetamines, a class of psychostimulants that include amphetamine, methamphetamine, and 3,4-methylenedioxy-

methamphetamine (Ecstasy). Natural amphetamines, such as components of the ephedra plant, have been used for thousands of years worldwide and are known for their stimulating properties. Japanese chemists studying ephedra first extracted l-ephedrine and r-pseudoephedrine from ephedra in 1885 and proposed its use for pupil dilation. Later studies revealed that ephedrine was a sympathomimetic and scientists sought to use ephedrine as a treatment for asthma (Lee 2011). Dr. Gordon Alles began focusing on amphetamine during the search for a better and cheaper drug with ephedrine-like actions (Rasmussen 2015). Smith, Kline, and French (currently GlaxoSmithKline) began marketing Benzedrine, a racemic mixture of d- and l-amphetamine, as a decongestant. Despite the failure with original intended use of amphetamine, asthma, researchers began looking at amphetamine to treat a variety of conditions such as narcolepsy, dysmenorrhea, and depression (Rasmussen 2006). Amphetamine gained popularity when it was discovered that it increased alertness and performance on intelligence tests (Davidoff et al. 1937; Nathanson 1937). Charles Bradley (M.D.) gave Benzedrine to children with behavioral disorders and found that Benzedrine subdued behaviors in some children and stimulated behavior others, findings that were important in the classification and treatment of attention deficit hyperactivity disorder (ADHD) (Strohl 2011). Amphetamines are used to this day in the treatment of ADHD, commonly in the form of Adderall (3:1 d-amphetamine to l-amphetamine).

Despite their medical importance, amphetamines have been historically misused worldwide. Misuse of Benzedrine was identified early among university students and misuse of Benzedrine inhalers were particularly an issue in the military during World War II (Davies 1939; Jackson 1971). During the 1960s, there were many reports on the increased use of amphetamines beyond therapeutic doses and for non-prescribed purposes (Kiloh and Brandon 1962; Rasmussen 2015). It was later found that extensive use of amphetamines led to an increased risk of

psychiatric disorders such as psychosis (Connell 1966). Currently, amphetamine misuse is still prevalent worldwide. The Monitoring the Future study, which evaluates drug use in high school students, found non-medical usage of amphetamines to be the highest reported drug used among high school students after alcohol and marijuana (Miech et al. 2018). Furthermore, a survey from 2017 estimated over 570,000 people in the United States with a stimulant-use disorder and over 960,000 people with a methamphetamine-use disorder (SAMHSA 2018). From the 2016 National Surveys on Drug Use and Health, Compton et al. (2018) showed that over 8% of adults who used prescription stimulants in the previous year had a substance-use disorder. From 2005 to 2016, there was a 35% increase in amphetamine confiscations globally along with a large increase in prescription stimulant consumption (Rasmussen 2006; UNODC 2018). Furthermore, diagnoses of ADHD are becoming more common (Xu et al. 2018), leading to more prescriptions of amphetamines. As extensive misuse of amphetamines has serious consequences on physical and mental health, efforts to decrease amphetamine use are important and necessary.

Mechanism of action

Amphetamines are considered indirect sympathomimetics and are structurally similar to dopamine and norepinephrine (Figure 1.3). They increase dopamine, norepinephrine, and to a lesser extent serotonin levels in the brain (Fleckenstein et al. 2007). Amphetamines are thought to act at monoamine transporters to increase extracellular dopamine (through DAT), norepinephrine, and serotonin levels. Due to their similar structure, amphetamines can act as substrates at DAT to enter the neurons along with sodium, competing with dopamine for uptake (Azzaro et al. 1974; Zaczek et al. 1991). Once in the neuron they cause changes that ultimately result in increased levels of extracellular dopamine, which are associated with the reinforcing effects of amphetamine.

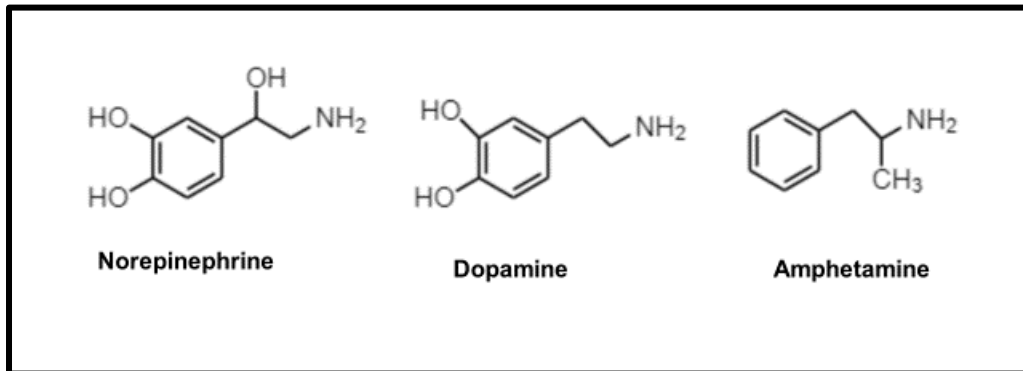


Figure 1.3 Structure of norepinephrine, dopamine, amphetamine

Amphetamine also alters extracellular dopamine levels by affecting machinery necessary for negative feedback. Studies have demonstrated that long exposures to amphetamine can decrease D₂/D₃ autoreceptor function, potentially through increased internalization of autoreceptors (Calipari et al. 2014; Luessen et al. 2016). This would lead to a decrease in the ability to regulate dopamine release. The findings on how amphetamine affects tyrosine hydroxylase activity have been mixed. Early studies showed that amphetamine exposure can lead to an increase in tyrosine hydroxylase activity, causing an increase in dopamine synthesis (Larsen et al. 2002; Mandell and Morgan 1970). Other studies found a decrease in tyrosine hydroxylase activity and suggested the differing results may be specific to tissue type or dose of amphetamine (Besson et al. 1973; Harris et al. 1975; Sulzer et al. 2005). Additional mechanisms may include activity at or regulation of trace amine-associated receptor-1 (TAAR1). TAAR1 is a GPCR coupled to G_s stimulatory proteins that can be activated by amphetamine. Amphetamine has a higher affinity for TAAR1 than DAT (K_i = 0.23 μM for rat TAAR1, 3.6 μM for rat DAT) (Ritz and Kuhar 1989; Simmler et al. 2016). Activation of TAAR1 decreases dopamine uptake through DAT (Xie and Miller 2007). While all the above may affect amphetamine-stimulated

dopamine levels, the canonical mechanism by which amphetamine increases extracellular dopamine levels is through its actions at the vesicular monoamine transporters (VMAT) and DAT.

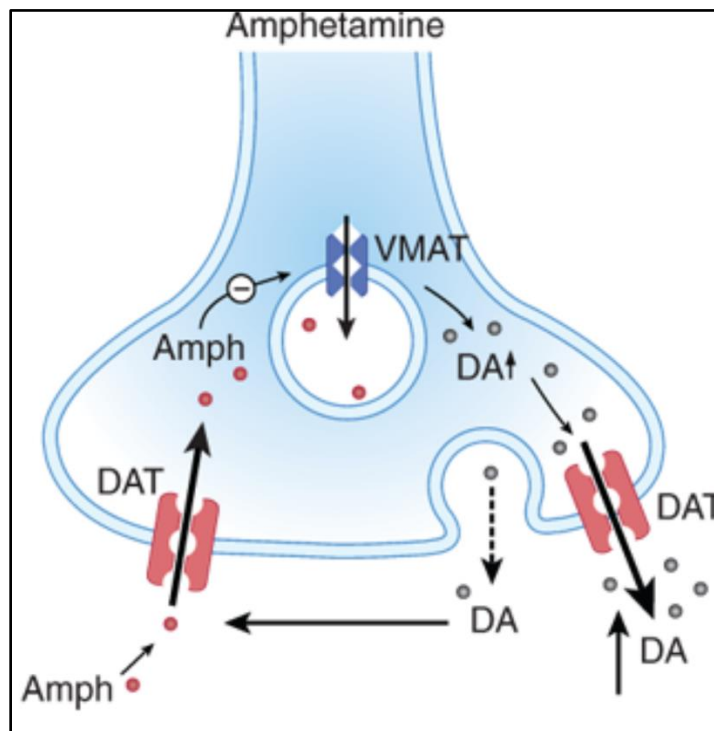


Figure 1.4 Mechanism of action of amphetamine

Adapted from “Drugs of Abuse”, Katzung BG, *Basic and Clinical Pharmacology* 2017. This image shows a dopaminergic neuron in the presence of amphetamine. Amphetamine (red circles) enters through DAT and causes the displacement of dopamine (white circles) from the vesicles and dopamine release through DAT (Lüscher 2017).

Years of research have suggested that amphetamine can enter a neuron via DAT, increase cytoplasmic dopamine, and ultimately reverse the direction of dopamine transport through DAT (Figure 1.4). The role of DAT in the action of amphetamine was determined in studies showing that DAT uptake blockers decrease the ability of amphetamine to increase extracellular dopamine (Raiteri et al. 1979; Sulzer et al. 1995). While amphetamine can elicit an increase in dopamine levels with DAT alone, the presence of VMAT enhances the effects of amphetamine (Pifl et al. 1995). The role of DAT in amphetamine’s action *in vivo* was further demonstrated by

Giros et al. (1996) who showed that amphetamine did not elicit an increase in locomotor activity in DAT knockout mice. The mechanisms by which amphetamine affects vesicular and cytosolic dopamine are regulated by factors such as dose of amphetamine. Butcher et al. (1988) utilized microdialysis to demonstrate that amphetamine preferentially releases newly synthesized dopamine found in the cytosol, however studies with reserpine also demonstrated the importance of VMAT in the mechanism of large doses of amphetamine. Cadoni et al. (1995) showed that reserpine, which decreases stores of dopamine in vesicles, blocked amphetamine-stimulated dopamine release with large doses of amphetamine (2 mg/kg) but not low doses of amphetamine (0.5 mg/kg). The “weak base” hypothesis is a widely accepted explanation for amphetamine-stimulated increases in cytosolic dopamine. As amphetamine is weak base, it is protonated and accumulates in vesicles. This leads to the alkalization of vesicles, which disrupts the proton gradient necessary for transport of dopamine into the neuron (Sulzer and Rayport 1990). Another theory suggests that the alkalization of the vesicles occurs when amphetamine is transported into the vesicles via VMAT and is exchanged for protons (Freyberg et al. 2016). Once in the cytoplasm, dopamine is released into the synapse through DAT. Multiple mechanisms have been proposed for the reversal of DAT function. One theory for efflux through DAT is called the “facilitated exchange model”. DAT has an outward- and inward-facing conformation that directs the direction of substrate movement. In the facilitated exchange model, transport of amphetamine into the neuron moves DAT from the outward-facing conformation to the inward-facing information and allowing for dopamine efflux (Sulzer et al. 2005). Harald Sitte and the Piffl group found efflux through DAT had a stronger correlation to currents induced by DAT substrates than substrate uptake, calling into question the facilitated exchange model (Sitte et al. 1998). Studies have also demonstrated that changes in the Na^+ gradient can drive dopamine

efflux (Khoshbouei et al. 2003). Post translational modifications such as phosphorylation have also been implicated in the reversal of DAT function (Vaughan and Foster 2013).

Protein kinase C

One of the most studied enzymes in relation to DAT phosphorylation is protein kinase C (PKC). PKC is a protein in the serine-threonine kinase family and is common in many signal transduction pathways. PKC is important for many different physiological processes such as regulation of angiogenesis and the immune response and can lead to the development of diseases such as diabetes and cancer (Mochly-Rosen et al. 2012). There are many different isoforms of PKC and they are divided into three different classes based on their structure: classical or conventional PKC (α , β I, β II, γ), atypical PKC (δ , θ , η , ϵ), and novel PKC (ζ , λ , ι) isoforms. All PKC isoforms contain a regulatory domain and a catalytic domain, but they differ primarily in their regulatory domain. The regulatory domain of classical PKCs contains a pseudo-substrate (important for keeping PKC inactive), two diacylglycerol binding domains, and a calcium binding domain (Figure 1.5). The catalytic domain is made up of an ATP-binding domain and a substrate binding domain. Novel PKCs do not contain a calcium-binding domain, and atypical PKCs contain neither a calcium-binding domain nor a diacylglycerol-binding domain. In their inactive conformation, the pseudo-substrate domain is bound to the substrate domain. Following binding of Ca^{2+} , PKC translocates to the plasma membrane, binds to diacylglycerol, and undergoes a conformational change causing the dissociation of the pseudo-substrate domain from the substrate binding domain, allowing actual substrates to bind to PKC and be phosphorylated (Callender and Newton 2017).

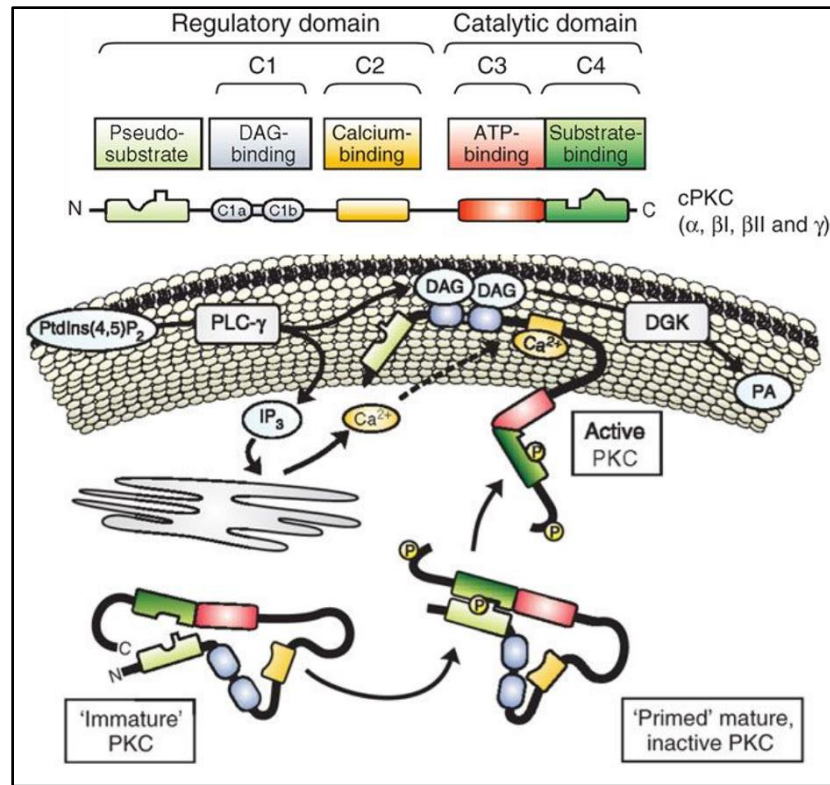


Figure 1.5 Protein kinase C activation

The structure of the classical PKCs is shown above an image demonstrating PKC activation. PKC in its active form is bound Ca^{2+} , diacylglycerol (DAG), and the plasma membrane (Spitaler and Cantrell 2004).

Classical isoforms of PKC are highly expressed throughout the brain and important for different functions within the brain (Kikkawa et al. 1983). Some substrates of PKC include growth-associated protein 43 (GAP43), a protein important for long term potentiation and memory, NMDA and AMPA receptors, proteins important for neurotransmission, and DAT (Callender and Newton 2017; Oehrlein et al. 1996; Vaughan et al. 1997). Early studies demonstrated that PKC activation alters neurotransmitter release (Zurgil and Zisapel 1985), but Cecilia Giambalvo provided some of the first direct evidence for a relationship between PKC and dopamine release, showing drugs that increase extracellular dopamine levels also increase PKC levels in membrane preparations, presumably indicating levels of active PKC (Giambalvo 1988).

She demonstrated that amphetamine (0.3-3.0 mg/kg) would increase PKC in membrane preparations and these effects were separate from autoreceptor activation, leading her to conclude that PKC modulates DAT (Giambalvo 1992a; b). Cloning of DAT in the early 1990s led to the discovery of multiple intracellular consensus sequences for PKC phosphorylation in the N-terminal region (Shimada et al. 1991). Activation of PKC with phorbol 12-myristate 13-acetate (PMA) increased phosphorylation of DAT and decreased dopamine uptake (Copeland et al. 1996; Huff et al. 1997; Kitayama et al. 1994), likely due to increased DAT internalization (Melikian and Buckley 1999).

In addition to showing PKC played a role in dopamine inward transport, many studies also elucidated its role in reverse-transport, particularly in relation to amphetamine. Iwata et al. (1997) demonstrated that amphetamine increased PKC activity *in vivo*. Increased Ca^{2+} levels in the cytosol in response to amphetamine is most likely responsible for an increase in PKC activation (Giambalvo 2004). PKC activation increased dopamine efflux through DAT, mimicking the effects of amphetamine (Cowell et al. 2000). Conversely, PKC inhibition decreased the effects of amphetamine *in vitro* (Giambalvo 1992b; Kantor and Gnegy 1998). Removal of the first 22 amino acids from the N-terminal region of DAT (containing the PKC phosphorylation sites) inhibited amphetamine-stimulated dopamine release (Khoshbouei et al. 2004), demonstrating that phosphorylation of DAT was likely important for the mechanism of action of amphetamine. Of all the PKC isoforms, PKC β II is the most important for amphetamine action. PKC β I and II are splice variants of the same protein differing in the C-terminal domain (Ono et al. 1986). Overexpression of PKC β II in cell models results in increased amphetamine-stimulated dopamine release, as compared with overexpression of PKC β I and PKC α (Johnson et al. 2005). Additionally, studies using PKC β knockout mice showed an attenuated response to

amphetamine-stimulated dopamine release in their striatal tissue and a decrease in amphetamine-stimulated locomotor activity (Chen et al. 2009). Based on this mechanism, PKC β has been proposed as a potential therapeutic target for treating amphetamine use disorder.

Therapeutics for amphetamine-use disorder

There is currently no known pharmacological therapeutic for the treatment of amphetamine-use disorder and the standard therapy currently only involves behavioral therapies. Thus, the development of new therapeutic options is a necessity. With the current knowledge about the mechanism of action of amphetamine, multiple targets have been proposed as potential therapeutics for amphetamine-use disorders. These targets include dopamine antagonists, VMAT inhibitors, DAT inhibitors and PKC inhibitors. The targets discussed in the following paragraphs are all in various stages of development.

One target for amphetamine-use disorder is to block signaling downstream induced by amphetamine. This has led to the testing of drugs targeting dopamine receptors as potential therapeutics. D₂ partial agonists act as an agonist in the presence of low dopamine levels and an antagonist in the presence of high dopamine levels and decrease amphetamine-stimulated locomotor activity (Clark et al. 1991). Aripiprazole decreased methamphetamine self-administration under an FR and PR schedule of reinforcement and attenuated subjective effects in human subjects (Stoops et al. 2006; Wee et al. 2007). Despite these promising results, aripiprazole was not significantly different from a placebo treatment in larger scale trials (Coffin et al. 2013). Another area that is currently being explored is the development of D₃ receptor antagonists. Amy Newman's group has done extensive work developing D₃-selective antagonists in rodent models. They have demonstrated that D₃ receptor antagonists decrease methamphetamine self-administration in rats under a PR schedule of reinforcement and block

cue-induced reinstatement (Higley et al. 2011a; Higley et al. 2011b). Despite the promising preclinical results, clinical studies investigating the role of D₃-selective antagonists for substance-use disorders have not shown any promise, though buspirone, a non-selective D₃ antagonist, may help patients undergoing withdrawal from different drugs of abuse. (Le Foll et al. 2014)

Another target for treating amphetamine use disorders is the monoamine transporters. Studies with VMAT2 inhibitors, such as lobeline, have shown that VMAT inhibitors can decrease amphetamine-stimulated locomotor activity. Lobeline decreases methamphetamine self-administration under an FR5 schedule of reinforcement, but an acute pretreatment also decreased sucrose self-administration (Harrod et al. 2001; Miller et al. 2001). Human trials with VMAT2 inhibitors are still in the early stages and have demonstrated lobeline is a safe drug with an aversive taste that could deter compliance (NIDA 2916). Other VMAT2 inhibitors for the treatment of substance-use disorders are still in the early stages of development (Nickell et al. 2014).

DAT inhibitors are another class of drugs that have been a focus of amphetamine-use disorder therapeutics. One DAT inhibitor that has been widely studied is modafinil, but it also has actions other than DAT inhibition. Modafinil decreases responding under an FR1 schedule of reinforcement for methamphetamine and also blocks cue- and drug-primed reinstatement (Reichel and See 2012). Small scale clinical trials showed some promise for the efficacy of modafinil to decrease methamphetamine use and craving (McElhiney et al. 2009) but larger trials showed only a modest decrease in methamphetamine use and no change in craving (Shearer et al. 2009). A concern about DAT inhibition is that a drug increasing extracellular dopamine (as modafinil does) may have similar reinforcing properties to cocaine. Modafinil partially

generalizes to cocaine in a discrimination assay and is self-administered by monkeys at high doses (Gold and Balster 1996). This has led to concerns that any therapeutic blocking DAT will contain its own abuse liability.

Additional targets for amphetamine-use disorder therapeutics are TAAR1 agonists. TAAR1 agonists block the development of methamphetamine sensitization and decrease methamphetamine self-administration, shifting the dose effect curve downward, and block cue- and drug-induced reinstatement (Jing et al. 2014). Despite their promise, the exact mechanism of TAAR agonist action has not been characterized (Liu and Li 2018). Outside of the dopamine system, gamma-aminobutyric acid (GABA) agonists, antidepressants, opioid antagonists, and selective serotonin reuptake inhibitors have also been considered as potential therapeutics for different aspects of amphetamine-use disorders (Cao et al. 2016; Morley et al. 2017).

A promising target for amphetamine-use disorder therapeutics is PKC inhibitors. As mentioned in the previous section, PKC plays an important role in the action of amphetamine, and multiple studies have demonstrated that inhibition of PKC blocks amphetamine-stimulated dopamine release (Johnson et al. 2005; Kantor and Gnegy 1998; Loweth et al. 2009). Additionally, direct injection of general PKC inhibitors into the NAc reduces amphetamine-stimulated locomotor activity (Browman et al. 1998). The role of PKC β specifically in amphetamine-stimulated dopamine release was further confirmed utilizing genetic models of PKC β inhibition and in microdialysis studies with PKC β -selective drugs, enzastaurin and ruboxistaurin (Chen et al. 2009; Zestos et al. 2016). The behavioral effects of PKC inhibition may be due to more than the ability of PKC to modulate DAT as PKC inhibition has also been shown to potentiate D₂ receptor function (Luderman et al. 2015; Namkung and Sibley 2004).

There are very few known isoform-selective PKC inhibitors. Enzastaurin and ruboxistaurin are two PKC β -selective inhibitors developed by Eli Lilly (Figure 1.6). These drugs inhibit PKC β with a K_i of 5-6 nM by binding to the ATP-binding site of the catalytic domain of PKC, preventing activation (Faul et al. 2003). These compounds were developed for the treatment of glioblastomas and diabetic retinopathy. Both drugs made it up to Phase III clinical trials, where they failed due to an inability to meet clinical endpoints (Bourhill et al. 2017). One important finding that these drugs displayed a good safety profile with little to no serious adverse events (Carducci et al. 2006; Javey et al. 2010). They have a limited therapeutic potential due to their low bioavailability; however, they make good tools to study PKC β inhibition in animal models and cellular studies. Enzastaurin and ruboxistaurin have been used in proof of principle studies that show the promise of PKC inhibitors as viable therapeutics (Zestos et al. 2016).

There is a dearth of bioavailable PKC inhibitors that can cross the blood-brain barrier (Chico et al. 2009). One brain-permeable PKC inhibitor that has been identified is tamoxifen. Tamoxifen is known for its effects as a selective estrogen receptor modulator and has many adverse effects that could limit compliance; however, it is also a non-selective PKC inhibitor. Repeated tamoxifen administration decreases amphetamine-stimulated locomotor activity (Einat et al. 2007) and has been investigated as a treatment for bipolar mania, which is commonly modeled by acute injections of amphetamine in rodents (Zarate et al. 2007). Recently there has been an effort to identify new PKC inhibitors, utilizing tamoxifen as a scaffold (Carpenter et al. 2016). The new PKC inhibitors have been designed to possess brain permeability and PKC inhibition with no affinity for estrogen receptors. The most promising of these drugs, **6c**, can successfully inhibit amphetamine-mediated behaviors but is still in the early stages of research.

Further development of **6c** and other compounds from the tamoxifen scaffold would pave the way for a new wave of potential therapeutics for amphetamine-use disorder.

While strides have been made to examine the effects of PKC inhibitors on amphetamine, at this point little has been done to examine whether these drugs alter the reinforcing effects of amphetamine. Inhibition of PKC decreases quinpirole-induced reinstatement of responding for cocaine (Ortinski et al. 2015) and amphetamine CPP (Aujla and Beninger 2003), suggesting that PKC plays a role in the rewarding properties of these stimulants. However, very little work has been done to investigate whether these drugs alter drug-taking behaviors. We have shown that the non-selective PKC inhibitor **6c** decreases amphetamine self-administration behavior under an FR5 schedule of reinforcement (Carpenter et al. 2017), but no work has been published with PKC β -selective inhibitors. Showing that PKC β -selective inhibitors decrease the reinforcing effects of amphetamine will go a long way towards demonstrating their usefulness as a therapeutic for substance-use disorders.

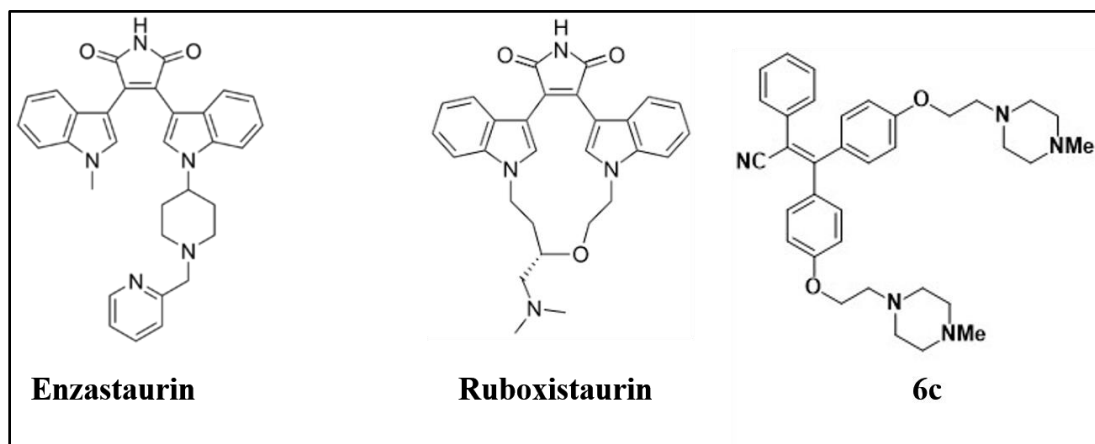


Figure 1.6 Chemical structures of enzastaurin, ruboxistaurin, and 6c

Summary and hypothesis

There are currently no good pharmacological interventions for the treatment of amphetamine-use disorder, despite the need. Studies examining the mechanism of action of

amphetamine have identified PKC β as a potential target for novel substance-use disorder therapeutics. The goal of my thesis is to characterize the effects of PKC β inhibitors on amphetamine-mediated behaviors, especially drug-taking behaviors, in order to determine their feasibility as therapeutic interventions. I will show that PKC β inhibitors decrease amphetamine-mediated behaviors and decrease the reinforcing effects of amphetamine. I will also propose a novel mechanism by which PKC β inhibitors are acting to reduce amphetamine-mediated behaviors. These findings strongly support the development of PKC β inhibitors for the treatment of amphetamine-use disorders.

In Chapter 2, I fully characterize the effects of enzastaurin, a PKC β -selective inhibitor, on amphetamine-mediated behaviors. Previous studies by Zestos et al. (2016), have shown that enzastaurin blocks amphetamine-mediated increases in extracellular dopamine levels. I sought to demonstrate that enzastaurin reduces amphetamine-stimulated locomotor activity and show for the first time that enzastaurin decreases amphetamine self-administration. In this chapter, the effect of enzastaurin on amphetamine-stimulated locomotor activity is characterized across different doses of enzastaurin, amphetamine, and pretreatment times. Enzastaurin (10 pmol) administered intracerebroventricularly (*i.c.v.*) shifts the dose-effect curve for amphetamine-stimulated locomotor activity to the right. More importantly, enzastaurin attenuates the reinforcing effects of amphetamine in a self-administration paradigm using an FR5 schedule of reinforcement in rats. Interestingly, these effects are only observed following an 18 hr pretreatment, and a shorter pretreatment time (3 hr) is not effective at altering amphetamine-maintained responding. Enzastaurin does not alter responding for sucrose under an FR5 schedule of reinforcement, demonstrating that the observed results with amphetamine are not due to behavioral suppression. Interestingly, I found that, when given *i.c.v.*, small doses of enzastaurin

(10-30 pmol) require an extended period of time to decrease amphetamine-stimulated locomotor activity. A large dose of enzastaurin (1 nmol) can acutely decrease amphetamine-stimulated locomotor activity. This provides the first hint that PKC β inhibitors decrease amphetamine-mediated behaviors through more than one mechanism. Altogether, these results support our hypothesis that PKC inhibition decreases amphetamine-mediated behaviors, especially the reinforcing effects of amphetamine.

In Chapter 3, I assess responding for amphetamine and sucrose under a PR schedule of reinforcement, comparing two different PKC inhibitors. Here, I compare responding for **6c** administered subcutaneously and enzastaurin administered through ICV injections. **6c** is a brain-permeable PKC inhibitor based on the tamoxifen scaffold. We previously demonstrated that **6c** (*s.c.*) alters responding for amphetamine under an FR5 schedule of reinforcement 18 hr after injection (Carpenter et al. 2017). I find that 18 hr after enzastaurin or **6c** pretreatment, the breakpoint for amphetamine responding decreases without altering responding for sucrose. A larger dose of enzastaurin is required to change responding under a PR schedule compared to the previous studies under an FR schedule. These findings further our conclusion that PKC inhibition alters the reinforcing properties of drugs and show that PKC inhibition will decrease the reinforcing strength of amphetamine.

The time course of PKC inhibitor effectiveness has raised the possibility of the inhibitors acting through multiple mechanisms to decrease amphetamine-mediated behaviors. In Chapter 4, I will discuss data further supporting the existence of multiple mechanisms. I found that if ruboxistaurin, another PKC β -selective inhibitor, is injected into the NAc, a short pretreatment (30 min) is enough to decrease locomotor activity, but the effect is gone 18 hr later. Conversely, if ruboxistaurin is injected into the VTA, amphetamine-stimulated locomotor activity is not

affected following an acute injection but is decreased 18 hr later. A decrease in PKC activity is seen in the NAc following an acute injection of a PKC β inhibitor into the NAc. Following an 18 hr pretreatment into the VTA, I observe a decrease in levels of PKC β II levels as well as PKC activity in the VTA. These findings suggest that PKC β inhibitors act directly in the NAc to decrease amphetamine-mediated behaviors but act indirectly in the VTA, likely by downregulating PKC β II.

In Chapter 5 of this thesis, I will discuss the implications of these findings and the future of PKC β inhibitors as therapeutics for amphetamine-use disorder. I will also examine additional factors that may be involved in the action of the PKC inhibitors. The work presented here will provide proof-of-concept work showing that PKC β inhibitors will decrease amphetamine-mediated behaviors and the reinforcing effects of amphetamine. Altogether, the findings of this thesis strongly support the further development of PKC β inhibitors as a new therapeutic for amphetamine-use disorders.

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Chapter 2 The protein kinase C β -selective inhibitor, enzastaurin, attenuates amphetamine-stimulated locomotor activity and self-administration behaviors in rats

Abstract

Pathological amphetamine (AMPH) use is a serious public health concern with no pharmacological treatment options. Protein kinase C β (PKC β) has been implicated in the mechanism of action of AMPH, such that inhibition of PKC β attenuates AMPH-stimulated dopamine efflux *in vivo*. With this in mind, inhibition of PKC β may be a viable therapeutic target for AMPH use disorder. The purpose of this study is to demonstrate that selective pharmacological inhibition of PKC β alters AMPH-stimulated behaviors in rats. Rats were administered intracerebroventricular (*i.c.v.*) injections of the PKC β -selective inhibitor enzastaurin 0.5, 3, 6, or 18 hr before evaluating AMPH-stimulated locomotion (0.32-3.2 mg/kg). Rats were trained to make responses for different doses of AMPH infusions or sucrose under a fixed ratio 5 schedule of reinforcement, and the effects of enzastaurin pretreatment 3 or 18 hr prior to a self-administration session were determined. Also, the effect of enzastaurin on AMPH-stimulated PKC activity in the ventral striatum was evaluated. Low doses of enzastaurin (10-30 pmol) attenuated AMPH-stimulated locomotor activity and shifted the AMPH dose-effect curve to the right following an 18-hr pretreatment. A high dose of enzastaurin (1 nmol) decreased AMPH-stimulated locomotor activity 0.5 hr following enzastaurin administration. Rats pretreated with enzastaurin 18, but not 3, hr prior to a self-administration session showed a

decrease in the number of responses for AMPH, shifted the ascending limb of the amphetamine dose effect curve, and produced no change in responses for sucrose. AMPH-stimulated PKC activity was decreased following a 0.5 or 18 hr pretreatment, but not a 3 hr pretreatment of enzastaurin. These results demonstrate that inhibition of PKC β decreases AMPH-stimulated behaviors and neurobiological changes and suggest that PKC β is potentially a viable target for AMPH use disorder.

Introduction

Amphetamines (AMPHs) are a class of stimulants that are highly abused worldwide; this class includes amphetamine, methamphetamine, and 3,4-methylenedioxymethamphetamine (UNODC 2017). AMPHs are commonly prescribed to children in the form of Adderall for the treatment of attention deficit/hyperactivity disorders (Lakhan and Kirchgessner 2012) but are also commonly misused for non-medical purposes (Johnston et al. 2016; SAMHSA 2014). Long term use of AMPH in patients with attention deficit/hyperactivity disorder has been shown to be relatively safe when properly prescribed. However, long term misuse of AMPH may lead to cognitive deficits and psychosis (Janowsky and Risch 1979; Lakhan and Kirchgessner 2012; Ornstein et al. 2000). Acute effects of AMPH in humans include enhanced attention, alertness, and euphoria (Seiden et al. 1993). Similarly, in laboratory animals, small doses of AMPH produce increased locomotor activity and sustained attention (Grilly et al. 1989; Randrup et al. 1963; Wise and Bozarth 1987). Rodents and monkeys will self-administer AMPH, demonstrating its ability to act as a reinforcer in animal models (Balster and Schuster 1973; Pickens and Harris 1968). Despite the prevalence of AMPH abuse, current treatments are primarily psychological/behavioral in nature and no approved pharmacological interventions are available.

AMPHs elicit their rewarding effects in part by increasing extracellular dopamine levels in the brain via competition with dopamine for uptake into dopaminergic terminal (e.g., dopamine transporter (DAT) substrates). Once in neurons, AMPH reverses the function of DAT, evoking a release of dopamine into the synapse, instead of the removal of dopamine from the synapse (Seiden et al. 1993). AMPH administration also results in an increase in protein kinase C (PKC) activity (Giambalvo 1992). Activation of PKC can lead to increased extracellular dopamine levels through exocytosis and/or via DAT (Cowell et al. 2000; Giambalvo 1988). Inhibiting PKC *in vitro*, *ex vivo*, and *in vivo* attenuates AMPH-stimulated increases in extracellular dopamine levels (Johnson et al. 2005; Kantor and Gnegy 1998; Loweth et al. 2009; Zestos et al. 2016).

Direct injection of PKC β -selective inhibitors into the nucleus accumbens or genetic deletion of PKC β demonstrated the importance of the β -isomer of PKC in dopamine efflux in response to AMPH as well as its effect on AMPH-stimulated locomotor activity (Chen et al. 2009; Zestos et al. 2016). While there are data demonstrating that PKC β modulates the neurochemical effects of AMPH, whether or not PKC β inhibition alters the reinforcing effects of AMPH is unknown. In this study, we characterized the effects of a PKC β inhibitor on AMPH-stimulated behaviors, namely locomotor activity and self-administration in rodents. We found that enzastaurin, a PKC β -selective inhibitor (Faul et al. 2003), effectively decreased AMPH-stimulated locomotor activity and AMPH-maintained responding in a surmountable manner and attenuated PKC activity in the presence of AMPH in the striatum. We believe that these data provide proof-of-concept evidence demonstrating the feasibility of selectively targeting PKC β for the treatment of AMPH abuse.

Methods

Subjects: Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing approximately 300-350g at the start of the experiments were single-housed in a temperature and humidity-controlled environment. Food was available *ad libitum*; however, rats used in AMPH and sucrose self-administration experiments were food restricted to approximately 80-90% of their free-feeding weight. All animals were on a 12-hr dark/light cycle with lights on at 0700 and all testing was done during the light phase. All animal procedures were designed within the rules and regulations of the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Michigan's Institutional Animal Care and Use Committee.

Drug Solutions and Delivery: D-AMPH (National Institute on Drug Abuse, Bethesda, MD) was dissolved in saline and administered subcutaneously (*s.c.*) for locomotor studies or intravenously (*i.v.*) for self-administration studies. Ketamine (Hospira, Lake Forest, IL) and xylazine (Akorn, Lake Forest, IL) were administered intraperitoneally. Enzastaurin was obtained from Cayman Chemicals and dissolved in a vehicle solution containing 0.005% dimethyl sulfoxide (DMSO) in artificial cerebrospinal fluid (Durect, Cupertino, CA).

Enzastaurin or vehicle was administered intracerebroventricularly (*i.c.v.*) via a programmable pump that administered 10 μ l over 1 hr at a rate of approximately 0.17 μ l/min. An infusion cannula (2mm C313I, Plastics One, Roanoke, VA) was connected by Tygon tubing to a single channel plastic swivel (375/22PS, Instech Laboratories, Plymouth Meeting, PA) suspended in a counter-balanced arm (PHM-110-SAI, Med Associates, Fairfax, VT), which was attached to a 10 μ l Hamilton syringe through Tygon tubing. The rats were awake and in their home cage throughout the course of the infusion.

Surgeries:

Cannula Implantation: All rats were implanted with a guide cannula (28 gauge with 1.7 mm projection C313GRL/SPC, Plastics One, Roanoke, VA) to allow for *i.c.v.* injections. Rats were given 5 mg/kg carprofen (*s.c.*) and ketamine/xylazine (90:10 mg/kg *i.p.*) and placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA). The cannula was implanted relative to bregma (AP = -0.8mm, ML=+1.5mm, DV=-2.8mm (Paxinos and Watson 1998)). The guide cannula was held in place with dental cement (OrthoJet-BCA, Lang Dental Manufacturing Co., Wheeling, IL) and anchored with two steel screws (19010-10, Fine Science Tools, Foster City, CA). The guide cannulae were fitted with dummy cannulae with no extension. Following all experiments, cannulae were infused with methylene blue *i.c.v.* while the rats were under heavy anesthesia for 5 min. The rats were decapitated, and brain tissue was collected and examined for methylene blue distribution throughout the ventricles.

Catheter Implantation: For AMPH self-administration studies, rats were also implanted with catheters into their left or right femoral vein during a separate surgery. Rats were anesthetized with ketamine/xylazine (90:10 mg/kg *i.p.*) and given 5 mg/kg carprofen (*s.c.*). Catheters made of Micro-Renathane tubing (MRE-040, Braintree Scientific, Braintree, MA) were attached to a backmount cannula guide (313-000BM-15-5UP/1/SPC, Plastics One, Roanoke, VA) that exited between the scapulae. Rats were allowed to recover for a minimum of 7 days. The catheters were flushed daily with 0.3-0.5 ml of heparinized-saline (50 U/ml), once daily during recovery and twice daily before and after each self-administration session. Intracranial cannulae were implanted approximately four weeks after catheter implantation.

Locomotor Activity: Locomotor activity was measured in an acrylic cage (14" x 14" x 8") containing infrared beams spaced 2.54 cm apart (Opto-M3 Activity Monitor). Experimental data were collected and analyzed using Multi Device Interface Software (Columbus Instruments,

Columbus, OH). Rats received an injection of saline (*s.c.*) and were habituated to the locomotor boxes for 60 min. Following habituation, the rats were given a second injection of saline (*s.c.*) and placed back in the box for 30 min. AMPH (0.32, 1, or 3.2 mg/kg) was then administered (*s.c.*) and activity was recorded for an additional 2.5 hr. The rats were pretreated with 0 (vehicle), 1, 10, or 30 pmol or 1 nmol enzastaurin *i.c.v.* in their home cage either 0.5, 3, 6, or 18 hr before AMPH administration. The number of beam breaks were recorded every min and summed into 10 min bins.

Self-Administration:

Apparatus: For self-administration studies, rats were placed in operant chambers (ENV-008CT, Med Associates, St. Albans, VT) inside sound-attenuating chambers (ENV-018CT, Med Associates). The operant boxes were outfitted with two nose poke devices each containing a yellow light (ENV-114BM), which were located on either side of a pellet receptacle (ENV-200R7M, Med Associates) attached to a dispenser (ENV-203-45, Med Associates) filled with 45 mg sucrose pellets. A white house light was on the wall opposite the nose poke devices. Drug solutions were delivered via a variable infusion rate syringe pump (PHM-107, Med Associates) connected by Tygon tubing to a single channel plastic swivel (375/22PS, Instech Laboratories, Plymouth Meeting, PA) on a counter-balanced arm (PHM-110-SAI, Med Associates). The Tygon tubing inside the operant chamber was protected with a stainless-steel spring. Data were collected using MED-PC Software (SOF-735, Med Associates).

AMPH Self-Administration: Rats with *i.v.* catheters were trained to respond in the nose poke device for infusions of AMPH (0.1 mg/kg/infusion) on a fixed-ratio 1 (FR1) schedule of reinforcement during 60 min daily sessions. Each session began with an infusion of 0.05 ml of

drug solution to fill the catheters. The “active” nose poke was illuminated by a yellow light and responses into the active nose poke were recorded. The light in the “inactive” nose poke was not illuminated and responses in the inactive nose poke were recorded but had no scheduled consequence. Completion of a FR in the active nose poke resulted in an infusion (100 μ l/kg over approximately 1 sec) with illumination of the house light. This was followed by a 10 sec blackout period during which all stimuli were turned off and responses during the blackout period were recorded but had no consequence. Once the animals responded in a consistent manner for AMPH infusions, the response requirement was gradually increased to an FR5 and the dose of AMPH was decreased to 0.032 mg/kg/infusion. Following stable AMPH self-administration (less than 20% variation in the number of responses and no increasing or decreasing trend in responding over 3 consecutive sessions), saline was repeatedly substituted for AMPH for 1-3 consecutive sessions until responding dropped to less than 30% of stable AMPH responding levels within a single session. All cues were present during the extinction tests. Once responding extinguished in the absence of AMPH, the rats were implanted with cannulae (as described above), then responding maintained by AMPH and extinction in the absence of AMPH were re-confirmed. The rats were pretreated with 10 pmol enzastaurin or vehicle (*i.c.v.*) 3 or 18 hr prior to an AMPH self-administration session.

A different group of rats were trained to self-administer 0.032 mg/kg/inf AMPH under an FR5 schedule of reinforcement as described above. Following stable AMPH self-administration, the rats were implanted with intracranial cannulae. Upon re-confirming stable responding for AMPH, the rats were switched to a multiple-dose self-administration session. Each daily session was comprised of five 25-min components with a 2-min blackout between each component. Responding during each component resulted in the delivery of different doses of amphetamine

delivered in ascending order by altering the infusion duration: 0 (responding recorded with no consequence), 0.0032, 0.01, 0.032, and 0.1 mg/kg/inf AMPH. Following 2-3 consecutive sessions of stable responding, saline was substituted for AMPH for all 5 components in one day to extinguish responding. The AMPH dose effect curve was re-established and then the rats were pretreated with vehicle or 10 pmol enzastaurin (*i.c.v.*) 18-hr prior to the self-administration session.

Food Self-Administration: Food self-administration studies were carried out with the same design as the AMPH self-administration sessions with a few exceptions. These rats had cannulae implanted but no catheters. The sessions lasted for 20 min and completion of a FR resulted in the delivery of a single 45 mg sucrose pellet. Instead of saline substitution, responding was extinguished by no delivery of the sucrose pellets. All other cues were present during the extinction test.

Protein Kinase C Activity:

Rats were administered 10 pmol or 1 nmol enzastaurin, or vehicle 0.5, 3, or 18 hr prior to an injection of 3 mg/kg AMPH (*s.c.*). The rats were euthanized 10-30 min following AMPH and the ventral striatum was dissected. The tissue was immediately frozen in liquid nitrogen, then 250 μ l boiling 1% SDS was added to each sample. The samples were sonicated for 5 pulses at frequency of 20 kHz, amplitude 50% (sonic dismembrator, Fisher Scientific, Pittsburgh, PA) and then spun at 14,000 rpm at 4°C, saving the supernatant. The samples (75 μ g) were separated by SDS-PAGE on a 12% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane at 100 mA for 16 hr. The membranes were blocked in a buffer (5% w/v milk, 150 mM NaCl, 10 mM Tris, 0.05% Tween20) before probing for rabbit anti-phosphoser⁴¹-growth

associated protein 43 (pGAP43) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and goat anti-GAP43 (Santa Cruz Biotechnology) antibodies for 24 hr at 4°C on two separate membranes. Primary antibody binding was detected with secondary antibodies for 1 hr at room temperature: antibodies for goat anti-rabbit for pGAP43 and donkey anti-goat for total GAP43 (Santa Cruz Biotechnology). The antibodies were imaged with Chemiluminescent Western Substrate (EMD Millipore, Darmstadt, Germany) and band densities were quantified using Image J software.

Statistical Analysis: Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc, San Diego, CA). Locomotor activity data are presented as beam breaks over time and as the area under the curve (AUC) of beam breaks over time for the first 40 min following AMPH administration. Comparisons were made with t-tests, one-way ANOVAs or the Kruskal-Wallis test (for nonparametric tests with uneven group size), two-way ANOVAs, and three-way ANOVAs, as indicated in the results section and/or figure legends. Alpha level was set at 0.05. A Dunnett's multiple comparison post hoc tests were performed for the dose-effect of enzastaurin studies and the time course of the different pretreatment times, Dunn's multiple comparison post hoc tests for the different pretreatment time AUC graph, and Sidak's multiple comparison post hoc tests were used for the locomotor activity time courses following different doses of AMPH, AMPH dose-effect curve for locomotor activity, and single-dose self-administration studies. A Tukeys multiple comparison post hoc test was used for the AMPH dose-effect curve with self-administration.

Results

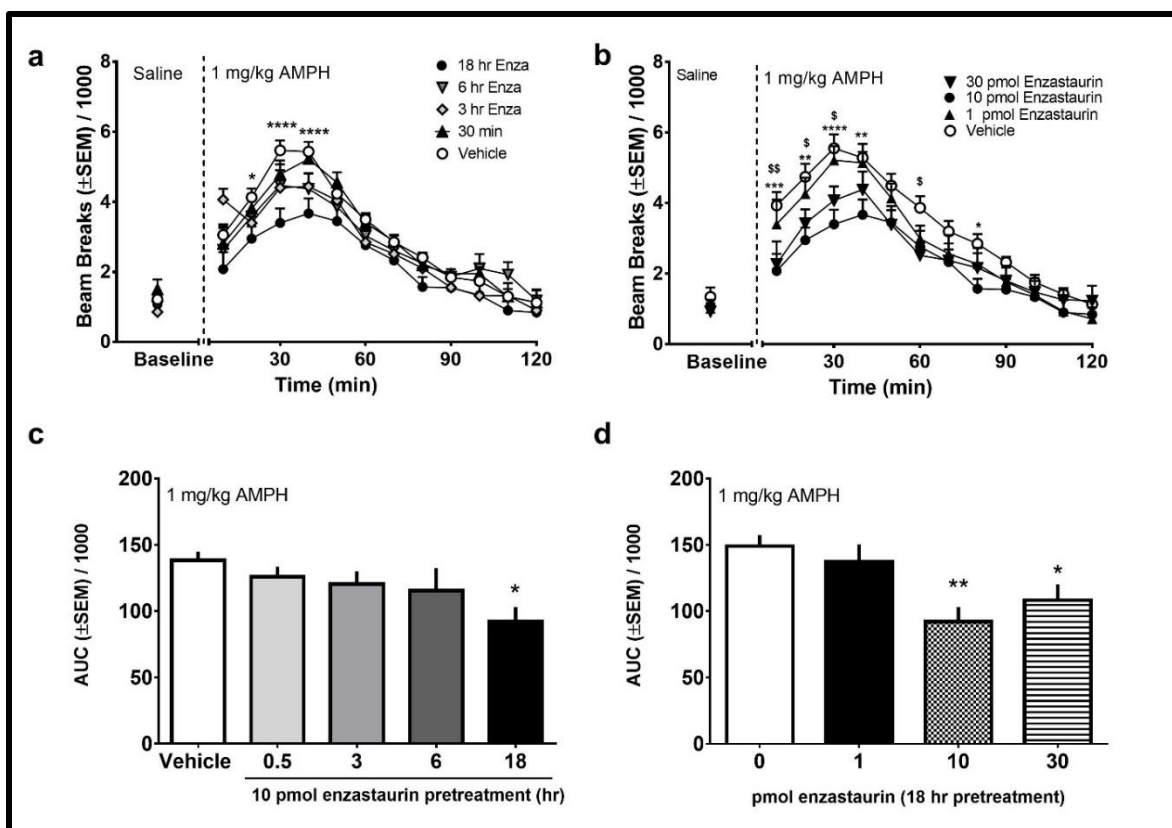


Figure 2.1 Time- and dose-dependent reduction of AMPH-stimulated locomotion by enzastaurin.

(a) Rats were given vehicle (open circles) or 10 pmol enzastaurin (*i.c.v.*) 0.5 (closed triangles), 3 (gray diamonds), 6 (gray triangle), and 18 (closed circles) hr prior to 1 mg/kg AMPH *s.c.* ($n = 6-7$ for all groups except vehicle $n = 20$). Baseline activity was calculated from the average number of beam breaks/10 min following an injection of saline prior to receiving AMPH. Data are presented as the number of beam breaks in 10 min bins over time. * $p < 0.05$, **** $p < 0.0001$ 18 hr vs. vehicle pretreatment (b) Rats were given 0 (vehicle-open circles), 1 (closed squares), 10 (closed circles), or 30 (closed diamonds) pmol of enzastaurin *i.c.v.* 18 hr prior to 1 mg/kg AMPH *s.c.* ($n = 6-7$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ 10 pmol vs. vehicle pretreatment, \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ 30 pmol vs. vehicle pretreatment (c) Locomotor activity over time is summarized as the AUC of beam breaks over time for 40 min following AMPH administration for the different pretreatment times. (d) Enzastaurin dose-effect summarized as AUC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs vehicle (c, d).

An 18 hr pretreatment of enzastaurin decreased AMPH-stimulated locomotor activity

To characterize the effect of PKC β inhibition on AMPH-stimulated behaviors, we examined whether enzastaurin altered AMPH-mediated locomotor activity. To assess the time

course of inhibition by enzastaurin, vehicle or 10 pmol enzastaurin was administered *i.c.v.* to rats 0.5, 3, 6, and 18 hr prior to injection of 1 mg/kg AMPH *i.p.* The data are expressed as the number of beam breaks over time (Figure 2.1a) and further summarized as the AUC for beam breaks over time (Figure 2.1c). The levels of AMPH-stimulated locomotor activity in vehicle-pretreated rats did not significantly change with the pretreatment time and were compiled into the group labeled vehicle. A two-way ANOVA indicated a significant interaction between the enzastaurin pretreatment time and experiment [$F(48,492) = 1.79, p = 0.001$] and a significant main effect of experiment time [$F(12,492) = 112.4, p < 0.0001$] and enzastaurin pretreatment time [$F(4,41) = 2.63, p = 0.05$] (Figure 2.1a). In data converted to AUC, locomotor activity in rats administered enzastaurin 18 hr prior to AMPH was significantly different ($p = 0.03$) from rats pretreated with vehicle, but a 0.5, 3, or 6 hr enzastaurin pretreatment had no effect compared to vehicle [Kruskal-Wallis test ($p = 0.05$)] (Figure 2.1c).

Different doses of enzastaurin (0-30 pmol) were given to rats *i.c.v.* 18 hr before 1 mg/kg AMPH *s.c.* The number of beam breaks over time for each dose is shown (Figure 2.1b) and further summarized as the AUC for beam breaks over time (Figure 2.1d). There was a significant interaction between dose and pretreatment (Figure 2.1b) [two-way ANOVA: $F(36,252) = 1.82, p=0.005$]. As compared with vehicle-treated rats (Figure 2.1d), enzastaurin given 18 hr prior to AMPH decreased AMPH-stimulated locomotor activity [one-way ANOVA: $F(3,21) = 6.02, p = 0.004$] at 10 pmol ($p=0.003$) and 30 pmol ($p=0.04$).

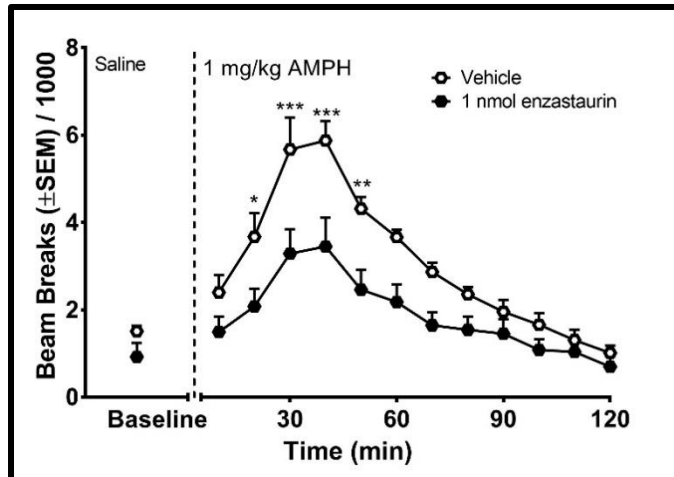


Figure 2.2 A large dose of enzastaurin decreased AMPH-stimulated locomotion.

Rats were given vehicle (open hexagons) or 1 nmol enzastaurin (*i.c.v.*) (closed hexagons) 0.5 hr prior to 1 mg/kg AMPH *s.c.* (n = 5-6). Baseline activity was calculated from the average number of beam breaks/10 min following an injection of saline prior to receiving AMPH. Data are presented as the number of beam breaks in 10 min bins over time. * p < 0.05, ** p < 0.01, *** p < 0.001 vehicle vs enzastaurin.

A large dose of enzastaurin decreased AMPH-stimulated locomotor activity acutely

Enzastaurin administered directly into the NAc can acutely reduce AMPH-stimulated locomotor activity (Zestos et al. 2016) but enzastaurin administered *i.c.v.* on the pmol level is not effective following a short pretreatment. We hypothesized that the concentration of enzastaurin *i.c.v.* was not high enough for an acute effect and, to test this, we performed the experiment using a higher dose of enzastaurin. Locomotor activity in rats administered 1 nmol enzastaurin following a 30 min pretreatment was significantly decreased compared to vehicle (Figure 2.2). A two-way ANOVA demonstrated a significant main effect of time [F (15, 150) = 22.82, p < 0.0001] and enzastaurin [F (1,10) = 11.42, p = 0.007].

Large doses of AMPH surmounted inhibition induced by enzastaurin

AMPH-stimulated locomotor activity displays an inverted U-shaped dose-effect curve (Rosenzweig-Lipson et al. 1997). The effect of enzastaurin on locomotor activity produced by multiple doses of AMPH was investigated in order to determine potential shifts in the AMPH dose-effect curve (Figure 2.3a, b, c, d). These data were further summarized as the AUC of the locomotor activity data over time to generate amphetamine dose-effect curves (Figure 2.3e). Enzastaurin significantly altered AMPH-stimulated locomotor activity at 1.0 mg/kg AMPH [$F(15,180) = 2.321, p = 0.0048$] (Figure 2.3c) and 3.2 mg/kg AMPH [$F(15,150) = 2.341, p = 0.0049$] (Figure 2.3d). A two-way ANOVA of the dose-effect curve (Figure 2.3e) determined that there was a significant interaction between dose of AMPH and enzastaurin pretreatment [$F(3,42) = 12.05, p < 0.0001$]. Enzastaurin did not alter locomotor activity in the absence of AMPH. Locomotor activity was significantly decreased at 1 mg/kg AMPH in rats pretreated with enzastaurin ($p < 0.0001$) and significantly increased at 3.2 mg/kg AMPH in rats pretreated with enzastaurin ($p = 0.006$) as compared with vehicle. Overall, enzastaurin appeared to produce a rightward shift in the AMPH dose response curve.

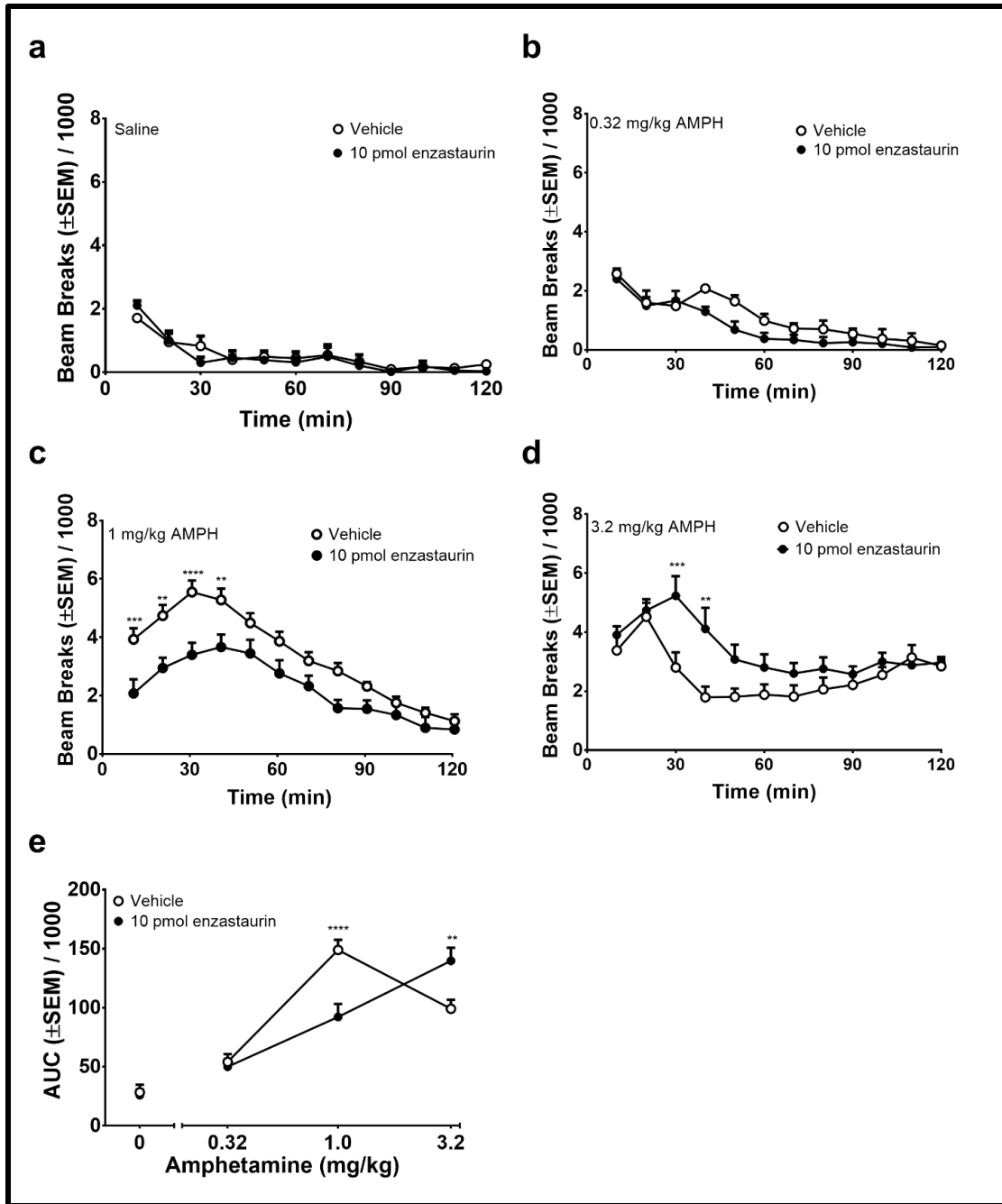


Figure 2.3 Enzastaurin pretreatment shifted the dose effect curve of AMPH.

Rats were pretreated with vehicle (open circles) or 10 pmol enzastaurin (closed circles) *i.c.v.* 18 hr prior to AMPH. Locomotor activity was measured for 2 hr following administration of (a) saline, (b) 0.32, (c) 1, or (d) 3.2 mg/kg AMPH (*s.c.*) (n = 6-7 per treatment). Data are presented in number of beam breaks in 10 min bins over time. (e) Beam breaks over time were converted to AUC following AMPH or saline administration. ** p < 0.01, *** p < 0.001, **** p < 0.0001 vehicle vs. enzastaurin.

Enzastaurin reduced AMPH-maintained responding without altering responding for sucrose

To determine if a PKC β inhibitor altered the reinforcing effects of AMPH, we examined the effect of enzastaurin on AMPH-maintained responding. Rats were trained to respond for a 0.032 mg/kg/infusion AMPH on an FR5 schedule of reinforcement and to extinguish responding in the absence of AMPH. Following acquisition of responding criteria, pretreatments of vehicle or 10 pmol enzastaurin (*i.c.v.*) were administered 3 and 18 hr prior to a self-administration session. Following an 18-hr pretreatment of enzastaurin (Figure 2.4a), the number of responses in the active nose poke were decreased by 80% when compared with vehicle pretreatment and with stable AMPH-maintained responding in the absence of a pretreatment. A two-way ANOVA demonstrated a significant interaction between enzastaurin pretreatment and self-administration condition/stage [$F(2,18) = 8.4, p = 0.003$]. In the absence of AMPH, responding significantly decreased ($p < 0.0001$). An 18 hr pretreatment of enzastaurin significantly decreased AMPH-maintained responding as compared with vehicle pretreatment ($p < 0.001$) to levels similar to that observed in the absence of AMPH. There were no significant differences in the number of active responses following a 3-hr pretreatment of enzastaurin or vehicle as compared with stable AMPH-maintained responding (Figure 2.4b). There were no significant differences in inactive responses between rats pretreated with enzastaurin or vehicle at 3 or 18 hr prior to the session (Figure 2.4 a, b).

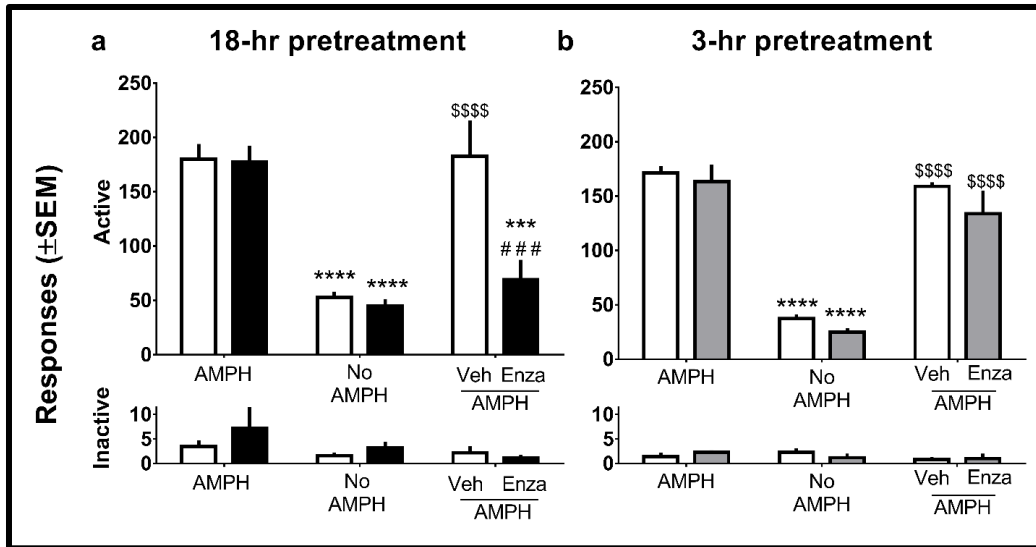


Figure 2.4 Enzastaurin decreased AMPH-maintained responding.

The figures demonstrate responding maintained by AMPH (0.03 mg/kg/injection) under an FR5 schedule of reinforcement and during saline substitution (no AMPH). After achieving stable responding for AMPH and rapid extinction of responding in the absence of AMPH, separate groups of rats were pretreated with vehicle (Veh) or 10 pmol enzastaurin (Enza) either 18 (a) or 3 (b) hr prior to an AMPH self-administration session. Active responses are shown in the top graphs and inactive responses are shown in the bottom graphs. ### $p < 0.001$ vs. vehicle *** $p < 0.001$ and **** $p < 0.0001$ vs. AMPH alone, \$\$\$\$ $p < 0.0001$ vs. No AMPH (n = 5-6).

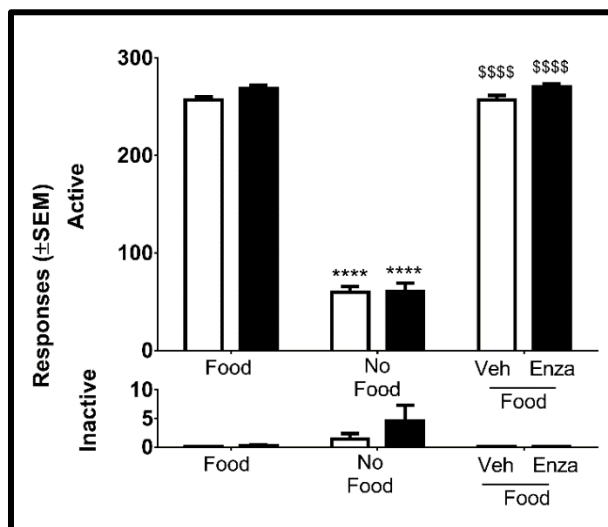


Figure 2.5 Enzastaurin did not decrease sucrose-maintained responding.

Figures show responding maintained by sucrose pellets (Food) under an FR5 schedule of reinforcement and during the absence of sucrose (No Food). After achieving stable responding for sucrose and rapid extinction of responding in the absence of sucrose, separate groups of rats

were pretreated with vehicle (Veh) or 10 pmol enzastaurin (Enza) 18 hr prior to a sucrose self-administration session. Active responses are shown in the top graph and inactive responses are shown in the bottom graph. **** p < 0.0001 vs. Food alone, \$\$\$\$ p < 0.0001 vs. No Food. n=5-6.

In control experiments, rats were trained to respond for sucrose pellets under similar conditions to the AMPH self-administration experiments, and we evaluated the effects of enzastaurin administered 18 hr prior to a sucrose self-administration session. A two-way ANOVA revealed a significant main effect of self-administration condition/stage [$F(2,20) = 125.8, p < 0.0001$] indicating that responding significantly decreased in the absence of sucrose (Figure 2.5). However, there was no significant interaction, demonstrating that enzastaurin pretreatment did not significantly alter sucrose-maintained responding as compared with vehicle pretreatment. There were also no significant differences in the number of inactive responses in rats pretreated with enzastaurin or vehicle.

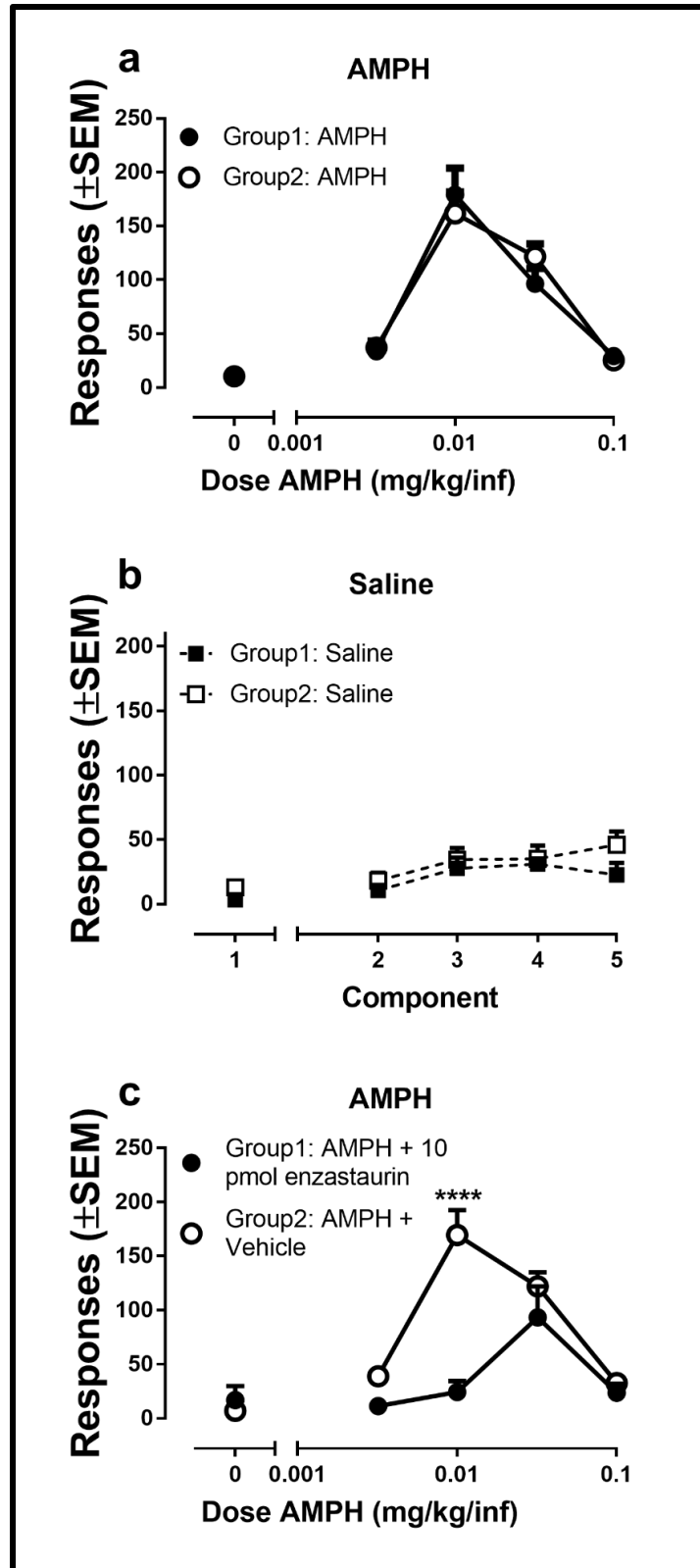


Figure 2.6 Enzastaurin altered the dose-effect curve for AMPH self-administration.

(a) Figure shows the number of responses maintained by AMPH under an FR5 schedule of reinforcement for three consecutive sessions prior to an enzastaurin (Group1-open squares) or vehicle (Group2-open circles) pretreatment. The data show responding over five 25 min components, separated by two min between each component. Responding during each component results in the delivery of 0, 0.0032, 0.01, 0.032, 0.1 mg/kg/inf AMPH, sequentially. (b) Saline was substituted in for AMPH for Group1 (half-filled squares) and Group2 (half-filled circles). (c) Group1 was pretreated with 10 pmol enzastaurin (closed squares) and Group2 was pretreated with vehicle (closed circles) 18 hr prior to responding for AMPH. **** $p < 0.0001$ enzastaurin vs. vehicle. $n=6$.

As with locomotor activity, the dose-effect curve for AMPH-maintained responding also displays an inverted-U shape, making it necessary to assess how enzastaurin alters the AMPH dose-effect curve. Two groups of rats were trained to self-administer 0-0.1 mg/kg/inf AMPH across five within-session, sequential components (Figure 2.6a, b). The rats were pretreated with vehicle or 10 pmol enzastaurin *i.c.v.* 18 hr prior to the subsequent self-administration session (Figure 2.6c). A three-way ANOVA demonstrated a significant interaction between dose, time (responding before and after the pretreatment within subject), and enzastaurin/vehicle pretreatment [$F(4,4) = 7.2, p < 0.0001$], and significant interactions between time and pretreatment [$F(1,4) = 10.6, p = 0.002$] and dose and pretreatment [$F(4,4) = 4.2, p = 0.004$]. Responding for 0.01 mg/kg/inf AMPH was significantly decreased in enzastaurin-treated rats compared to vehicle ($p < 0.0001$) and compared to levels of responding prior to the pretreatment ($p < 0.0001$). The peak level of responding in rats pretreated with enzastaurin was significantly different before and after the enzastaurin pretreatment ($p = 0.002$). These results indicate that enzastaurin pretreatment results in a rightward and downward shift the dose-effect curve for AMPH self-administration.

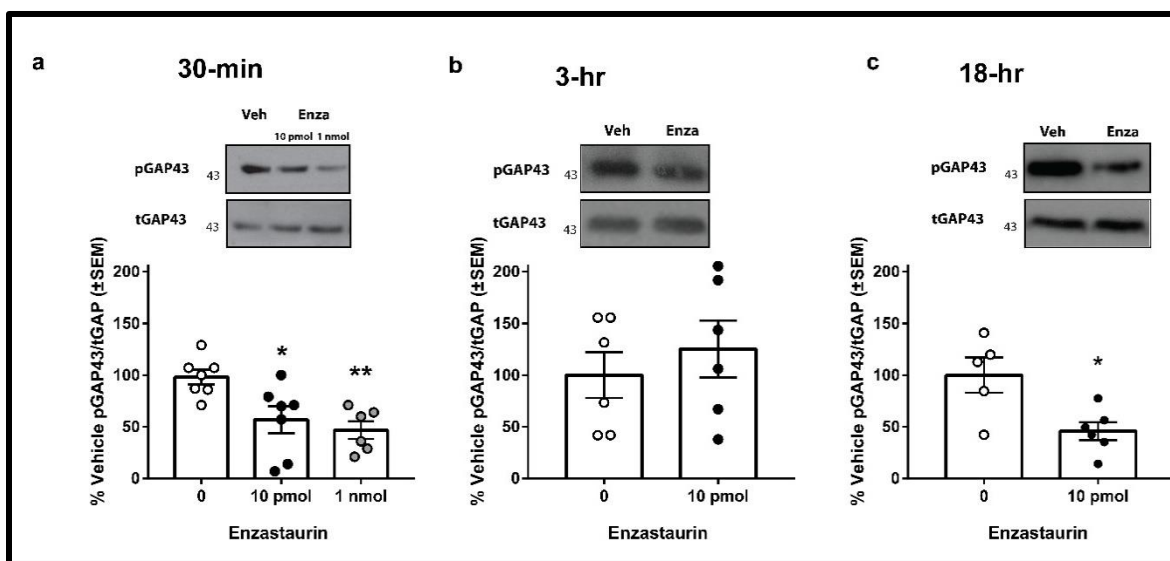


Figure 2.7 A 30 min and 18-hr, but not a 3-hr, pretreatment with enzastaurin decreased PKC activity in the ventral striatum of AMPH-treated rats.

Levels of phosphoserine⁴¹GAP43 (pGAP43) over total GAP43 (tGAP43) are shown in the ventral striatal tissue of AMPH-treated rats given vehicle/enzastaurin (10 pmol or 1 nmol) 30 min (a), 3 hr (b), or 18 hr (c) prior to collecting the tissue. Data are presented as O.D. pGAP43 / tGAP43 (% vehicle). * p < 0.05, ** p < 0.01 vehicle vs. enzastaurin. n=6-7.

Enzastaurin decreased PKC activity in AMPH-treated rats

We wanted to determine if PKC activity was altered at time points corresponding to the enzastaurin-induced changes in AMPH-stimulated locomotor activity and responding for AMPH. Rats were pretreated with vehicle, 10 pmol, or 1 nmol enzastaurin (*i.c.v.*) at 0.5, 3 or 18 hr prior to receiving AMPH *s.c.* (Figure 2.7). PKC activity was determined by phosphorylation of GAP43 at serine⁴¹ (pGAP43), a substrate site selective for PKC (Oehrlein et al. 1996). pGAP43 levels were significantly decreased following 10 pmol by more than 40% and 1 nmol enzastaurin by more than 50% compared to vehicle [one-way ANOVA: $F(2,17) = 7.44$, $p = 0.005$] (Figure 2.7a). pGAP43 was decreased by over 50% in AMPH-treated rats following an 18-hr pretreatment of 10 pmol enzastaurin as compared with vehicle pretreatment (Figure 2.7b) [t-test: $p = 0.02$]. We did not observe a significant change in GAP43 phosphorylation following a 3-hr

pretreatment of 10 pmol enzastaurin as compared with vehicle (Figure 2.7c). Enzastaurin did not alter total levels of GAP43 in the striatum.

Discussion

A role for PKC β in the behavioral effects of AMPH has been demonstrated using PKC inhibitors in rats (Browman et al. 1998; Carpenter et al. 2017; Zestos et al. 2016) and genetic deletion of PKC β in mice (Chen et al. 2009). In this study, we sought to establish the consequences of selective inhibition of the PKC β isoform on the reinforcing effects of AMPH. A highly soluble, brain permeable PKC β inhibitor would be an ideal tool, however due to a dearth of such drugs, we chose to administer enzastaurin centrally (*i.c.v.*) to evaluate its effects on amphetamine-induced locomotor activity, amphetamine-maintained behavior, and to take note of any adverse events. We performed an investigation of dose and time dependence of PKC β inhibition on AMPH-stimulated locomotor activity and found that an 18-hr pretreatment of 10 pmol enzastaurin *i.c.v.* was most effective. We then demonstrated that this same dose of enzastaurin decreased AMPH self-administration without generally suppressing behavior. All together, we have shown pharmacological inhibition of PKC β is effective at decreasing AMPH-stimulated behaviors.

First, we showed that pharmacological inhibition of PKC β produced a rightward shift in the AMPH dose effect curve without altering baseline locomotor activity. Vehicle-pretreated rats displayed the typical inverted-U shaped AMPH dose-effect curve typically seen in adult rats (Campbell et al. 1969). At the measured times, 10 pmol enzastaurin *i.c.v.* was only effective at decreasing AMPH-stimulated locomotor activity 18 hr following administration. Notably, the normal inverted U-shaped dose effect curve was not evident in enzastaurin-treated rats. Increased locomotor activity with the highest dose of AMPH in these rats was likely due to decreased

levels of stereotypy in the enzastaurin-treated rats (Del Rio and Fuentes 1969). A higher dose of AMPH might reveal the inverted U-shaped curve. These results suggest that 10 pmol enzastaurin shifts the dose-effect curve of AMPH-stimulated locomotor activity to the right, consistent with data from PKC β deletion or inhibition (Chen et al. 2009).

We previously showed that a novel non-selective PKC inhibitor decreased AMPH-maintained responding in rats (Carpenter et al. 2017). The present study demonstrated for the first time that the selective PKC β inhibitor enzastaurin, at a dose that reduced AMPH-stimulated locomotor behavior, decreased the reinforcing effects of AMPH without altering responding for non-drug rewards. Enzastaurin decreased AMPH-maintained responding to levels observed with saline substitution. An 18-hr pretreatment of enzastaurin did not alter sucrose-maintained responding in a separate group of rats. We evaluated the dose-effect curve for AMPH-maintained responding under an FR5 schedule of reinforcement and found that enzastaurin shifted the ascending limb of the curve to the right without increasing AMPH intake on the descending limb. These findings strongly support the hypothesis that inhibition of PKC β reduces AMPH-mediated behaviors and reinforcing effects without altering sucrose-maintained responding.

We initially observed that an 18 hr pretreatment with enzastaurin decreased responding for AMPH, possibly indicating that enzastaurin decreased the reinforcing effects of AMPH. To further probe this interpretation, we evaluated the effects of enzastaurin on an AMPH dose-effect curve determined within session. Similar to that observed in the locomotion experiments, the ability of enzastaurin to decrease AMPH-maintained behavior was surmountable. This was not a parallel rightward shift in the amphetamine dose-effect curve but more a shift in the ascending limb. This complex shift in the AMPH dose-effect curve may be due to the within session design, leading to accumulation of large concentration of AMPH in circulation. Alternatively,

the shift in the AMPH dose-effect curve may be due to the complex interaction between the site of action of AMPH and PKC β . Few studies have evaluated or characterized shifts in drug dose-effect curves following inhibition of one component of the intracellular signaling pathway. These interactions are likely to be multifaceted and potentially dose-dependent. For example, small doses of AMPH may be more dependent on PKC β signaling mechanisms, whereas large AMPH doses may invoke multiple signaling molecules, cellular actions, and neurocircuits overwhelming the effects of PKC β . However, future studies would need to explore these effects and shifts in the AMPH dose effect curve in more detail.

A possible alternative explanation for the effect of PKC β inhibition on the reinforcing effects of AMPH is through the impairment of memory. PKC β is important for memory through its role in promoting LTP (Colley and Routtenberg 1993; Lovinger et al. 1986; Nogues 1997; Routtenberg et al. 1986; Weeber et al. 2000), and pharmacological inhibition of PKC can cause memory impairment, especially with drug-associated memories (Aujla and Beninger 2003; Cervo et al. 1997; Takashima et al. 1991). This raises the possibility that decreased responding for AMPH in the presence of enzastaurin may be due to impairment of memories associated with self-administration training (e.g., operation of the nose poke device and/or reinforcer contingencies). However, we did not observe alterations in responding for sucrose under similar experimental conditions, suggesting that we are not impairing memory retrieval under these experimental conditions.

Our data repeatedly demonstrated that low doses of enzastaurin, given *i.c.v.*, must be given a substantially long time before AMPH to observe an effect on AMPH-mediated behaviors. In contrast to our current results with *i.c.v.* enzastaurin administration, previous studies showed that enzastaurin and other PKC inhibitors have immediate effects when delivered

directly into the nucleus accumbens (Browman et al. 1998; Loweth et al. 2009; Zestos et al. 2016). One possible explanation for the lack of immediate drug effect in this study could be due to low levels of enzastaurin in the nucleus accumbens following *i.c.v.* administration. Although the administered concentrations of enzastaurin are calculated to be similar, the direct application of concentrated drug in the nucleus accumbens (Zestos et al. 2016) might produce more immediate effects as compared with the slower diffusion of the drug when given *i.c.v.* (Luger et al. 2005). To test this, we administered a large dose of enzastaurin (1 nmol) 30 min prior to AMPH administration and saw a decrease in locomotor activity. An injection of 10 pmol enzastaurin at the same timepoint did not affect AMPH-stimulated locomotor activity. This raises the possibility that different mechanisms may be behind the decrease in AMPH-mediated behaviors observed at 18 hr and acutely.

In order to take a closer look at drug action following the different pretreatment times, we looked at PKC activity following enzastaurin administration. Previous studies demonstrated that AMPH increases phosphorylation of GAP43, a substrate of PKC, in rat striatal tissue and that PKC inhibitors block this effect (Iwata et al. 1997a, Iwata et al. 1997b). We used AMPH-stimulated GAP43 phosphorylation as a readout of PKC activity to determine how long and short pretreatments of enzastaurin effect PKC activity. Although most PKC isozymes will phosphorylate GAP43, phosphorylation of the protein is especially robust with PKC β (Oehrlein et al. 1996, Sheu et al. 1990, Young et al. 2002). While it might be assumed that a PKC inhibitor would inhibit all substrates of PKC equally, that is not necessarily true (Carpenter et al. 2017). Because GAP-43 is readily phosphorylated in response to AMPH treatment both *in vivo* and *in vitro* (Iwata et al. 1997a, Iwata et al. 1997b), inhibition of AMPH-stimulated PKC activity may be more directed to GAP-43 than to other substrates, as demonstrated by Carpenter et al. (2017).

We found that AMPH-stimulated PKC phosphorylation of GAP43 is decreased by *i.c.v.* enzastaurin administration following a 30 min pretreatment and an 18 hr pretreatment, but not following a 3 hr pretreatment. One thing to note is that, while 30 min pretreatment of 10 pmol enzastaurin decreased PKC activity in the ventral striatum, it has no effect on amphetamine-stimulated locomotor activity. There are a couple possible explanations for these unusual results. First, PKC β inhibition alone may not be sufficient to decrease amphetamine-stimulated locomotor activity or that 10 pmol enzastaurin does not produce a robust decrease in PKC β to have an effect on behavior. Although the 10 pmol enzastaurin statistically decreased phosphorylation of GAP43, the effect was quite variable, suggesting that the effect is not robust or that the assay is not sensitive enough to differentiate fine changes in PKC activity. Second, it is also possible that PKC β inhibition in the nucleus accumbens is necessary, but not sufficient, to decrease amphetamine-stimulated behaviors. Locomotor activity is regulated by neurocircuitry on many levels and the ventral striatum is only a small snapshot of the brain, therefore the actions of large and small doses of enzastaurin in the nucleus and/or other brain regions may be important for modulating locomotor activity.

Another conundrum raised by these data is the question of why PKC β inhibitors act 18 hr after administration. Enzastaurin decreased PKC activity 18 hr, but not 3 hr after administration. These findings support the earlier point that PKC inhibitors may decrease AMPH-mediated behaviors through a secondary mechanism. As inhibition of PKC activity is again evident at a later timepoint, it is possible that the mechanism underlying the long pretreatment effect is due to changes downstream of PKC signaling that ultimately affect PKC activity. Some groups have demonstrated that PKC β inhibition with enzastaurin can decrease PKC β promoter activity and

PKC β mRNA levels, which is one possible explanation for a PKC inhibitor's secondary effect on AMPH-mediated behaviors (Liu et al. 2004).

Another explanation for time course of enzastaurin could be due to its metabolism and potential active metabolites. Human studies indicated enzastaurin has long-lasting, active metabolites (Carducci et al. 2006). Multiple-dose pharmacokinetic studies in Fischer 344 rats following oral administration found the half-life of enzastaurin to be around 2 hr and the half-life of its active metabolite to be 3.6 hr with the lowest dose tested. The half-life increased with dose, possibly due to metabolic capacity (personal communications with Denovo Biopharma and Eli Lilly). However, as another structurally unrelated PKC inhibitor also displays a similar time course (Carpenter et al. 2017), we believe that the time course is more related to the mechanism of action of these drugs and not their pharmacokinetics.

Taken together, our results show that PKC β inhibition may be a viable therapeutic option to treat AMPH abuse. A major concern in developing a PKC β inhibitor as a therapeutic is potential toxic effects due to the ubiquitous nature of PKC. However, clinical studies with enzastaurin and other PKC β inhibitors demonstrated good profiles (Vinik et al. 2005; Welch et al. 2007) and our own studies have shown that enzastaurin does not suppress generally elicited or conditioned behaviors. Other isozymes of PKC may be compensating for the reduction in PKC β activity in ways that lessen the impact and toxicity.

While we have shown that enzastaurin decreased AMPH-taking behaviors, we did not test for the effect it would have on AMPH-seeking behaviors and motivation for AMPH. Future work will utilize reinstatement procedures and progressive ratio schedules of reinforcement to further evaluate the behavioral effects PKC β inhibition. More work will also need to be done to further understand the time course and mechanism of PKC β inhibitors. Additional studies to

assess the effect of PKC β inhibition on AMPH sensitization, additional schedules of reinforcement, and extended-access self-administration will also be useful in assessing PKC β inhibitors as potential therapeutics. In conclusion, the present findings provide proof-of-concept selective PKC β inhibitors may be useful for therapeutic interventions for AMPH abuse.

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Chapter 3 PKC inhibition decreases responding for amphetamine under a progressive-ratio schedule of reinforcement

Abstract

Amphetamine (AMPH) are a class of stimulants that are widely misused. They can act in the brain disrupting dopamine regulation in the brain, resulting in an increase in extracellular dopamine levels. Protein kinase C (PKC) has been shown to be important for these actions; inhibiting PKC can block increases in AMPH-stimulated extracellular dopamine levels. Inhibition of PKC can also attenuate certain behavioral effects of AMPH such as AMPH-stimulated locomotor activity. In this study, we wish to examine whether PKC inhibition can alter the reinforcing properties of AMPH. Male Sprague-Dawley rats were trained to self-administer 0.032 mg/kg/infusion AMPH or sucrose pellets under a progressive-ratio (PR) schedule of reinforcement. Number of infusions earned, breakpoints, and session duration were recorded over consecutive sessions. Once AMPH-maintained responding stabilized, rats were pretreated with 0, 10, or 30 pmol of enzastaurin, a PKC β -selective inhibitor, or 6 mg/kg **6c**, a brain-permeable PKC inhibitor, 18 hr prior to a self-administration session. A pretreatment of 30 pmol enzastaurin or 6 mg/kg **6c** decreased the number of AMPH infusions earned and breakpoints without altering sucrose-maintained behaviors. These data suggest that PKC inhibition can selectively alter the reinforcing properties of AMPH and are worth pursuing as potential therapeutics for the treatment of AMPH-use disorder.

Introduction

Amphetamines (AMPH) are a class of stimulants that are commonly prescribed for the treatment of attention deficit/hyperactivity disorder (Lakhan and Kirchgessner 2012). They are also among the most highly abused classes of drugs in the world and present a serious public health risk (McCabe et al. 2017; SAMHA 2014; UNODC 2010). Furthermore, nearly one in ten people who misuse amphetamines during adolescence later develop substance-use disorders (Compton et al. 2018). There are currently no approved pharmacological interventions for treating AMPH-use disorder.

AMPHs can act by dysregulating dopamine levels in the brain; they enter dopaminergic neurons through the dopamine transporter (DAT) and reverse the function of DAT, resulting in a release of dopamine from the neuron into the synapse (Seiden et al. 1993). Protein kinase C (PKC), a common signaling protein, has been shown to play a role in AMPH action. AMPH administration can activate PKC (Giambalvo 1992; Iwata et al. 1997) and PKC activation can further increase AMPH-induced dopamine release into the synapse (Cowell et al. 2000; Giambalvo 1988). Conversely, inhibition of PKC blunts an increase in AMPH-stimulated extracellular dopamine levels *ex vivo* and *in vivo* (Johnson et al. 2005; Zestos et al. 2016). Furthermore, pharmacological inhibition of PKC can decrease AMPH-stimulated locomotor activity (Browman et al. 1998; Carpenter et al. 2017; Zestos et al. 2016). Additional studies have indicated β isoform of PKC to be particularly important for the action of AMPH (Chen et al. 2009; Johnson et al. 2005).

Our studies have demonstrated that pharmacological inhibition of PKC β can reduce AMPH-stimulated locomotor activity as well as ongoing AMPH self-administration under a fixed-ratio (FR) schedule of reinforcement following an 18-hr pretreatment. We have also

shown a novel brain-permeable, non-selective PKC inhibitor, **6c**, can decrease responding for AMPH under an FR ratio, also after an 18-hr pretreatment (Carpenter et al. 2017). While an FR schedule of reinforcement is a simple measure of drug-taking behavior and can be the first step in screening the effectiveness of a novel therapeutic, other schedules of reinforcement can provide additional information. A progressive-ratio (PR) schedule of reinforcement increases the ratio of responses required for the delivery of a reinforcer; response ratios can increase within or between self-administration sessions. One output of a PR session is the breakpoint: the final ratio completed in the session (Hodos 1961). A PR schedule of reinforcement is thought to evaluate quantitatively the reinforcing strength of drugs of abuse (Hodos 1961; Richardson and Roberts 1996). Thus, looking at the effect of these drugs on AMPH self-administration under a PR schedule of reinforcement can measure whether a PKC inhibitor affects the reinforcing strength of AMPH.

In this study, we investigated whether or not the PKC β inhibitor, enzastaurin, or the general PKC inhibitor, **6c**, would alter responding for AMPH under a PR schedule of reinforcement. We pursued this goal to determine if PKC inhibition alters the motivation for and the reinforcing strength of AMPH, with the long-term goal of developing a therapeutic for AMPH-use disorder. We found that enzastaurin and **6c** decreased the breakpoint for AMPH self-administration compared to vehicle, without affecting the breakpoint for sucrose self-administration. These data suggest that PKC inhibition decreases the reinforcing efficacy of AMPH.

Methods

Subjects: Male Sprague-Dawley rats (Envigo Laboratories, Indianapolis, IN) were single-housed in a temperature- and humidity-controlled environment. The rats were food-

restricted to 80-90% of their body weight. The rats were on a 12-hr light/dark cycle with lights on at 0700 and all testing was done during the light phase. The animal procedures were designed within the rules and regulations of the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Michigan Institutional Animal Care and Use Committee.

Drugs and solutions: d-AMPH was dissolved in saline and administered intravenously. Artificial cerebral spinal fluid (aCSF) was prepared according to a recipe produced by ALZET Osmotic Pumps (Durect Corporation, Cupertino, CA). Enzastaurin (Cayman Chemical, Ann Arbor, MI) was dissolved in a vehicle solution containing 0.005% dimethyl sulfoxide in aCSF and administered (*i.c.v.*). **6c** (6c·2.5 HCl) was synthesized by the Vahlteich Medicinal Chemistry Core at the University of Michigan and dissolved in a solution containing 5% Tween-80 in saline and administered *s.c.*

Catheter Implantation: Rats were implanted with intravenous catheters into their left or right femoral vein. They were first anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) *i.p.* and administered 5 mg/kg carprofen *s.c.* Catheters were prepared in-house from Micro-Renathane tubing (MRE-040, Braintree Scientific, Braintree, MA) with a Tygon tubing (ND 100-80, Saint-Gobain, Malvern, PA) sleeve. The catheters exited between the scapulae and were attached to a backmount cannula guide (313-0090BM-15-5UP/1/SPC, Plastics One, Roanoke, VA). Rats were flushed with 0.3-0.5 ml of heparinized saline (50 U/ml) before and after each self-administration session.

Cannula Implantation: Rats administered enzastaurin were implanted with a guide cannula to allow for direct injections into their lateral ventricles (*i.c.v.*). The rats were anesthetized (procedure described in “Catheter Implantation”) and placed in a stereotaxic

instrument (Kopf Instruments, Tujunga, CA). The guide cannula (28 gauge with 1.7 mm projection C313GRL/SPC, Plastics One, Roanoke, VA) was implanted at AP = -0.8 mm, ML = 1.5 mm, DV = -2.8 mm relative to bregma (Paxinos and Watson 1998) and held in place with dental cement (OrthoJet-BCA, Lang Dental Manufacturing Co., Wheeling, IL) and two anchor screws (19010-10, Fine Science Tools, Foster City, CA). Placement of the guide cannula was verified following completion of the experiments by infusing methylene blue dye into the guide cannula and examining the brain for dye distribution in both sides of the lateral ventricles and the third and fourth ventricles.

Self-Administration Apparatus: In the self-administration studies, operant chambers (ENV-008CT, Med Associates, St. Albans, VT) in sound-attenuating cubicles (ENV-018CT, Med Associates) were used. Chambers had two nose poke devices (ENV-114BM, Med Associates) on either side of a pellet receptacle (ENV-200R7M, Med Associates) and a white house light on the opposite wall. Data were recorded using MED-PC Software (SOF-735, Med Associates). Reinforcers were delivered through a dispenser for sucrose pellets (ENV-203-45, Med Associates) or a variable infusion rate syringe pump for AMPH (PHM-107, Med Associates). The syringe pump was connected to Tygon tubing by a single channel, plastic swivel (375/22PS, Instech Laboratories, Plymouth Meeting, PA) held in place by a counter-balanced arm (PHM-110-SAI, Med Associates).

AMPH Self-Administration: The rats were trained to respond in the nose poke devices for intravenous infusions of AMPH (0.1 mg/kg/infusion) on an FR 1 schedule of reinforcement during daily 60 min sessions. Sessions began with an infusion of 0.5 ml of AMPH to prefill the catheter and illumination of a single nose poke device only (active nose poke). Responding in the active nose poke resulted in an infusion of AMPH (100 μ l/kg over 1 sec), turning off the nose

poke light, and illumination of the house light, followed by a 10 sec blackout period in which responses were recorded but had no consequence. Responses in the inactive nose poke (not illuminated) were recorded but had no scheduled consequence. Once the rats reliably responded for more than 20 infusions per session, the AMPH dose was decreased to 0.032 mg/kg/infusion. Some rats were implanted with cannulas. After recovery from surgery, the rats continued daily AMPH self-administration sessions under an FR1 schedule until consistent responding was stable. Once this was achieved, both groups began training under a PR schedule, in which the response requirement for a single infusion increases in an exponential manner as described in Robert and Richardson (1992). Self-administration sessions under the PR schedule of reinforcement could last 180 min but was terminated if a rat did not complete a ratio within 30 min. The final ratio completed is referred to as the breakpoint (Roberts and Goeders 1989). The number of responses, the number of reinforcers, session duration, and the breakpoint were recorded at the end of each sessions. Stable responding was determined as a change in one or less infusions between sessions. Once stable responding was achieved, cannulated rats were given vehicle (aCSF), 10, or 30 pmol of enzastaurin (*i.c.v.*), and the non-cannulated rats were given vehicle (5% Tween-80 in saline) or 6 mg/kg **6c** (*s.c.*) 18 hr prior to a test session. During the test sessions, rats responded for AMPH infusions under a PR schedule as usual and all cues remained present. In a separate group of rats, vehicle pretreatments (*i.c.v.*) were administered and saline was substituted for AMPH for a single session to determine breakpoints for AMPH-paired cues alone in the absence of AMPH. After a single test session, AMPH-maintained responding was evaluated for an additional 4 days.

Sucrose Self-Administration: The sucrose self-administration studies were carried out under the same design as the AMPH self-administration, with a few differences. Completion of a

response requirement resulted in the delivery of a 45 mg sucrose pellet. The training sessions under an FR schedule of reinforcement lasted for 20 min, but the sessions under a PR schedule could last for 180 min and were terminated if a ratio was not completed in 30 min. On the testing days, rats were pretreated with vehicle or 30 pmol enzastaurin (*i.c.v.*) 18 hr prior to a test session where responding resulted in the delivery of a sucrose pellet or the conditioned stimuli alone.

Statistics: Data were analyzed, and statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA). Comparisons were made with unpaired t-tests, one-way ANOVAs, and two-way ANOVAs as indicated in the results section. *Post hoc* analyses were completed with Sidak's multiple comparisons test and Tukey's multiple comparisons test.

Results

Enzastaurin decreased responding for AMPH but not sucrose under a PR schedule of reinforcement

We evaluated whether or not the PKC β -selective inhibitor, enzastaurin, altered AMPH-maintained responding under a PR schedule of reinforcement. Figure 3.1 shows data collected from the 3 sessions prior to a test session in which responding under the PR schedule was evaluated 18 hr following a pretreatment with vehicle, 10, or 30 pmol enzastaurin. A two-way ANOVA comparing pretreatments across the self-administration sessions (Figure 3.1a) demonstrated a significant interaction between pretreatment and session [$F(9, 60) = 3.803, p = 0.0008$] and a significant main effect of session [$F(3, 60) = 21.46, p < 0.0001$]. *Post hoc* analyses comparing across the different groups demonstrated no significant difference in the number of infusions during sessions 1-3, but a significant decrease in the number of infusions earned following pretreatment with 30 pmol enzastaurin as compared with vehicle and 10 pmol

pretreatments (Tukey's multiple comparisons test $p = 0.004, .03$ respectively). In one group of rats pretreated with vehicle, saline was substituted for AMPH to evaluate responding for AMPH-paired cues in the absence of AMPH. Under these conditions, the number of infusions earned decreased by approximately 50% as compared with infusions earned when AMPH was available (statistically different from sessions 1-3 (Tukey's multiple comparisons test $p < 0.0001$)). Responding for saline infusions was not statistically different from responding for AMPH following pretreatment with 30 pmol enzastaurin. A pretreatment with 10 pmol enzastaurin resulted in little change to the number of infusions of AMPH earned. A one-way ANOVA looking at the number of infusions alone (Figure 3.1b) showed a significant effect of pretreatment [$F(3, 20) = 4.558, p = 0.0137$] with the vehicle + saline group and the 30 pmol pretreatment group being significantly different from vehicle + AMPH (Sidak's multiple comparisons test $p = 0.03$ for both).

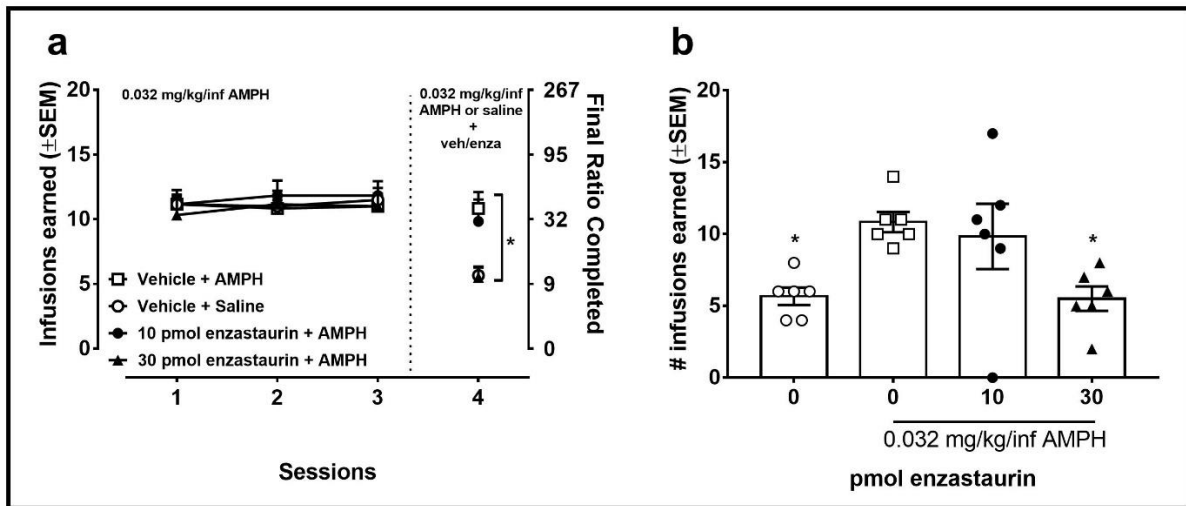


Figure 3.1 Enzastaurin decreased responding for AMPH under a PR schedule of reinforcement.

(a) demonstrates the number of infusions earned (left axis) and the final ratio completed (right axis) during the test day (session 4) and the three consecutive sessions prior to the test day. aCSF (*i.c.v.*) was given to the rats 18 hr prior to responding for AMPH (open squares) or saline (open circles) or rats were administered 10 (closed circles) or 30 pmol (closed triangles) of enzastaurin

(*i.c.v.*) 18 hr prior responding for AMPH. The test day data are further summarized in a bar graph (b) showing individual data. * $p < 0.05$ vehicle + saline/30 pmol enzastaurin vs vehicle + AMPH (a, b)/10 pmol enzastaurin (a). $n=6$.

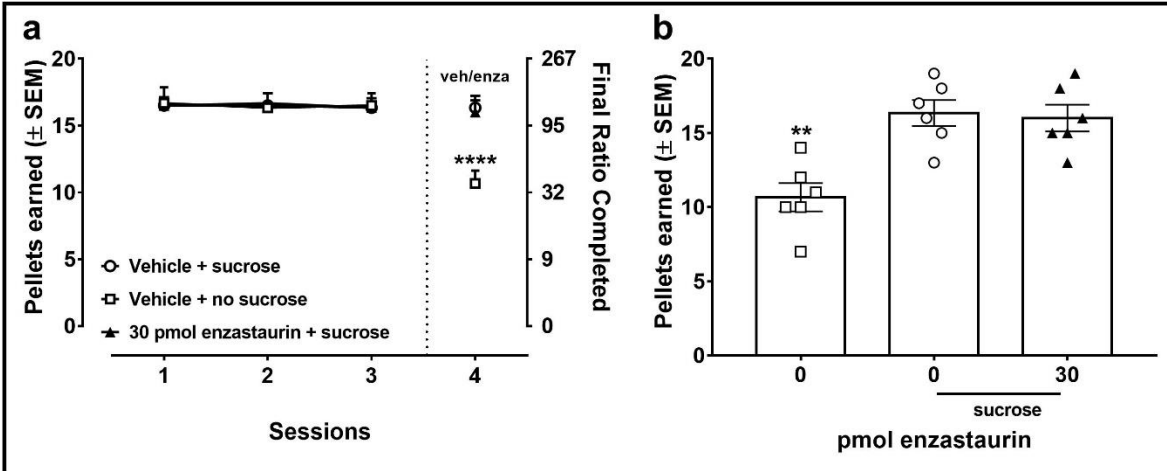


Figure 3.2 Enzastaurin did not alter responding for sucrose under a PR schedule of reinforcement

(a) demonstrates the number of sucrose pellets earned (left axis) and the final ratio completed (right axis) during the test day (session 4) and the three consecutive sessions prior to the test day (sessions 1-3). Rats were given aCSF (*i.c.v.*) 18 hr prior to responding for sucrose pellets (open circles) or responding for sucrose cues alone (open squares) or they were administered 30 pmol (closed triangles) of enzastaurin (*i.c.v.*) 18 hr prior responding for sucrose. The test day data are further summarized in a bar graph (b) showing the individual data. ** $p < 0.01$, **** $p < 0.0001$ vehicle + no sucrose vs. vehicle + sucrose (a, b)/30 pmol enzastaurin (a). $n=6$.

To determine if this dose of enzastaurin would decrease any operant behavior, rats were trained to self-administer sucrose pellets under a progressive ratio schedule of reinforcement before a pretreatment with vehicle or enzastaurin (Figure 3.2). A repeated measure across time two-way ANOVA showed a significant interaction of pretreatment and session [$F(6,45) = 14.04$, $p < 0.0001$] with a significant main effect of session [$F(3, 45) = 18.99$, $p < 0.0001$]. Responding in the absence of a reinforcer was decreased compared with responding for sucrose regardless of the pretreatment condition (Tukey's multiple comparisons test $p < 0.0001$). A pretreatment of 30

pmol enzastaurin did not significantly affect the number of sucrose pellets earned compared to vehicle. A one-way ANOVA comparing the test day alone (Figure 3.2b) showed a significant effect of pretreatment [$F(2, 15) = 12.19, p = 0.0007$] with the vehicle + no sucrose being significantly different from vehicle + sucrose and 30 pmol enzastaurin + sucrose ($p = 0.001, 0.002$ respectively).

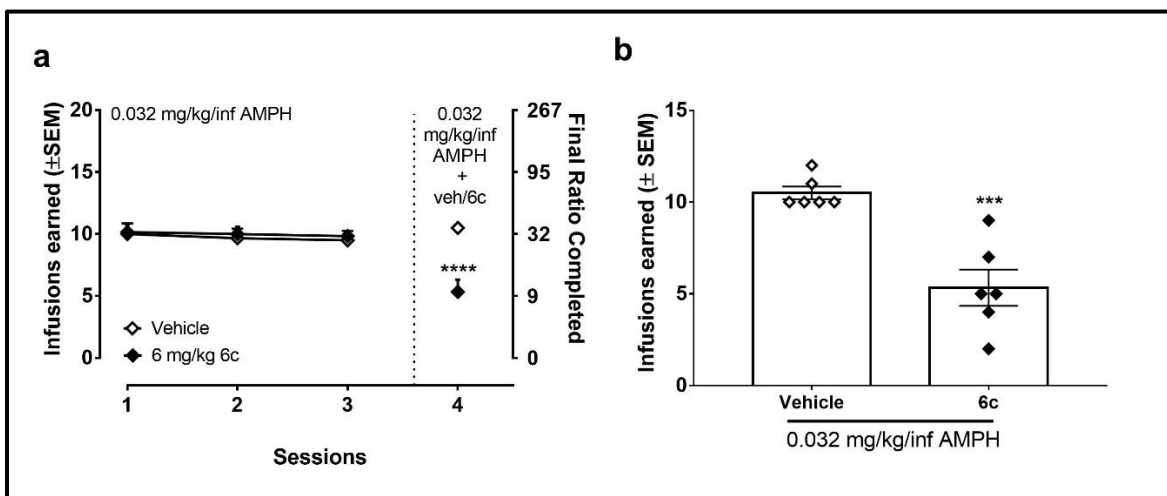


Figure 3.3 The brain permeable PKC inhibitor 6c decreased responding for AMPH under a PR schedule of reinforcement.

The graph demonstrates number of infusions earned (left axis) and the final ratio completed (right axis) during the test day (session 4) and the three consecutive sessions prior to the test day (a). Rats were administered **6c** (*s.c.*) or vehicle 18 hr prior to self-administering AMPH. The test day data are further summarized in a bar graph (b) showing the individual data. *** $p < 0.001$, **** $p < 0.0001$ vehicle vs 6c. $n=6$.

6c decreased responding for AMPH under a PR schedule of reinforcement

Previous data demonstrated that the nonselective, brain penetrant PKC inhibitor **6c** can decrease AMPH self-administration under an FR schedule of reinforcement (Carpenter et al. 2017), thus we sought to determine the effects of **6c** on AMPH-maintained responding under a PR schedule of reinforcement. Rats trained to self-administer AMPH under a PR schedule of

reinforcement were given vehicle or 6 mg/kg **6c** (*s.c.*); data from the three sessions prior to the test and the test session are shown in Figure 3.3a. An injection of **6c** significantly decreased responding for AMPH under a PR schedule of reinforcement (repeated measures by session two-way ANOVA [$F(3, 30) = 21.42, p < 0.0001$] with a significant main effect of session [$F(3, 30) = 11.26, p < 0.0001$]). Sidak's *post hoc* analysis showed **6c** significantly decreased the number of responses on the test day compared to the vehicle pretreatment on the test session ($p < 0.0001$) and compared to responding during sessions 1-3 ($p < 0.0001$). A t-test comparing the number of infusions earned on the test day alone (Figure 3.3b) showed a significant decrease in infusions ($p = 0.0006$). There was no significant change in the number of infusions earned during sessions 1-3 between the two groups of rats.

Discussion

Previously it was shown that these PKC inhibitors can decrease ongoing AMPH self-administration under an FR schedule of reinforcement (Carpenter et al. 2017). In this study, we sought to evaluate further the effects of PKC inhibitors on AMPH-maintained behaviors under a different schedule of reinforcement. Our findings show for the first time that the PKC β -selective inhibitor enzastaurin and the brain-permeable PKC inhibitor **6c** can alter responding for AMPH under a PR schedule of reinforcement without altering responding for other reinforcers (sucrose). Rats pretreated with enzastaurin or **6c** earned less infusions and had a lower breakpoint for AMPH compared with their previous levels of AMPH-maintained responding and compared with rats that received vehicle pretreatments. This suggests that PKC inhibition may change the reinforcing strength of AMPH and/or the motivation to work for AMPH infusions (Hodos 1961; Richardson and Roberts 1996; Roberts and Richardson 1992).

One notable finding is that the dose of enzastaurin previously shown to be effective at decreasing AMPH-stimulated locomotor activity and self-administration under an FR schedule, 10 pmol enzastaurin, is not effective at altering the breakpoint under a PR schedule. The PR schedule is considered a higher effort task than responding under an FR schedule, so it is possible that higher doses of enzastaurin are necessary to alter those behaviors. Another interesting note is that both enzastaurin and **6c** can alter AMPH-maintained responding following a long pretreatment time, regardless of their route of administration. This is consistent with our previous studies demonstrating that an 18 hr pretreatment of enzastaurin or **6c** is required to decrease AMPH self-administration (Carpenter et al. 2017). While little is known about the pharmacokinetics of **6c**, microdialysis studies have demonstrated that **6c** is present in minimal concentrations in the nucleus accumbens following administration of the drug (Carpenter et al. 2017). Furthermore, the half-life of enzastaurin following oral administration is 2 hr (personal communications with Denovo Biopharma and Eli Lilly). While these studies have not been repeated following *i.c.v.* administration, it is unlikely that metabolism will drastically differ between oral and *i.c.v.* administration. The possibility of a metabolite being responsible for the long-lasting effects of either drug cannot be ruled out, but due to the differences in structures and routes of administration. This could indicate a second mechanism, indirectly due to or unrelated to direct PKC inhibition, is responsible for decreasing AMPH self-administration.

One concern about a within session design for measuring breakpoint is the amount of AMPH consumed could affect responding for the breakpoint. We do not believe this is a major concern here as the number of infusions earned is less than half of the infusions typically earned when the animals self-administered AMPH under an FR schedule. Another limit of this study is that a single dose of AMPH was tested. More doses should be tested in the future but based on

our previous findings, we highly expect that enzastaurin would shift the dose-effect curve for AMPH self-administration under a PR schedule down and to the right as it does in Chapter 2.

Altogether, these studies demonstrate two structurally different PKC inhibitors previously shown to decrease AMPH self-administration under an FR schedule of reinforcement also decrease self-administration under a PR schedule of reinforcement. These findings suggest that PKC inhibition may be decreasing the reinforcing strength of AMPH in rats or the motivation to work for AMPH infusions. As these drugs do not decrease responding for sucrose, this suggests that the doses tested are not inhibiting general behavior and may be somewhat selective for AMPH. Future studies will evaluate the effect of PKC inhibition on additional doses of AMPH, other drugs of abuse, and other models of drug seeking behaviors such as the extinction/reinstatement paradigm. These findings are a promising step towards the development of PKC inhibitors as therapeutic targets for AMPH-use disorder.

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Chapter 4 PKC inhibition attenuates amphetamine-stimulated locomotor activity through a direct and indirect mechanism

Abstract

Protein kinase C β (PKC β) inhibitors have been shown to decrease the behavioral effects of amphetamine (AMPH). Previous studies demonstrated that a PKC β inhibitor acutely decreased AMPH-stimulated locomotor activity when injected into the nucleus accumbens (NAc), but the same dose (10 pmol) injected into the ventricles only decreased locomotor activity 18 hr after administration. From these studies, we hypothesized that PKC β inhibitors act to decrease AMPH-stimulated locomotor activity directly in the NAc and indirectly in a different brain region. To test this hypothesis, rats were implanted with cannulas to allow for direct injections into the NAc or the VTA. They were pretreated with 10 pmol ruboxistaurin 30 min or 18 hr before receiving 1 mg/kg AMPH and recording locomotor activity. PKC β levels and activity were assessed in the VTA and NAc following an injection of ruboxistaurin to the NAc 30 min prior to tissue collection or into the VTA 18 hr prior to tissue collection. A 30 min pretreatment of ruboxistaurin in the NAc attenuated AMPH-stimulated locomotor activity, but not an 18 hr pretreatment. An 18 hr pretreatment of ruboxistaurin, but not a 30-min pretreatment, in the VTA attenuated AMPH-stimulated locomotor activity. PKC β and pGAP43 levels were decreased in the VTA following an 18 hr pretreatment into the VTA. Following a 30 min injection into the NAc, pGAP43 was decreased in the NAc but not the VTA. These data suggest PKC β inhibitors act directly in the NAc to decrease AMPH-stimulated behaviors. PKC β

inhibitors act indirectly in the VTA to decrease behaviors, likely through a downregulation of PKC β .

Introduction

Amphetamines (AMPH) are a class of stimulants that are commonly used worldwide. AMPHs increase alertness and attention and are often prescribed for attention deficit hyperactivity disorder in the form of Adderall, but long-term misuse of AMPH can lead to cognitive deficits and psychosis (Heal et al. 2013; Janowsky and Risch 1979). AMPHs' reinforcing effects often lead to their misuse; they are the second most common class of drug abused worldwide (UNODC 2018). Furthermore, misuse of prescription stimulants during adolescence is correlated with higher rates of drug use in adulthood (McCabe et al. 2017). Despite their prevalence, there is currently no good therapeutic option for AMPH-use disorder beyond behavioral therapy (UNODC 2016b).

The reinforcing effects of AMPH are due to the dysregulation of dopamine primarily in dopaminergic neurons extending from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Di Chiara and Imperato 1988). These neurons are part of the mesolimbic or "reward" pathway; dopamine release along this pathway regulates locomotor activity, motivation and reinforcement (Volkow et al. 2004). Extracellular dopamine levels in the NAc are regulated by the dopamine transporter (DAT), which removes dopamine from the synapse to terminate dopaminergic signaling in the brain. AMPHs enter neurons through DAT and reverse the direction of the transporter, resulting in the release of dopamine into the extracellular space and an increase in dopaminergic signaling (Seiden et al. 1993).

Protein kinase C β (PKC β) is a signaling protein that is important for AMPH's action. Previous studies have demonstrated that inhibition of PKC blocks AMPH-stimulated dopamine

release (Giambalvo 1992a; b; Kantor and Gnegy 1998). PKC β , in particular, is the isoform of PKC necessary for AMPH action (Chen et al. 2009; Johnson et al. 2005). Furthermore, pharmacological or genetic inhibition of PKC attenuates AMPH-stimulated locomotor activity (Carpenter et al. 2017; Chen et al. 2009; Zestos et al. 2016). Our group has also demonstrated that PKC β inhibition decreases responding for AMPH in a self-administration paradigm (Chapter 2). All together, these studies suggest that PKC β inhibitors are viable therapeutic options for the treatment of AMPH-use disorder.

One conundrum is that the time course of action for the PKC inhibitors differs depending on the route of administration. When tamoxifen, a brain-permeable PKC inhibitor, was administered peripherally, a single acute injection was not sufficient to decrease AMPH-mediated behaviors (Einat et al. 2007; Mikelman et al. 2018). Interestingly, a peripheral injection of **6c**, a tamoxifen analog, decreased amphetamine-stimulated dopamine release when administered simultaneously with AMPH and when administered 18 hr before AMPH, however only an injection followed by an extended period of time was sufficient to decrease AMPH self-administration (Carpenter et al. 2017). Our studies with enzastaurin, a PKC β -selective inhibitor, that both dose and time after treatment were crucial for its ability to reduce the behavioral effects of AMPH. A high dose of enzastaurin (1 nmol) was sufficient to decrease AMPH-stimulated locomotor activity acutely, but a low dose (10-30 pmol) required 18 hr before effectively attenuating locomotor activity. When low doses of a PKC inhibitor (9-30 pmol) were injected directly into the NAc, they acutely decreased AMPH-stimulated behaviors (Browman et al. 1998; Zestos et al. 2016). The discrepancy in action at different time points suggests that PKC β inhibitors may alter AMPH action through more than one mechanism.

Understanding these mechanisms is a necessary step in moving PKC β inhibitors forward as therapeutic options and gaining a greater understanding of regulation of dopaminergic activity. Additionally, knowledge behind the action of these drugs may provide useful information for the treatment of other psychiatric disorders such as bipolar disorder and post-traumatic stress disorder (Mochly-Rosen et al. 2012). The goal of this study is to investigate whether PKC β inhibitors decreased AMPH-mediated behaviors through different mechanisms depending on the brain region or time course of PKC β administration. Phosphorylation-related alterations in protein levels and function will be assessed to track the biological function of the inhibitors and provide mechanistic information. We found that AMPH-stimulated locomotor activity was decreased when ruboxistaurin, a PKC β -selective inhibitor, was injected acutely in the NAc but only after 18 hr when injected into the VTA. Conversely, no change in AMPH-stimulated locomotor activity was evident 18 hr after injection in the NAc or acutely after injection in the VTA. As expected, an acute treatment of ruboxistaurin in the NAc inhibited PKC activity. However, there was a decrease in PKC levels in the VTA following the extended time period, which mirrored the changes in locomotor behavior. These studies suggest PKC β inhibition directly decreases AMPH-stimulated locomotor activity in the NAc but acts through an indirect mechanism (likely the downregulation of PKC β) in the VTA.

Methods

Subjects: Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were single-housed in a humidity and temperature-controlled environment. All animals were on a 12-hr light/dark cycle and all testing was done during the light phase. Food was available to the rats *ad libitum*. All animal procedures were approved by the University of Michigan's Institutional Animal Care

and Use Committee and designed within the rules and regulations of the National Research Council Guide for the Care and Use of Laboratory Animals.

Drug Solutions: D-AMPH (National Institute on Drug Abuse, Bethesda, MD) was dissolved in saline and administered subcutaneously (*s.c.*). Carprofen (Zoetis, Parsippany, NJ) was diluted in saline and administered *s.c.* Ketamine (Hospira, Lake Forest, IL) and xylazine (Akorn, Lake Forest, IL) were administered intraperitoneally. Ruboxistaurin (National Institute of Health, Bethesda, MD) was dissolved in artificial cerebral spinal fluid (Durect, Cupertino, CA) and 0.005% dimethyl sulfoxide (DMSO) and administered directly into the brain.

Ruboxistaurin was administered over a programmable pump that administered 0.5 μ l at a rate of 0.25 μ l/min bilaterally into the NAc core or the VTA. The rats were awake throughout the course of drug administration. Tygon tubing connected an infusion cannula (NAc: 8IC313ISPCXC 28 GA 6.4 mm with a 2 mm projection, VTA: C232I/SPC 28 GA 7.5 mm with a 2 mm projection, Plastics One, Roanoke, VA) to a single channel plastic swivel (275/22PS, Instech Laboratories, Plymouth Meeting, PA) suspended on a counter-balanced arm (PHM-110-SAI, Med Associates, Fairfax, VT). A 10 μ l Hamilton syringe was attached via Tygon tubing to the other end of the plastic swivel.

Cannula implantation: Rats were implanted with a guide cannula (NAc: C313G-L14/SP 22 GA with 6.4 mm projection, VTA: C232G-1.0/SPC 22 GA with 7.5 mm projection, Plastics One) to allow for direct injections. The rats received 5 mg/kg carprofen and ketamine/xylazine (90:10 *i.p.*). They were placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA) and cannulae were implanted relative to bregma [NAc: AP = +1.4 mm, ML = \pm 1.5 mm, DV = -5.5 mm, VTA: AP = -5.7 mm, ML = \pm 0.5 mm, DV = -6.9 mm (Paxinos and Watson 2005)]. The cannulae were fitted with a dummy cannula (NAc: C313IDC/SPC, VTA: C232DC/SPC, Plastics

One) and covered with a dust cap (303DC/1, Plastics One). Everything was anchored in place with dental cement (OrthoJet-BCA, Lang Dental Manufacturing Co., Wheeling, IL) and two steel screws (19010-10, Fine Science Tool, Foster City, CA). Placement was confirmed in locomotor activity rats by anesthetizing the rats and infusing Fast Green FCF (Millipore Sigma, Darmstadt, Germany) into the cannula. Their brain was collected and frozen in isopentane and dry ice. The brains were sliced with a cryostat (Leica CM1850, Buffalo Grove, IL) and mounted on slides, then examined for dye distribution.

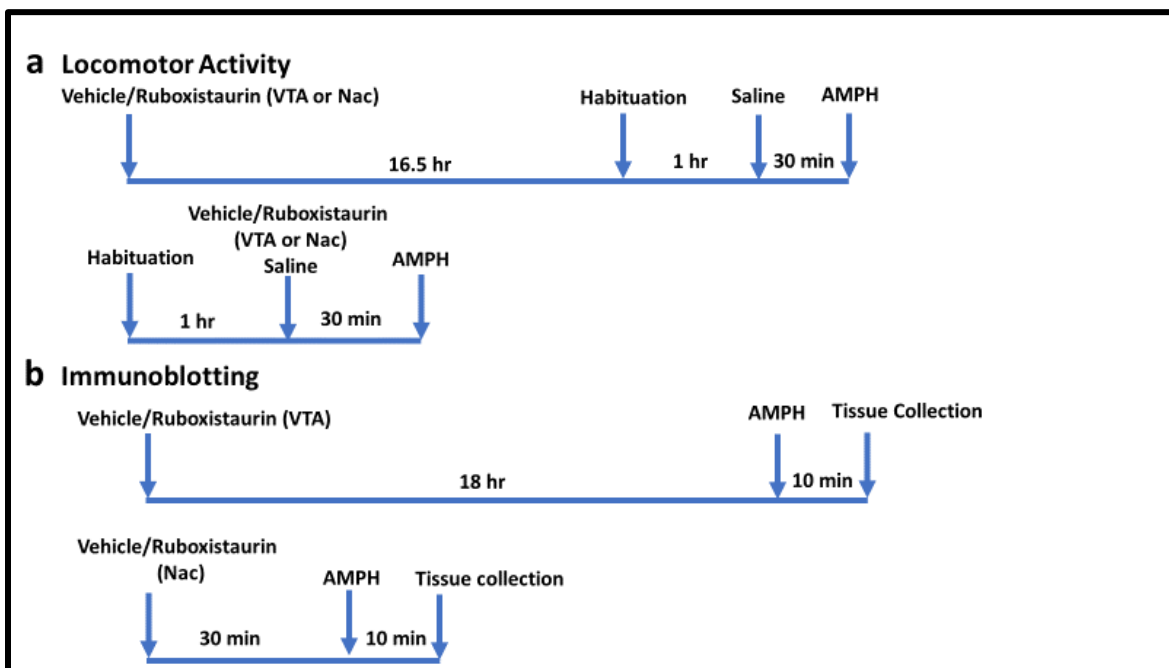


Figure 4.1 Schematic representing experimental timeline

(a) Demonstrates the time line for locomotor activity assays. Four groups of rats were tested in the locomotor activity assays. The first and second group were administered vehicle or ruboxistaurin into the VTA or the NAc 18 hr before AMPH. The third and fourth group received vehicle or ruboxistaurin into the VTA or NAc 30 min before AMPH administration. All groups were habituated to the locomotor activity cages 1.5 hr before AMPH administration and received an injection of saline 30 min before AMPH administration. (b) One group of rats received vehicle or ruboxistaurin into the VTA 18 hr before administering AMPH. The second group of rats received vehicle or ruboxistaurin in the NAc 30 min before administering AMPH. Tissue was collected in both groups after 10 min.

Locomotor activity: Rats were placed in an acrylic cage containing infrared beams (Opto-M3 Activity Monitor) as described in Chapter 2. Rats were injected with saline (*s.c.*) and habituated to the cage for 60 min. They were then given a second saline injection followed 30 min later by 1 mg/kg AMPH (*s.c.*). Activity for the rats was recorded for 2.5 hr following the AMPH injection. Two groups of rats were injected with vehicle or 10 pmol ruboxistaurin directly into the NAc or the VTA (10 pmol / 2 min) 18 hr prior to AMPH administration. Two more groups were injected with vehicle or ruboxistaurin directly into the NAc or VTA (10 pmol / 2 min) directly before the second saline injection, 10 min prior to AMPH administration (Figure 4.1).

Immunoblotting: Rats were administered vehicle or 10 pmol ruboxistaurin directly into the NAc or VTA 30 min or 18 hr prior to an injection of 1 mg/kg AMPH (*s.c.*). VTA and striatal tissue were collected 10 min following the AMPH or saline injection and the samples were immediately frozen in isopentane. Boiling 1% SDS was added to each sample and the samples were sonicated at a frequency of 20 kHz (sonic dismembrator, Fisher Scientific, Pittsburgh, PA), then centrifuged at 14,000 rpm, 4°C. The samples were separated by gel electrophoresis on a 12% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane at 1 A for 1 hr. The membranes were blocked in a buffer (5% w/v milk, 150 mM NaCl, 10 mM Tris, 0.05% Tween20) before probing for anti-growth associated protein 43 (GAP43) and anti-phosphoserine⁴¹-GAP43 (pGAP43) (both anti-goat, Santa Cruz Biotechnology, Santa Cruz, CA), PKC β II (anti-rabbit, Santa Cruz Biotechnology) and GAPDH (anti-rabbit, Cell Signaling Technology, Danvers, MA) overnight at 4°C. The membranes were washed with a wash buffer (150 mM NaCl, 10 mM Tris, 0.05% Tween20) three times before being incubated with secondary antibodies for 1 hr at room temperature: horseradish peroxidase (HRP)-tagged goat anti-rabbit for PKC β II and GAPDH (Invitrogen, Carlsbad, VA) and HRP-tagged donkey anti-goat for

GAP43 and pGAP43 (Santa Cruz Biotechnology). The antibodies were imaged with Chemiluminescent Western Substrate (EMD Millipore, Darmstadt, Germany) and band densities were quantified using ImageJ software (Schneider et al. 2012).

Statistical Analysis: Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc, San Diego, CA). Two-way ANOVAs were used to compare locomotor data with Dunnett's multiple comparisons *post hoc* tests. Immunoblotting data were analyzed by a parametric t-test. Alpha level was set at 0.05.

Results

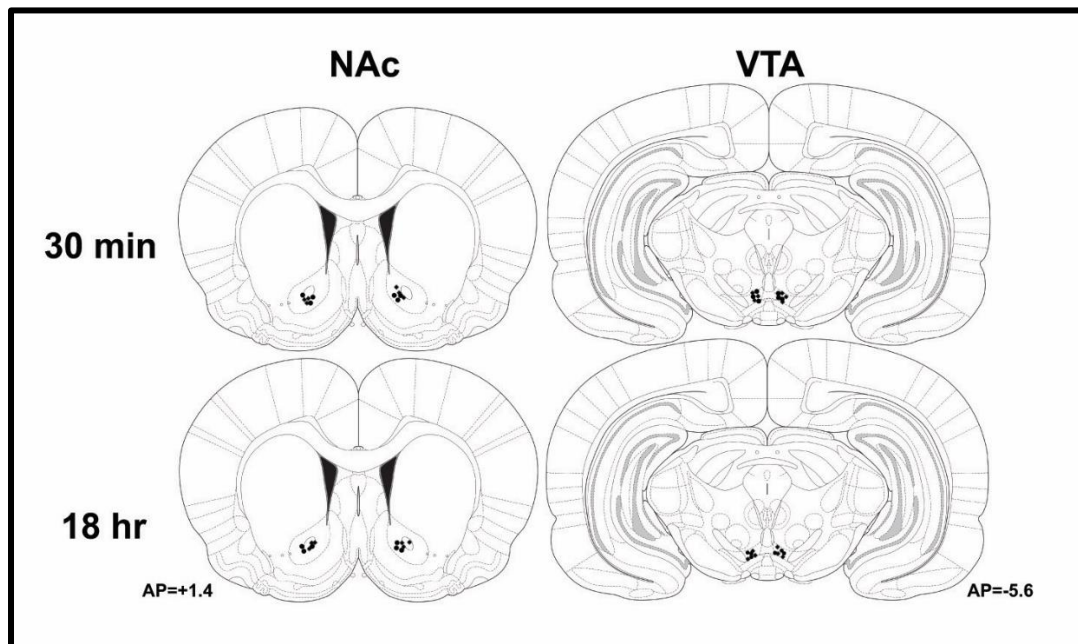


Figure 4.2 Verification of cannula placement into the NAc and VTA.

Panels display coronal sections (adapted from The rat brain in stereotaxic coordinates, 5th edition) from the NAc (1a) and the VTA with black circles indicating cannula placement in rats injected with vehicle or ruboxistaurin (Paxinos and Watson 2005).

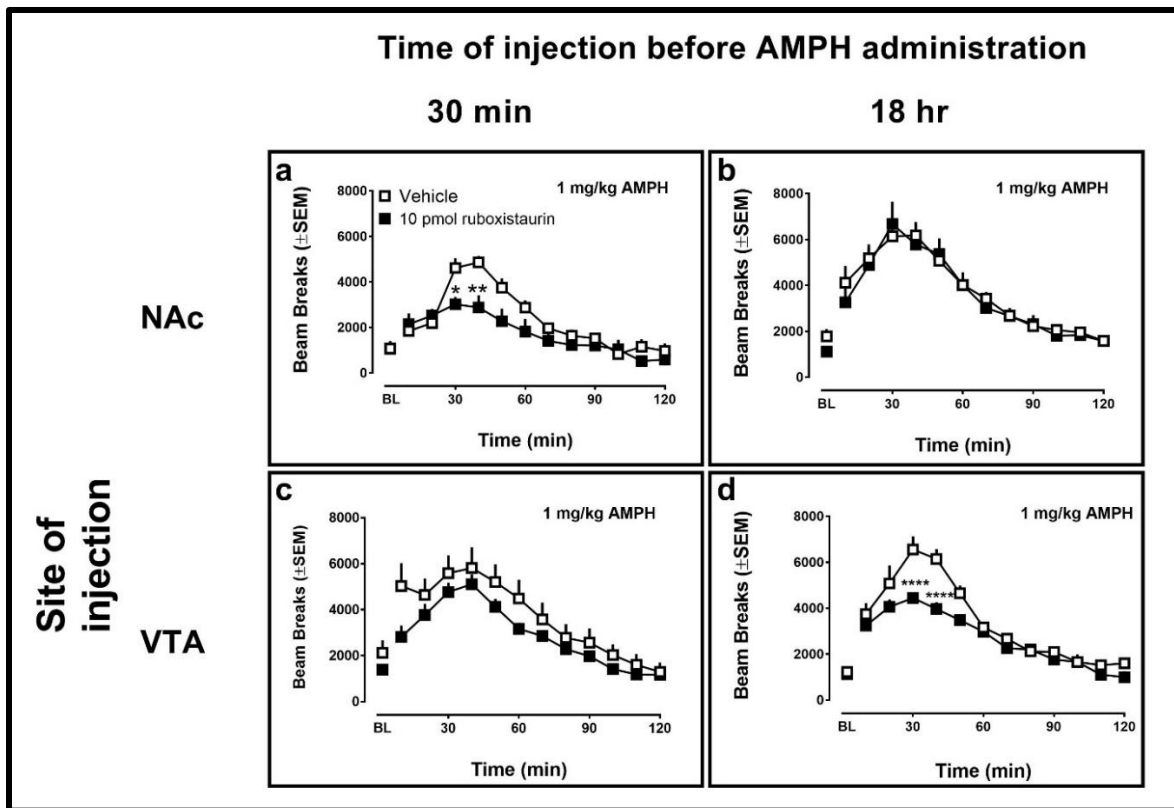


Figure 4.3 Ruboxistaurin attenuated AMPH-stimulated locomotor activity when administered into the NAc after 30 min and into the VTA after 18 hr.

The effects of ruboxistaurin on locomotor activity was assessed in rats 30 min or 18 hr after injections into different brain regions. Their baseline (BL) is calculated as the average number of beam breaks over 30 min following a saline injection (*s.c.*). At the end of the 30 min of saline, rats were injected with 1 mg/kg AMPH (*s.c.*). The number of beam breaks are summed into 10 min bins. Rats were pretreated with vehicle (open squares) or 10 pmol ruboxistaurin (closed squares). Ruboxistaurin was administered directly into the NAc 30 min (a) or 18 hr (b) before administration of AMPH or into the VTA 30 min (c) or 18 hr (d) before administration of AMPH. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ ruboxistaurin vs. vehicle pretreatment. $n=6$

The effect of ruboxistaurin on AMPH-stimulated locomotor activity is time and location dependent.

In order to determine the role that time course and brain region played in the behavioral effects of ruboxistaurin, we administered 10 pmol ruboxistaurin directly into the NAc (Figure 4.3 a, b) or VTA (Figure 4.3 c, d) of rats 30 min or 18 hr prior to administering 1 mg/kg AMPH (*s.c.*). A

30-min pretreatment of ruboxistaurin into the NAc (Figure 4.3 a), but not an 18-hr pretreatment (Figure 4.3 b) significantly decreased AMPH-stimulated locomotor activity, without altering locomotor activity [two-way ANOVA: significant interaction between time and vehicle/ruboxistaurin pretreatment $F(15, 120) = 2.802$, $p = 0.0009$ and a significant main effect of time $F(15, 120) = 31.47$, $p < 0.0001$]. Conversely, an 18-hr pretreatment of ruboxistaurin into the VTA (Figure 4.3 d), but not a 30-min pretreatment (Figure 4.3 c), significantly decreased AMPH-stimulated locomotor activity [two-way ANOVA: significant interaction between time and vehicle/ruboxistaurin pretreatment $F(15, 120) = 2.08$, $p = 0.0152$ and significant main effects of time $F(15, 120) = 48.95$, $p < 0.0001$, and vehicle/ruboxistaurin pretreatment $F(1, 8) = 7.169$, $p = 0.0280$]. There was no significant change in locomotor activity before AMPH administration.

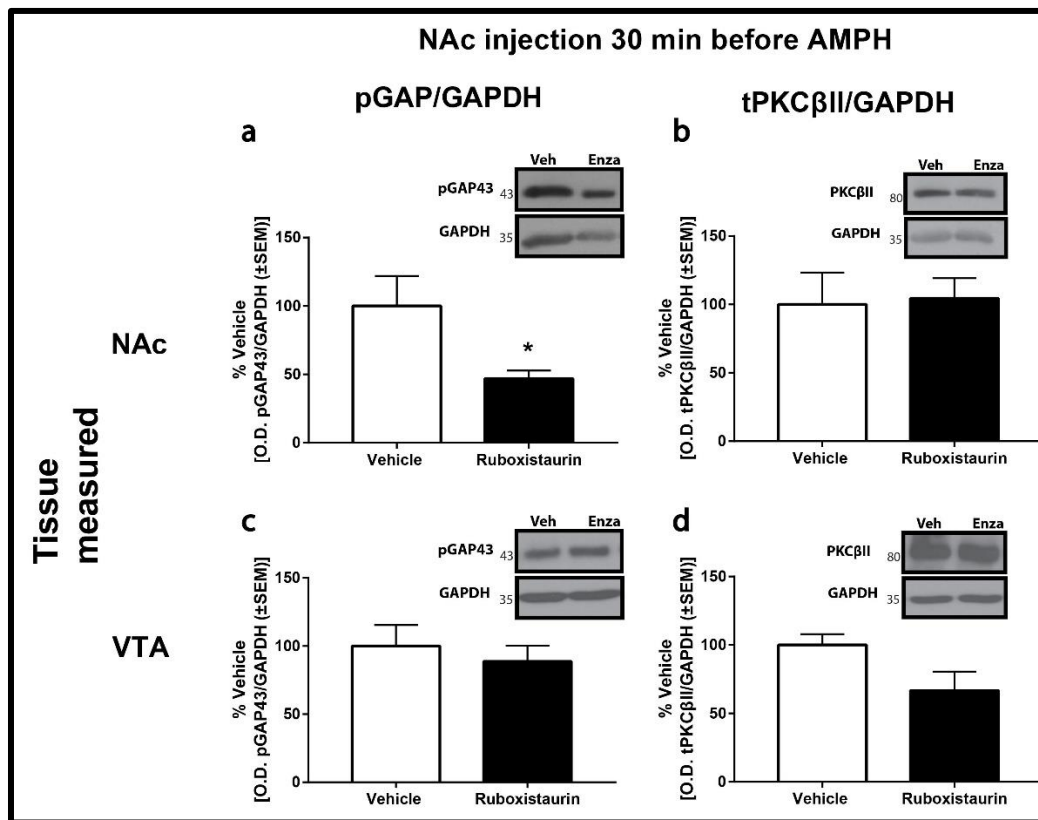


Figure 4.4 Decreased PKC activation in the NAc following a 30 min pretreatment of ruboxistaurin into the NAc.

NAC (**a, b**) and VTA (**c, d**) tissue from rats treated with vehicle or 10 pmol ruboxistaurin into the NAC 30 min before AMPH were collected and probed for phosphoser⁴¹-GAP43 (pGAP43) and total PKC β II (tPKC). The data are presented as the ratio of the optical density of pGAP43/GAPDH (**a, c**) and tPKC/GAPDH (**b, d**) \pm SEM (n=5-6). * $p < 0.05$ vs. vehicle pretreatment. n=6.

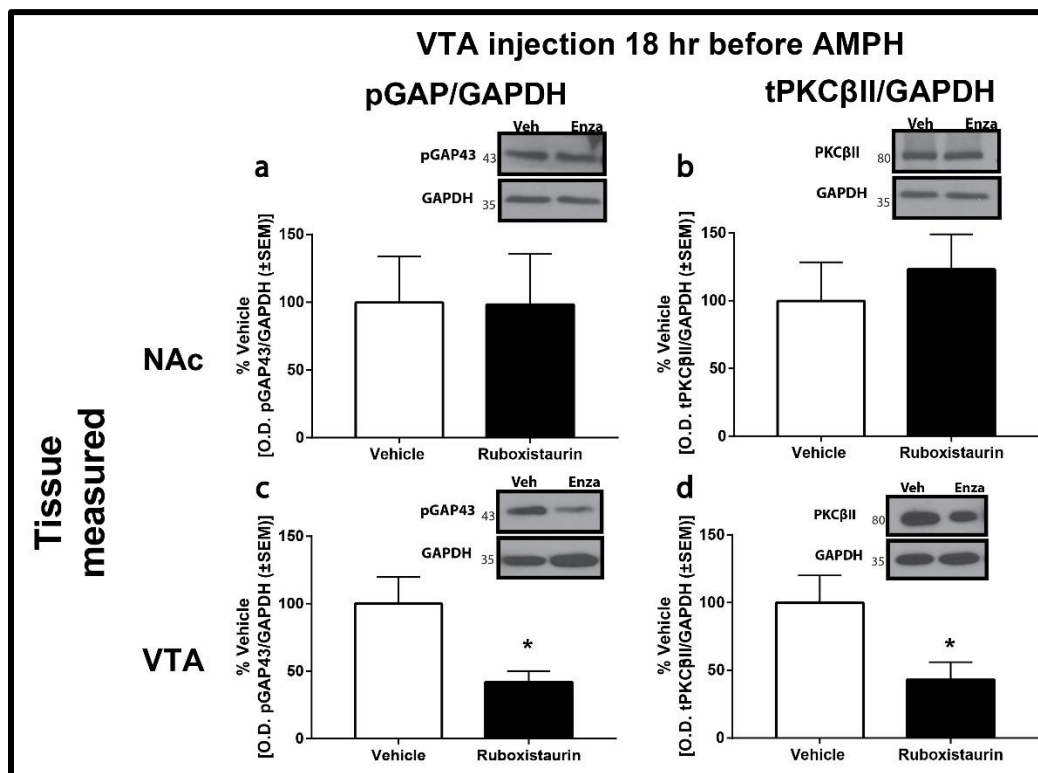


Figure 4.5 Decreased PKC levels and activation in the VTA following an 18 hr pretreatment of ruboxistaurin into the VTA.

NAC (**a, b**) and VTA (**c, d**) tissue from rats treated with vehicle or 10 pmol ruboxistaurin into the VTA 18 hours before AMPH were collected and probed for phosphoser⁴¹-GAP43 (pGAP43) and total PKC β II (tPKC). The data are presented as the ratio of the optical density of pGAP43/GAPDH (**a, c**) and tPKC/GAPDH (**b, d**) \pm SEM (n=6). * $p < 0.05$ vs. vehicle pretreatment. n=6

Ruboxistaurin administered into the VTA 18 hr before AMPH administration decreased PKC levels and activity in the VTA

We sought to identify changes in the brain that corresponded with a decrease in locomotor activity. Rats were administered vehicle or ruboxistaurin (10 pmol) into the NAc 30 min before 1 mg/kg AMPH *s.c.* A second group of rats were administered VTA vehicle or ruboxistaurin 18 hr before 1 mg/kg AMPH *s.c.* NAc and VTA tissue were collected from both groups 10 min after AMPH administration and blotted for PKC β II and pGAP43 and tGAP43 (data not shown). As might be expected, a 30 min treatment with the PKC inhibitor ruboxistaurin into the NAc significantly decreased pGAP43 levels in the NAc (t-test $p = 0.04$) as compared to vehicle (Figure 4.4a). There were no changes in pGAP43 in the VTA, nor in total PKC β II content in either the NAc or VTA following the 30 min injection into the NAc (Figure 4.4 b, c, d). In the second group of rats, a t-test determined that ruboxistaurin treatment given 18 hours prior to AMPH significantly decreased pGAP43 ($p = 0.02$) in the VTA as compared to vehicle (Figure 4.5 c, d) but there was also a significant decrease in total PKC β II ($p = 0.04$). There were no significant changes in pGAP43 or PKC β II in the NAc when ruboxistaurin was injected into the VTA (Figure 4.5 a, b). There were no changes in total GAP43 in any group (data not shown).

Discussion

In this study, we demonstrate for the first time that ruboxistaurin inhibits AMPH-mediated behavior through both direct and indirect mechanisms depending on the brain region. Previous studies have shown that ruboxistaurin and other PKC inhibitors injected directly in the NAc 15-30 min before AMPH administration decrease AMPH-stimulated locomotor activity (Browman et al. 1998; Loweth et al. 2009; Zestos et al. 2016). On the contrary, PKC inhibitors decrease AMPH-mediated behaviors following extended pretreatment times when administered *i.c.v.* or *s.c.*, indicating that PKC inhibition may elicit a secondary mechanism that can alter behavior (Carpenter et al. 2017). To further probe the different mechanisms, we chose to

compare the actions of ruboxistaurin in two brain regions of the mesolimbic dopamine pathway, the NAc and VTA. Ruboxistaurin decreased AMPH-stimulated locomotor activity when administered into the NAc 30 min or into the VTA 18 hr before AMPH, while the converse, 18 hr in the NAc or 30 min in the VTA before AMPH was ineffectual. Biochemical analysis of these different brain regions demonstrated the expected decrease in local PKC activity following a short (30 min) pretreatment into the NAc. Notably, however, following a long pretreatment in the VTA, we found a decrease in both pGAP43 and total PKC β II levels rather than merely an inhibition of PKC activity. These findings indicate that ruboxistaurin acts directly in the NAc to decrease PKC activity and locomotor activity but acts indirectly in the VTA to reduce locomotor activity and PKC activity by decreasing PKC β II levels.

The effect of ruboxistaurin on AMPH-stimulated locomotor activity differed depending on the brain region and pretreatment time. The finding that a short pretreatment time (30 min) was effective in the NAc but a long pretreatment time (18 hr) was only effective in the VTA indicates that the mechanisms of ruboxistaurin action are different in the NAc compared to the VTA. Most likely, acute ruboxistaurin in the NAc is inhibiting AMPH-stimulated locomotor activity by decreasing AMPH-stimulated dopamine efflux through DAT, following the known mechanism of PKC inhibition of AMPH action (Chen et al. 2009; Giambalvo 1988; Johnson et al. 2005; Kantor and Gnegy 1998).

A longer pretreatment time of PKC inhibitor is likely acting through a secondary mechanism downstream of PKC. To further probe the action of ruboxistaurin in the VTA, we examined how PKC β II levels and activity were affected 18 hr following ruboxistaurin administration into the VTA. We looked at PKC β II in particular because PKC β II has been implicated to be more important for AMPH-stimulated dopamine efflux than other isoforms of

PKC (Johnson et al. 2005), and this isoform is known to be expressed around the NAc and the VTA (O'Malley et al. 2010; Saito et al. 1989). A long pretreatment saw a decrease in PKC activity and PKC β II levels in the VTA raising the possibility that a decrease in PKC levels may account for the behavior.

Interestingly, the administration of a PKC inhibitor into the VTA did not alter PKC activity in the NAc despite the extended pretreatment. This was unexpected as acute effects of AMPH on locomotor activity occur when AMPH is injected into the NAc but not the VTA (Perugini and Vezina 1994; Vezina and Stewart 1990). Furthermore, we previously demonstrated that PKC β inhibitors administered *i.c.v.* 18 hr before tissue collection decrease PKC activity in the NAc (Chapter 2). It is possible that there was a decrease in PKC activity in dopaminergic neurons in the NAc, but since the NAc contains a heterogenous mix of cells, any decrease in PKC activity may be obfuscated by the other cells within the samples. This was likely not an issue following *i.c.v.* administration of a PKC inhibitor, as we were not targeting specific brain regions. Additional studies examining changes specifically in presynaptic dopamine neurons in the NAc would be able to verify a decrease in PKC activity.

Multiple mechanisms can lead to a decrease in PKC levels. One possibility is that PKC β II is downregulated. The VTA contains the cell bodies of the dopaminergic neurons in the mesolimbic pathway, therefore changes in protein transcription occurs in the VTA. PKCs interact with many different transcription factors to regulate gene expression (Ventura and Maioli 2001). It is well known that activators of PKC increase degradation of PKC and downregulate the enzyme (Ballester and Rosen 1985; Lum et al. 2013), but downregulation of PKC following administration of an inhibitor is not entirely unprecedented. One group demonstrated that enzastaurin decreases PKC β II promoter activity and mRNA levels (Liu et al.

2004) and another group has shown that increased PKC β II increases promoter activity and mRNA levels (Cejas et al. 2005). Together, these studies indicate that PKC β II acts in positive and negative directions to affect its own synthesis. Additional studies have also shown that tamoxifen or enzastaurin downregulated different isoforms of PKC in cell-based assays (Gundimeda et al. 1996; Jane and Pollack 2008). An additional mechanism that may regulate PKC levels in the VTA is through increased degradation of PKC. Previous studies have demonstrated that an acute pretreatment with an active site inhibitor structurally similar to ruboxistaurin, bisindoylmaleimide I, protects PKC from degradation (Gould et al. 2011), however the stability of PKC following metabolism or elimination of the inhibitor is not understood.

Outside of changes in PKC levels, PKC inhibitors may alter AMPH-mediated behaviors through other means. PKC is important for the trafficking and regulation of many proteins that may play a role in AMPH's action. The trafficking of D₂ and D₃ autoreceptors in particular are regulated by PKC β (Cho et al. 2007; Luderman et al. 2015; Namkung and Sibley 2004). PKC is also known to affect DAT internalization (Sorkina et al. 2005) so PKC β inhibition may be changing surface levels of DAT. It is also possible that PKC may regulate gene expression of proteins that interact with dopamine receptors or DAT or their downstream effectors.

Additional effects of ruboxistaurin may underlie its actions on AMPH-mediated behaviors following a long pretreatment. One potential mechanism is that an active metabolite of ruboxistaurin could decrease AMPH-mediated actions in the brain. A study looking at [¹⁴C]-ruboxistaurin in a rat following oral administration determined the half-life for ruboxistaurin and its active metabolite to be 2.5 and 4.3 hr, respectively; remaining levels of ¹⁴C could indicate additional metabolites with a longer half-life (Campanale et al. 2002). While we cannot rule out

metabolism as a factor on AMPH-stimulated behaviors, the fact that the prolonged time course is seen across a variety of structurally unrelated PKC inhibitors (bisindolmaleimides such as enzastaurin and ruboxistaurin and tamoxifen analogs) indicate that the time course may be due to the mechanism of the drugs rather than their pharmacokinetics.

In conclusion, we have demonstrated that ruboxistaurin, a PKC β selective inhibitor, may be altering AMPH-stimulated behaviors through a direct mechanism and an indirect mechanism. A potential explanation behind the indirect mechanism may be by decreasing PKC β II levels in the mesolimbic pathway. This does not mean that a decrease in PKC levels is the only effect of a long pretreatment with PKC inhibitors; additional proteins that may be affected and can play a role in AMPH-mediated behaviors include dopamine receptors and their downstream effectors. Future studies should identify additional mechanisms and explore the specific proteins downstream of PKC that may play a role in this effect. These studies provide the first step in understanding an additional mechanism important for the action of PKC inhibitors and add to our understanding of the therapeutic potential of such inhibitors for the treatment of substance-use disorders.

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Chapter 5 Discussion

Amphetamine abuse is prevalent throughout the United States and the world and presents a serious public health concern (Miech et al. 2018; UNODC 2016a; 2018). Despite the widespread use of amphetamine, the only treatments for amphetamine-use disorder are cognitive or behavioral therapies (UNODC 2016b), and a large percentage of patients treated for misuse of amphetamine-type stimulants relapse within a year (Brecht and Herbeck 2014). This highlights the unmet need for a pharmacological intervention to supplement treatment for amphetamine-use disorders. Protein kinase C β (PKC β) is a potential target for a novel therapeutic. Years of research have demonstrated that PKC, in particular PKC β , inhibitors block the actions of amphetamine *ex vivo* and *in vivo* (Browman et al. 1998; Chen et al. 2009; Johnson et al. 2005; Kantor and Gnegy 1998).

In this thesis, I demonstrate that PKC β inhibitors decrease amphetamine-mediated behaviors, particularly locomotor activity and reinforcing effects. Furthermore, the experiments presented here suggest that PKC β inhibitors decrease amphetamine-stimulated behaviors through more than one mechanism. These studies expand our understanding of PKC β actions in the brain and support the development of PKC β inhibitors as therapeutics for amphetamine use-disorder.

PKC β inhibition and locomotor activity

Locomotor activity is a simple, rapid way to investigate amphetamine-induced behavior, making it a good first step in characterizing the actions PKC β inhibitors. Locomotion is an unconditioned behavior, thus requires no training and allows for the rapid testing of drug

conditions (Nader 2016). Stimulants such as amphetamine increase locomotor activity in rats by increasing extracellular dopamine levels and dopaminergic signaling in the nucleus accumbens. Locomotor activity has long been tied to dopamine transmission in the brain (Beninger 1983; Kelly and Iversen 1976; Schindler and Carmona 2002). This led to the hypothesis that PKC inhibitors, which attenuate amphetamine-stimulated dopamine efflux, will decrease amphetamine-stimulated locomotor activity. In fact, multiple studies have demonstrated that administration of a PKC inhibitor directly into the nucleus accumbens blocks amphetamine-stimulated locomotor activity (Browman et al. 1998; Loweth et al. 2009; Zestos et al. 2016). In this thesis, I used locomotor activity to further characterize the effectiveness of PKC β -selective inhibitors.

In Chapter 2, I showed that enzastaurin, a PKC β -selective inhibitor, decreased amphetamine-stimulated locomotor activity. Enzastaurin has a poor bioavailability but good safety profile, thus it serves as a proof-of-concept to demonstrate the actions of PKC β inhibition on amphetamine-stimulated locomotor activity. I found that small doses of enzastaurin (10-30 pmol) effectively attenuated amphetamine-stimulated locomotor activity. These doses of enzastaurin did not depress locomotor activity on its own, suggesting that the enzastaurin only acts when locomotor activity is enhanced. I also demonstrated that enzastaurin shifted the dose-effect curve for amphetamine-stimulated locomotor activity to the right. At the largest dose tested (3.2 mg/kg amphetamine), locomotor activity for rats pretreated with enzastaurin was significantly higher than for the vehicle pretreated rats. This is likely due to the onset of stereotypy; short, repetitive movements that interfere with ambulatory locomotor activity, often seen with large doses of stimulants (Del Rio and Fuentes 1969). Stereotypy was not directly

quantified in these experiments, but the enzastaurin-pretreated rats appeared to display less stereotyped behaviors and more ambulatory activity than the vehicle-pretreated rats.

The most unexpected finding in these studies was the time course of enzastaurin. Previous studies looking at PKC inhibitors, including enzastaurin, that were delivered directly to the NAc saw a decrease in amphetamine-stimulated activity within 30 min of PKC inhibitor administration (Browman et al. 1998; Loweth et al. 2009; Zestos et al. 2016). Our studies found that a large dose of enzastaurin given *i.c.v.* has an acute effect on amphetamine-stimulated locomotor activity; however small doses of enzastaurin given *i.c.v.* needed to be administered 18 hr before amphetamine to decrease amphetamine-stimulated locomotor activity. These findings were not exclusive to enzastaurin; an 18-hr pretreatment was necessary for *i.c.v.* ruboxistaurin to be effective at decreasing locomotor activity (Figure 5.1). The long pretreatment time was unexpected, given that *i.c.v.* administration should bypass pharmacokinetic parameters such as absorption and distribution. Furthermore, given the known pharmacokinetics of these drugs, the likelihood that enzastaurin or ruboxistaurin or their active metabolites are present at 18 hr is minimal. However, a prolonged time course for a PKC inhibitor is not unprecedented. Tamoxifen, a brain permeable PKC inhibitor, did not decrease amphetamine-mediated locomotor activity acutely and required repeated dosing (Einat et al. 2007; Mikelman et al. 2018). One explanation for this effect is that tamoxifen required repeated dosing to accumulate in the brain. However, it is not known if a single injection of tamoxifen would attenuate amphetamine-stimulated locomotor activity following a long pretreatment. As multiple PKC inhibitors have been shown to reduce amphetamine action at later time points, it is possible that the prolonged time course is not due to the pharmacokinetics of these drugs but rather an effect downstream of

PKC inhibition. The time course of PKC inhibitors will be addressed further in a later section of this chapter.

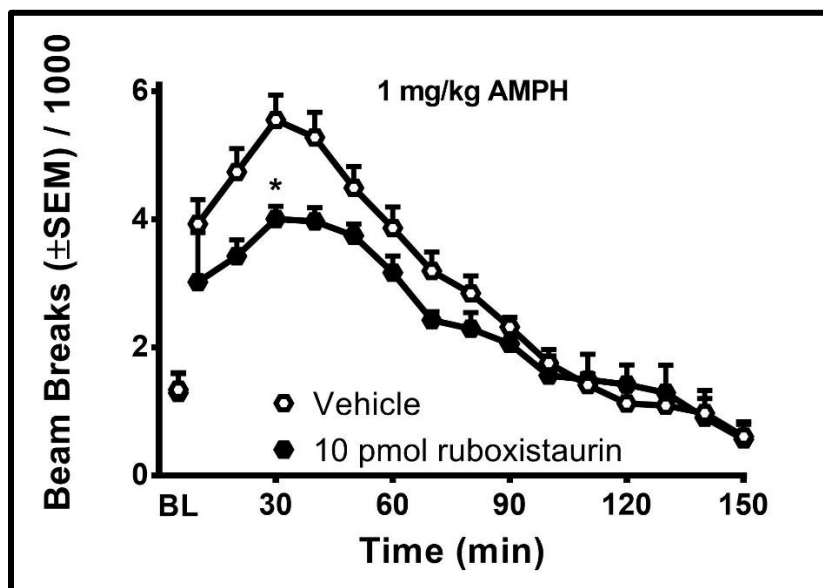


Figure 5.1 Ruboxistaurin decreased amphetamine-stimulated locomotor activity.

Rats were administered vehicle or 10 pmol ruboxistaurin *i.c.v.* 18 hr before receiving 1 mg/kg AMPH *s.c.* Locomotor activity is presented as the number of beam breaks over 10 min bins. Baseline activity (BL) was calculated from the average number of beam breaks/10 min bin 30 min prior to amphetamine administration. A two-way ANOVA showed a significant interaction between vehicle/ruboxistaurin and time [F (15, 150) = 1.9, $p = 0.03$] as well as a significant main effect of time [F (15, 150) = 45.43, $p < 0.0001$] and vehicle/ruboxistaurin [F (1, 10) = 6.03, $p = 0.03$]. * $p < 0.05$. $n = 5-7$.

While PKC β inhibitors successfully decrease amphetamine-stimulated locomotor activity, it is not known if these effects are selective for amphetamine or affect the activity of other drugs of abuse. One drug of abuse that may be affected by PKC inhibition is cocaine. Zestos et al. found that ruboxistaurin injected directly into the NAc reduced cocaine-stimulated (15 mg/kg *i.p.*) dopamine overflow and locomotor activity (Zestos et al. 2018). Despite these findings, an 18 hr pretreatment of 10 pmol enzastaurin *i.c.v.* had no significant effect on cocaine-

stimulated locomotor activity (15 mg/kg *i.p.*) (Figure 5.2a). There appears to be a trend towards *i.c.v.* enzastaurin increasing cocaine-stimulated locomotor activity, thus a full dose effect curve needs to be evaluated for cocaine-stimulated locomotor activity to determine if enzastaurin has any effect. Additionally, the time course of enzastaurin action on cocaine should be considered. I also examined whether *i.c.v.* enzastaurin would alter SKF-81297 (D₁-selective agonist) locomotor activity (Figure 5.2b). Again, a full dose-response curve should be evaluated with SKF-81297 at different time points. More studies evaluating dose-effects curves with different direct and indirect dopamine agonists are necessary to determine if PKC β selectively attenuates amphetamine-stimulated behaviors or will also decrease behaviors stimulated by other drugs of abuse.

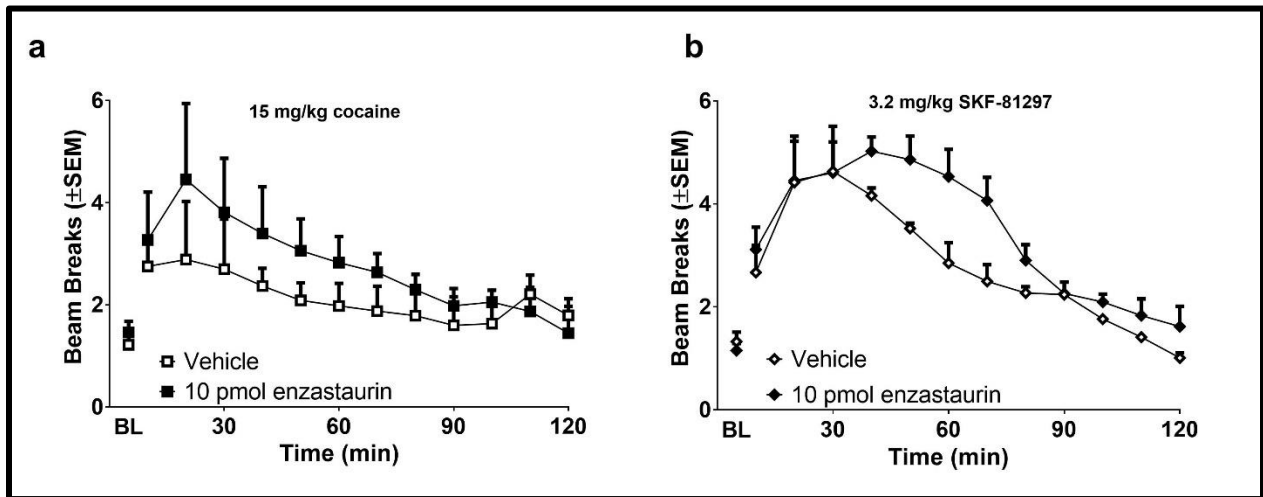


Figure 5.2 Enzastaurin did not significantly attenuate cocaine- or SKF-81297-mediated locomotor activity.

Rats were pretreated with vehicle or 10 pmol enzastaurin 18 hr prior to receiving (a) 15 mg/kg cocaine *i.p.* (n = 5-6) or (b) 3.2 mg/kg SKF-81297 *s.c.* (n = 3). Data are presented as the number of beam break over time in 10 min bins. Baseline (BL) is calculated from the average number of beam breaks/10 min 30 min before cocaine or SKF-81297 administration. A two-way ANOVA did not reveal a significant interaction between time and pretreatment (vehicle vs enzastaurin) or a significant main effect of pretreatment.

PKC β inhibition and self-administration

Locomotor activity assays are perfect for the initial studies characterizing new therapeutics, but these assays have many limitations. The biggest limitation to locomotor activity as a model for studying drugs of abuse is that it does not model behaviors associated with substance-use disorder and is not a measure of drug reinforcement. Drug self-administration assays are the most common model to assess drug reinforcement as they directly model drug-taking and drug-seeking behaviors. These models have been used to accurately predict drug self-administration in humans (Griffiths et al. 1979) and have predictive validity in assessing new medications for certain substance-use disorders (Egli et al. 2016). Predictive validity has yet to be shown for amphetamine-use disorders due to a dearth of clinically-approved therapeutics (Egli et al. 2016). In order to evaluate a pharmacological intervention/treatment for amphetamine-use disorder, the effect of PKC β inhibitors on the reinforcing effects of amphetamine must be evaluated. Through self-administration models, I have determined that PKC β inhibitors decrease the reinforcing properties of amphetamine in rats.

In Chapter 2, I demonstrated that 10 pmol enzastaurin (the most effective dose in locomotor activity assays) *i.c.v.* attenuates responding for amphetamine (0.032 mg/kg/inf) under a fixed-ratio (FR) 5 schedule of reinforcement. This dose of enzastaurin was only effective when administered 18 hr prior to the self-administration session. A 3 hr pretreatment had no effect on responding for amphetamine, and preliminary data suggest that a 1 hr pretreatment also has no effect (data not shown). As the dose-effect curve for amphetamine self-administration is an inverted U-shaped curve, it is important to evaluate whether enzastaurin attenuates responding for multiple doses of amphetamine (Mello and Negus 1996). An ideal therapeutic for substance-use disorders will shift the dose-effect curve downwards, demonstrating decreased drug-intake.

A rightward shift in the dose-effect curve could be interpreted as decreased sensitivity to the self-administered drug but leads to concerns about increased drug intake at higher doses (Piazza et al. 2000). When I tested the full dose-effect curve for amphetamine self-administration, I found 10 pmol enzastaurin shifted the ascending limb of the dose-effect curve without changing the descending limb. This shift in the dose-effect curve is not a typical downward or rightward shift, suggesting decreased drug-intake at lower doses of amphetamine without a corresponding increase in drug-intake at higher doses. An explanation for the direction of this shift may be due to experimental design. The dose-effect curve for amphetamine was assessed within a single session so it is possible that enzastaurin was surmounted by high doses of amphetamine in the later components of the session.

One concern about using a PKC β inhibitor as a therapeutic is that the inhibitor would decrease all operant responding due to adverse effects, so sucrose self-administration was used as a control. Enzastaurin did not alter responding for sucrose under an FR5 schedule of reinforcement. These studies demonstrate that enzastaurin does not suppress all behaviors and functions necessary for operant responding. While these findings may also indicate a selectivity for amphetamine over non-drug reinforcers, a concentration-effect curve with sucrose would need to be evaluated in order to fully assess enzastaurin selectivity.

The studies in Chapter 2 demonstrated that a PKC β inhibitor affects drug-taking behaviors. To further corroborate my findings that PKC β inhibitors decrease the reinforcing properties of amphetamine, I used the progressive-ratio (PR) schedule of reinforcement (Arnold and Roberts 1997). In Chapter 3, I demonstrated a decrease in the reinforcing strength of amphetamine following a pretreatment with a PKC β inhibitor. It has also been suggested that a decrease in responding under a PR schedule represents a decrease in motivation for seeking or

craving amphetamine (Markou et al. 1993). Furthermore, as with the previous sucrose studies, the PKC β inhibitor did not affect responding for sucrose under a PR schedule, suggesting that PKC β inhibition does not affect the rewarding strength of a non-drug reinforcer.

These proof-of-concept studies with enzastaurin and ruboxistaurin overall demonstrate that PKC β is a reasonable target to attenuate amphetamine-stimulated behaviors, including amphetamine-taking behavior. These compounds cannot be repurposed or developed for the treatment of amphetamine use disorder because these compounds do not cross the blood brain barrier following peripheral administration. Since there are few to no studies published on brain-permeable PKC inhibitors (Mochly-Rosen et al. 2012), efforts were made to use the tamoxifen scaffold to develop a blood-permeable PKC inhibitor without the anti-estrogenic properties of tamoxifen (Carpenter et al. 2016). The lead compound from these studies, **6c**, successfully attenuates amphetamine-stimulated dopamine release *in vivo* following peripheral administration. More importantly, it decreases amphetamine self-administration under an FR schedule (Carpenter et al. 2017) and a PR schedule (Chapter 3), without affecting responding for sucrose. **6c** needs to be examined with a full amphetamine dose-effect curve, but these initial findings are a promising start in identifying new compounds. Interestingly, **6c** also displayed the same delayed activity as the structurally unrelated compounds enzastaurin and ruboxistaurin and required long pretreatments to have any effect in the self-administration models. These findings are consistent with my hypothesis that the extended time necessary for action may be more related to the pharmacodynamic actions of PKC inhibitors as opposed to pharmacokinetic measures.

PKC β inhibitors and time course

A common theme throughout these studies is that PKC β inhibitors require long pretreatments before effectively decreasing amphetamine-mediated behaviors. This has raised two main, albeit related, questions:

- (1) Why do PKC β inhibitors given *i.c.v.* not act acutely?
- (2) Why do PKC β inhibitors require long pretreatment times?

Other studies have already demonstrated that PKC β inhibitors act acutely (Loweth et al. 2009; Zestos et al. 2016), however a common feature among these studies is that the drugs were administered directly into the NAc. In trying to reconcile these findings, what stands out most is the differences between routes of administration. A direct drug injection into the NAc will concentrate the drug within the NAc whereas an *i.c.v.* injection would distribute a drug throughout the brain (Luger et al. 2005), resulting in less drug accumulation in the NAc. Therefore, I hypothesized that giving a larger amount of PKC β inhibitor *i.c.v.* should decrease amphetamine-stimulated activity. Consistent with this prediction, I found that a large dose (1 nmol enzastaurin *i.c.v.*) could decrease amphetamine-stimulated locomotor activity following a 30 min pretreatment (Chapter 2). I also demonstrated that 10 pmol ruboxistaurin directly into the NAc decreased amphetamine-stimulated locomotor activity following a 30 min pretreatment (Chapter 4). These findings support the hypothesis that drug concentration in the NAc is important for the behavioral effects of these inhibitors although future studies quantifying drug concentration in these brain regions are necessary.

To further probe the mechanism involved in the effects observed following the long pretreatment time, I compared drug action in the NAc to a different region in the mesolimbic pathway, the ventral tegmental area (VTA). Direct injections of a PKC β inhibitor into the NAc acutely decreased amphetamine-stimulated locomotor activity, however long pretreatments in the

NAc were ineffective at modulating locomotor activity. PKC β inhibition had the opposite effects in the VTA; long pretreatments, but not acute pretreatments, of ruboxistaurin attenuated amphetamine-stimulated locomotor activity. These findings have two main implications: (1) the brain region is an important determinant of PKC β inhibitor action and (2) a long pretreatment with PKC β inhibitors may decrease amphetamine action through a different mechanism as compared to an acute pretreatment.

There are many potential explanations behind the effectiveness of a long pretreatment. Drug metabolism is one such explanation, however the pharmacokinetic studies for the half-lives of enzastaurin (personal communications with Eli Lilly and Denovo Biopharma) and ruboxistaurin (Campanale et al. 2002) do not support an 18 hr time course. One caveat is that the pharmacokinetic studies were performed following oral administration of the drugs, not *i.c.v.* Further complicating this explanation, the effectiveness of a long pretreatment time was also established with the **6c** and it is unlikely that the structurally unrelated compound has a similar pharmacokinetic profile to enzastaurin and ruboxistaurin.

Another explanation for the prolonged time course is that changes in protein expression due to PKC inhibition are responsible for the decrease in amphetamine-mediated behaviors. PKC activity is an important factor in many signaling pathways and can control expression of different genes and proteins (Ventura and Maioli 2001). Furthermore, the cell bodies of the neurons in the mesolimbic pathway are in the VTA, highlighting the importance of drug activity in that brain region. To support this explanation, I found that PKC β II levels and activity in the VTA were decreased following the 18 hr pretreatment with a PKC β inhibitor. This was initially a surprising find given that multiple studies have demonstrated that activation of PKC downregulates the PKC (Newton 2018). Some studies, however, found that PKC β activation or inhibition

autoregulates activity of the PKC β promotor (Cejas et al. 2005; Liu et al. 2004). For instance, Liu et al. found inhibition of PKC β with enzastaurin decreased expression of PKC β II mRNA in cell cultures (Liu et al. 2004). Taken together, these findings suggest that acute inhibition of PKC β in the NAc decreases amphetamine-mediated behaviors but over time the inhibition of PKC β in the VTA results in the downregulation of PKC β II which contributes to the decrease in amphetamine-mediated behaviors 18 hr later.

Additional targets of PKC inhibitors

A downregulation of PKC β is one likely explanation for the prolonged time course of PKC inhibitors, but other targets must also be taken into consideration. Due to the ubiquitous nature of PKC, it is likely that inhibition will have effects on expression, activity, and trafficking of other proteins, including some that are important for amphetamine action. One clear target to investigate is the dopamine receptor. The studies with the dopamine D₁ receptor agonist SKF-81297 (Figure 5.2) were not conclusive in determining if there is any change in D₁ receptor function. Another group found that PKC inhibitors had no effect on D₁-agonist-induced reinstatement of cocaine seeking (Ortinski et al. 2015), suggesting that PKC inhibition may not have much of an effect on D₁ receptor activity. Multiple groups have shown that PKC regulates trafficking and function of D₂-like receptors (Luderman et al. 2015; Namkung and Sibley 2004). Zestos et al. found that acute enzastaurin decreased cocaine-stimulated locomotor activity through the activation of D₂ autoreceptors (Zestos et al. 2018). While an 18 hr pretreatment of *i.c.v.* 10 pmol enzastaurin had no significant effect on cocaine-stimulated locomotor activity (Figure 5.2), evaluating a full dose-effect curve for cocaine may reveal a decrease in locomotor activity through D₂-like activity.

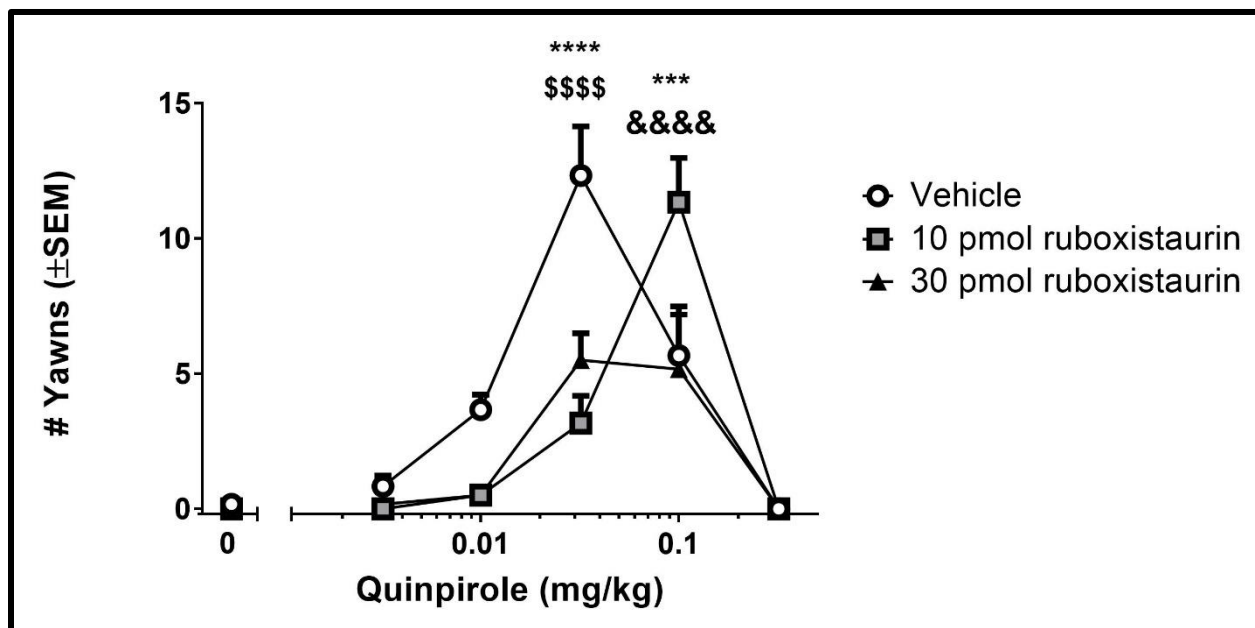


Figure 5.3 Ruboxistaurin shifted the yawning dose-effect curve for quinpirole to the right.

Rats were pretreated with vehicle (open circles), 10 pmol (grey squares), or 30 pmol (closed triangles) ruboxistaurin *i.c.v.* 18 hr before receiving cumulative doses (0.0032-0.32 mg/kg) of quinpirole (*s.c.*) every 30 min. The number of yawns over a 10 min period were recorded 20 min after each injection. A two-way ANOVA demonstrated a significant interaction between dose of quinpirole and pretreatment. *** $p < 0.001$, **** $p < 0.0001$ vehicle vs 10 pmol ruboxistaurin, &&&& $p < 0.0001$ 10 pmol vs 30 pmol ruboxistaurin, \$\$\$ $p < 0.0001$ vehicle vs 30 pmol ruboxistaurin. $n=6$.

To assess whether D_2 -like receptor activity was changed with a long pretreatment time with a $PKC\beta$ inhibitor, I turned to yawning as a readout of D_2 and D_3 receptor activity. Low doses of quinpirole, a D_2/D_3 agonist, induce yawning in rodents and high doses decrease yawning. Collins et al. (2007) further examined the specific contributions of D_2 and D_3 receptor activation to the induction of yawning and found that D_3 receptor activation induces yawning and controls the ascending limb of yawning dose-effect curve while D_2 activation inhibits yawning inducing the descending limb. I pretreated rats with 10 or 30 pmol ruboxistaurin *i.c.v.* 18 hr before administering cumulative doses of quinpirole (Figure 5.3). I found an 18 hr pretreatment of ruboxistaurin shifted the ascending limb of the dose-effect curve to the right, suggesting a

decrease in potency at the D₃ receptor. A decrease in D₃-receptor activity could modulate amphetamine-mediated behaviors as D₃ receptor antagonists have been shown to decrease self-administration (Higley et al. 2011). A 10 pmol dose of ruboxistaurin appears to shift the descending limb to the right, indicating a decrease in potency at the D₂ receptor. The higher dose of ruboxistaurin, however, does not show a downward shift, perhaps due to less induction of yawning by D₃ receptors. Further studies with quinpirole-induced hypothermia, a state controlled entirely through D₂-receptor activation, would further enhance our understanding of whether PKC β inhibition over time affects D₂ receptors. Additional studies could also examine dopamine receptor activation, dopamine receptor trafficking, or downstream effectors of dopamine receptor signaling to determine how PKC β inhibition decreases D₂-like receptor activity.

Conclusions

PKC β inhibition is a promising target as a therapeutic for amphetamine-use disorders. I have demonstrated that PKC β inhibitors decrease amphetamine-stimulated behaviors. More importantly, PKC β inhibitors decrease the reinforcing properties of amphetamine in rodents, a necessary outcome in the development of therapeutics for substance-use disorders. Finally, I showed that PKC β inhibitors decrease locomotor activity acutely, but then induce a secondary effect that decreases amphetamine-mediated behaviors 18 hr after administration. These actions likely occur through separate mechanisms. These findings are the first steps or proof of concept studies necessary for the development of PKC β inhibitors for amphetamine use disorder. Further studies should examine the effects of repeated dosing over time as well as whether PKC β inhibitors will decrease reinstatement for amphetamine in a relapse model. Finally, further characterization of the proteins downstream of PKC signaling will provide important information for the development of additional therapeutics and Altogether, the findings of this thesis warrant

further investigation and development of PKC β inhibitors as a therapeutic for amphetamine-use disorder.

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