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Modulators of behavioral sensitivity to cocaine following dopamine β-hydroxylase (DBH) inhibition

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Neuroscience

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Abstract

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By Meriem Gaval

Dopamine β-hydroxylase (DBH) converts dopamine (DA) to norepinephrine (NE), playing a direct role in determining the DA/NE ratio in noradrenergic neurons. DA and NE are both involved in the modulation of reward and reinforcement of natural stimuli and drugs of abuse. Recent evidence from human laboratory studies and animal studies suggests that DBH inhibition results in alterations in behavioral responses to psychostimulants. Here, we discuss the individual contributions of DA and NE to psychostimulant-induced behaviors in animal models, as well as the functional interactions between the dopaminergic and noradrenergic system that may underlie the altered responses to psychostimulants following DBH inhibition. The experiments described in this dissertation describe the changes in cocaine-induced behaviors following pharmacological and genetic DBH inhibition as well as examine the molecular and cellular consequences of this manipulation, which may underlie the behavioral effects on psychostimulant-induced behaviors.

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1.2 Cocaine addiction

The most potent stimulant of natural origin, cocaine, is also the most abused illicit stimulant in America. The Office of National Drug Control Policy estimates that 3.6 million Americans fit the criteria for chronic cocaine dependence. In 2009, approximately 11.3 percent of those seeking treatment for an addiction disorder in publicly-funded facilities did so for their addiction to cocaine. In the same year, out of almost one million visits to the emergency room involving an illicit drug, 422,896 of them involved problems associated with cocaine use (SAHMSA Treatment Episode Data). Given its abuse liability and the debilitating nature of cocaine addiction, research has been committed to understanding its mechanism of action, identifying the neurobiological underpinnings of its abuse, and developing pharmacotherapies for the treatment of addiction.

The mechanism of action of cocaine has been well-described. Cocaine binds to the monoaminergic transporters in neurons and prevents the reuptake of dopamine (DA), norepinephrine (NE) and serotonin (5-HT) into pre-synaptic neurons (Heikkila et al., 1975; Reith et al., 1986; Ritz et al., 1987), thus increasing the extracellular concentrations of each of these neurotransmitters (Jones et al., 1995; Stamford et al., 1989; Lee et al., 2001) (Figure 1.1). Specifically, the psychostimulant effects of cocaine are mediated primarily by its ability to enhance dopaminergic signaling in the brain (Roberts et al., 1977; Di Chiara and Imperato, 1988). In addition to its direct effects on monoaminergic neurotransmitters, cocaine also acts indirectly (i.e. via monoamines) to regulate glutamate

and GABAergic drive in mesolimbic circuits that drive addiction (Cameron and Williams, 1994; Wolf, 2010).

1.3 Catecholamines and cocaine addiction

1.3.1 Dopamine and psychostimulant-induced behaviors

DA is critical for the reinforcing effects of rewarding stimuli (Pifl et al., 1995; White and Kalivas, 1998; Spanagel and Weiss, 1999; Kelley and Berridge, 2002; Salamone and Correa, 2002). Specifically, dopaminergic transmission in the mesocorticolimbic DA pathway is implicated in the modulation of reward signals to both natural stimuli and drugs of abuse (as reviewed by Koob, 1996; Wise, 2000; Nestler, 2004). The mesocorticolimbic pathway is comprised of dopaminergic cell bodies in the ventral tegmental area (VTA) in the midbrain, which project to areas of the limbic system such as the ventral striatum (including the nucleus accumbens (NAc) and olfactory tubercle), the amygdala, the hippocampus and the prefrontal cortex (PFC) (Lindvall et al., 1974; Moore and Bloom, 1978; Lindvall et al., 1983) (Figure 1.2). Once DA is released from presynaptic terminals, in activates members of a family of G-protein-coupled dopamine receptors that are divided into two major groups, the D1 class and the D2 class (Andersen et al., 1990; Sibley and Monsma, 1992; Sokoloff et al., 1992; Civelli et al., 1993). This classification is based on their activation of different intracellular signaling cascades upon ligand binding. The D1-like class, comprised of D1 and D5 receptors, is coupled to $G\alpha_{s/olf}$ and agonist binding results in stimulation of adenyl cyclase (AC), which leads to the production and accumulation of cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA), which then phosphorylates several

target proteins. Among other effects, PKA modulates the release of extracellular calcium stores while inhibiting potassium currents, both which result in depolarization of the cell and in facilitation of transmission (Kitai and Surmeier, 1993; Missale et al., 1998; Gainetdinov et al., 2004; Beaulieu and Gainetdinov, 2011). D1-like receptors are primarily expressed postsynaptically on DA-receptive cells such as medium spiny neurons in the striatum, as well as in olfactory tubercle, hypothalamus, hippocampus, substantia nigra, and the limbic, premotor and entorrhinal cortices, where it is present mostly post-synaptically though not exclusively so (Dearry et al., 1990; Fremeau et al., 1991; Huntley et al., 1992; Rappaport et al., 1993; Choi et al., 1995).

D2-like receptors, comprised of D2, D3 and D4, have an effect opposite to that of D1-like receptors. D2-like receptors are coupled to $G\alpha_{i/o}$ and their activation leads to inhibition of AC and decreased production of cAMP. Binding of an agonist to these receptors decreases intracellular calcium and increases in outward potassium currents, leading to hyperpolarization of the cell and inhibition of neural transmission. D2-like receptors are expressed by DA neurons in the substantia nigra pars compacta and VTA, where they function as inhibitory autoreceptors that decrease DA neuron firing and DA release, as well as by DA target cells in the striatum, the prefrontal, cingulate, and entorrhinal cortices, the olfactory tubercle, amygdala, hippocampus (Bouthenet et al., 1991; O'Malley et al., 1992; Kitai and Surmeier, 1993; Levey et al., 1993; Hersch et al., 1995; Missale et al., 1998; Gainetdinov et al., 2004; Sokoloff et al., 2006; Rankin et al., 2010; Rondou et al., 2010; Beaulieu and Gainetdinov, 2011).

Given the robust expression of both types of DA receptors in areas of the mesocorticolimbic pathway and their opposing molecular and cellular effects, disruption

of DA transmission in this pathway using dopaminergic agents is a very useful tool for elucidating the specific role of DA signaling in behaviors elicited by psychostimulants. Next, I will discuss the role of DA transmission in cocaine-induced behaviors such as locomotion, behavioral sensitization, stereotypy, place conditioning and operant self-administration.

Dopamine and acute psychostimulant-induced locomotion

Most drugs of abuse, including cocaine, lead to acute locomotor hyperactivity in experimental animals as measured by horizontal ambulation and stereotypic behaviors. This motor-activating effect often correlates with an individual's accumbal DA response to the drug and their propensity to self-administer it (Piazza and Deminiere, 1989; Hooks et al., 1991 and 1992). In fact, systemic administration of a monoamine-depleting agent or a DA receptor antagonist prevents the acute psychomotor stimulant effects of cocaine (Van Rossum et al., 1962; Scheel-Kruger et al., 1977). As a result, the euphoric properties of cocaine are thought to be related to its ability to enhance dopaminergic neurotransmission. Because acute locomotor responses are a simple, robust, and reliable measure of the behavioral and neurochemical effects of a drug, and can be a predictor of its abuse liability, the role of DA transmission in cocaine-induced locomotion has been widely studied.

Locomotion in response to cocaine, as measured by horizontal ambulation, increases in a dose-dependent manner and peaks between 5-10 minutes following cocaine administration. Simultaneously, it has the neurochemical effect of increasing in vivo extracellular DA levels in the nucleus accumbens in an impulse-dependent manner (Di

Chiara and Imperato, 1988; Carboni et al., 1989; Broderick, 1991; Cass et al., 1992; Bradberry et al., 2000; Wu et al., 2001). Increases in extracellular DA in this area are thought to primarily underlie the reinforcing and locomotor-activating properties of cocaine, and are mediated by the D1 receptor. For example, a systemic injection of the D1 antagonist SCH233390, but not the D2 antagonist raclopride, dose-dependently inhibits cute locomotion to cocaine (Starr and Starr, 1989; Ushijima et al., 1995).

Dopamine and locomotor behavioral sensitization to psychostimulants

Repeated administration of a psychostimulant, such as cocaine, results in a progressive and long-lasting enhancement of the motor stimulant effects of the drug. This effect continues throughout the administration of the drug and persists following presentation of the drug after a period of abstinence. This phenomenon is known as behavioral sensitization (BS) (Robinson and Becker, 1986) and has been extensively characterized in rodent models. However, clinical data show that humans do not reliably experience increases in the behavioral effects of psychostimulants following their repeated use. Several studies have shown that both chronic users of psychostimulants and healthy controls chronically treated with repeated amphetamine fail to consistently describe an enhancement in their subjective reports of euphoria, energy or enhanced mood, nor do their speech or eye blink responses sensitize (Rothman et al., 1994; Strakowski et al., 1996, 2001; Gorelick and Rothman, 1997; Strakowski and Sax, 1998; Wachtel and deWit, 1999; Boileau et al., 2006). However, three human responses do increase with chronic psychostimulant abuse: anxiety, paranoia and psychosis (Ellinwood, 1968; Angrist and Gershon, 1970; Post, 1975), suggesting the existence of

sensitization to the aversive effects of psychostimulants. However, most often patients report a tolerance to the behavioral effects of these drugs (reviewed by Narendran and Martinez, 2008). In fact, following repeated administration of cocaine and amphetamine, the neural changes seen in rodents are profoundly different than those seen in humans. Given this contrast, there are limitations to using BS as an animal model of addiction. Thus, BS is now most often used as a measure of long-lasting drug-induced synaptic plasticity. Studying the activity-dependent molecular and cellular changes that underlie this long-term change in behavioral response contributes to understanding the neural underpinnings of addiction, as this disorder is thought to arise from drug-induced neuroadaptations in reward-related learning. Similar to acute locomotor responses, the neural substrates that mediate BS are also thought to overlap with those responsible for the rewarding effects of drugs of abuse (Wise and Bozarth, 1987).

Although the exact neurochemical mechanisms underlying BS to cocaine are not completely understood, the induction of this phenomenon arises, at least in part, from changes in dopaminergic neurotransmission along the mesocorticolimbic pathway, starting with changes in VTA neurons. DA neurons in the VTA possess somatodendritic D2 autoreceptors. Following repeated treatment with cocaine, these autoreceptors begin to desensitize, leading to increased tyrosine hydroxylase levels in the VTA, enhancement of neurotransmission in the terminal fields of these neurons, and an increase in DA signaling in these brain regions (see Figure 1.1)(reviewed by Wolf et al., 1998). The VTA is crucial for psychostimulant BS, as local amphetamine infusions in this area are sufficient to induce the behavioral response (Vezina, 1996), while local administration of the protein synthesis inhibitor anysomycin prevents sensitization (Sorg and Ulibarri,

1995). In order to better understand the role of DA in the VTA in BS, it is necessary to discuss the interplay between the DA and glutamate systems in this area. In addition to increasing monoamine levels, cocaine indirectly increases extracellular glutamate in the VTA, the striatum and the PFC via monoaminergic signaling (Kalivas and Duffy, 1995; Smith et al., 1995; Reid and Berger, 1996; Reid et al., 1997). Activation of both NMDA and D1-like receptors in the VTA are necessary for the development of BS to psychostimulants. For example, rodents pretreated with NMDA or D1 receptor antagonists in the VTA prior to systemic cocaine or amphetamine injections fail to show BS (Kalivas and Alesdatter, 1993; Vezina, 1996). Furthermore, NMDA antagonists are also known to prevent the development of some of the cellular neuroadaptations previously described during BS, such as D2 autoreceptor desensitization and the increase in VTA tyrosine hydroxylase levels.

The NAc is also a site of long-lasting adaptations following chronic cocaine. In this area, the increase in DA release is dependent on the time of withdrawal from the last injection. Increased, decreased and no changes in DA release have been reported following a seven day withdrawal period since the last injection (Izenwasser et al., 1990; Segal and Kuczenski, 1992a,b; Weiss et al., 1992; Kalivas and Duffy, 1993; Heidbreder et al., 1996). However, withdrawals longer than 14 days consistently increase DA transmission in this area and are correlated with the behavioral expression of sensitization (Kolta et al., 1985; Kalivas and Duffy, 1993; Hooks et al., 1994; Paulson and Robinson, 1995; Heidbreder et al., 1996). This increase in DA neurotransmission leads to enhanced D1 receptor sensitivity in the NAc (Henry and White, 1991; De Vries et al., 1998). Activation of post-synaptic D1 receptors in the NAc is also important for BS to cocaine,

as local delivery of a D1 antagonist prevents its expression. As a result of this prolonged activation of sensitized D1 receptors, several proteins downstream of the D1 signaling pathway are also altered following BS to psychostimulants. For example, production and activation of adenyl cyclase (AC), protein kinase A (PKA), extracellular-signal protein kinase (ERK) and delta FosB (ΔFosB) are all increased following repeated administration of cocaine (Terwilliger et al., 1991; Hope et al., 1994; Kim and Kim, 2008; Boudreau et al., 2009; Schumann and Yaka, 2009).

Dopamine and psychostimulant-induced stereotypy

In addition to increasing locomotor activity, psychostimulant administration may result in dose-dependent increases in stereotypy in rodents. Stereotypy consists of behaviors that are highly repetitive, purposeless, and compulsive and include intermittent rearing and sniffing, intense sniffing in one location, continuous circling/pivoting, and excessive grooming. They typically manifest following high doses of psychostimulants and their severity may be strain-dependent (Morse et al, 1993; Schlussman, 1998; Kelley, 2001). These behavioral patterns are also dependent on the time course of drug action, with stereotypy usually expressed immediately following administration of the psychostimulant, subsiding over time, and replaced with increased horizontal ambulatory activity (Randrup and Munkvad, 1974; Cheal et al., 1978; Kuczenski and Segal, 1999). These behaviors are accompanied by enhanced dopaminergic neurotransmission in the striatum. Infusion of DA agonists into the ventrolateral striatum is sufficient to elicit stereotypic behaviors and requires concurrent activation of D1 and D2 receptors in the

striatum, as pretreatment with either a D1 or a D2 antagonist dose-dependently antagonizes amphetamine-induced stereotypy. (Delfs and Kelley, 1990).

Dopamine and psychostimulant place conditioning

Place conditioning (PC) is a behavioral paradigm classically used to measure the positive, negative or neutral interoceptive properties of drugs. In this paradigm, animals are first exposed to a chamber with compartments that can be distinguished based on distinct sets of contextual cues. Several conditioning sessions are then performed in which the animal is injected with vehicle and restricted to one compartment, then injected with drug and restricted to the other compartment. Following conditioning, the animal is again allowed to explore both contexts in a drug-free state. An increase in time spent in the drug-paired context is interpreted as drug-associated reward, while avoidance of the drug-paired context is interpreted as a drug-associated aversion. Disruptions of PC can take place during the conditioning trials (thereby impairing the acquisition process) or immediately prior to the test (impairing the expression of the conditioned behavior).

DA neurotransmission is strongly implicated in the acquisition of PC. For example, DAT KO mice have impaired conditioned place preference to cocaine, as they are only able to form one at a very narrow range of doses (Medvedev et al., 2005). D1 receptors are necessary for the induction (acquisition) of cocaine PC as well as the extinction of cocaine-cue memories. D2 like receptors are not critical for cocaine PC acquisition or expression but are important for extinction (Cervo and Samanin, 1995; Chen and Xu, 2010; Fricks-Gleason et al., 2012). The role of DA in acquisition of PC speaks to its importance in appetitive learning and in assigning value to a reward. Its role

in PC extinction indicates its importance in context learning, even if the context is no longer rewarded.

The NAc is a region of great importance for this paradigm given its role in both context learning and modulation of behavioral responses to rewards. Because of its extensive and varied innervations from the PFC, hippocampus, thalamus, amygdala, and VTA (McGeer et al., 1977; Lindvall and Bjorklund, 1978; Walaas and Fonnum et al., 1979; Young and Bradford, 1986; Robinson and Beart, 1988), the NAc is a perfect position to recognize context and reward information and relay this to planning and motor regions. Infusions of a psychostimulant directly into the NAc can induce PC (Beninger et al., 2003), and D1-associated signaling proteins such as cAMP-response element binding protein (CREB) and dopamine-and-cAMP-regulated phosphoprotein (DARPP-32) are elevated in this region following PC training.

Dopamine and psychostimulant self-administration

The self-administration paradigm is widely used for assessing the reinforcing properties of a drug. In this task, subjects are trained to perform an operant task that is followed by an intravenous infusion of a drug and the presentation of a conditioned stimulus. There are four distinct stages to this paradigm. First is the acquisition of the operant behavior, when the animal learns to perform the operant task to receive drug. Next is the maintenance phase, when operant responses and drug-intake stabilize. After the subject reaches stable responding, the next step is extinction. During this phase, operant responding is not reinforced with a drug infusion, and the operant response diminishes to a very low level. The last phase, reinstatement, is a model for relapse in

human addicts. During this step, the operant behavior is restored by an external stimulus such as a drug priming injection, the presentation of a drug-associated cue or exposure to a stressor, despite the fact that operant responses still do not result in a drug infusion.

Since 1977, when it was first reported that lesions that reduce DA levels in terminals in the nucleus accumbens decrease cocaine self-administration in the maintenance phase (Roberts et al., 1977), the role of DA in cocaine self-administration has been widely studied. In rodents, a systemic injection of a DA antagonist prevents the acquisition of cocaine self administration if it is administered during the training phase of the procedure (Di Chiara et al., 1995). DA antagonists administered to rodents or humans after they have reached a stable level of responding initially increase response rate for amphetamine and cocaine, presumably because the subjects "experience" a lower dose of stimulant (Yokel and Wise, 1975, 1976; Woolverton, 1987; Di Chiara, 1995). DAT KO mice, which lack the DAT and thus do not respond to psychostimulants with an increase in DA, do not acquire cocaine self administration reliably (Thomsen et al., 2009). Similarly, pharmacological or genetic reduction in D1 receptor signaling decreases acquisition and maintenance of cocaine self-administration (Koob et al., 1987; Bergman et al., 1990; Caine et al., 2007). Conversely, pharmacological or genetic inhibition of D2 receptors results in increased rates of cocaine self-administration during maintenance, an effect likely mediated by heteroreceptor activation (Caine et al., 2002).

The NAc is heavily implicated in drug-seeking behavior. Lesions of DA terminals or cell bodies in the VTA disrupts self-administration of cocaine during the maintenance phase (Roberts and Koob, 1982; Pettit et al., 1984; Zito et al., 1985; Caine and Koob, 1994), while amphetamine, cocaine, DA, or a mixture of D1 and D2 agonists is directly

self-administered in the NAc (Hoebel et al., 1983; Carlezon et al., 1995; Ikemoto et al., 1997; McKinzie et al., 1999; Cornish and Kalivas, 2000).

Cortical DA is also important for the maintenance of lever pressing for cocaine. Rats will self administer cocaine directly into the medial PFC (Goeders and Smith, 1993; Carlezon et al., 1995) and dopaminergic lesions of this area decrease responding for cocaine (Goeders and Smith, 1986).

DA neurotransmission is also implicated in animal models of relapse (i.e. reinstatement of drug-seeking following protracted abstinence). Infusion of a D1 or a D2 agonist into the NAc is sufficient to reinstate drug seeking (Anderson et al., 2003, 2006), while infusion of a D1 or D2 antagonist blocks cocaine-primed reinstatement (e.g. Anderson et al., 2003). Cocaine, amphetamine, or DA infusions into the dorsal PFC reinstate cocaine seeking and can be blocked by D1 or D2 antagonists in the medial PFC (McFarland and Kalivas, 2001; Capriles et al., 2003; Sun and Rebec, 2005). Alone, inactivation of the dorsal PFC is sufficient to prevent cocaine-primed reinstatement (McFarland and Kalivas, 2001). DA transmission in the basolateral amygdala, a region of importance in conditioned incentive properties, is also implicated in reinstatement. Lesions of this region can block cue-induced reinstatement (Meil and See, 1997), while local application of D1 antagonists block cue- and cocaine-primed reinstatement (Norman et al., 1999; See et al., 2001). Finally, manipulation of proteins that are downstream of DA receptor activation can also affect reinstatement of drug-seeking.

1.3.2 Norepinephrine and psychostimulant-induced behaviors

The brain noradrenergic system consists of two ascending projections, the dorsal noradrenergic bundle and the ventral noradrenergic bundle. The dorsal projection arises from the locus coeruleus (LC) and projects to the hippocampus, cerebellum and forebrain. The ventral projection arises from nuclei in the pons and medulla, such as the A1 and A2 nuclei, and projects to the hypothalamus, midbrain and extended amygdala (reviewed by Moore and Bloom, 1979). NE is synthesized from DA by the enzyme dopamine β-hydroxylase (DBH). In noradrenergic neurons, DBH is present inside of synaptic vesicles that contain the vesicular monoamine transporter (VMAT). DA is taken up into the vesicles by the VMAT and within the vesicle DA is converted into NE. NE is also taken back up into the cell by NET, where it can be directly transported into vesicles. Once released from the terminal, NE activates three G-protein receptor families, its reuptake is modulated by NET, and it is metabolized by monoamine oxidase A and catechol-O-methyltransferase (Kopin, 1968; Anden et al., 1969; Carlsson, 1969; Weiner, 1970).

The three G-protein receptor families activated by NE are α_1, α_2, and β, each consisting of various subtypes (Bloom, 1979). In summary, α_1 and β receptors are primarily postsynaptic and α_2 receptors are both pre- and postsynaptic and function as autoreceptors. α_1 receptors are (α_{1A}, α_{1B} and α_{1D}) G_q-coupled and the activate phospholipase C (PLC)- inositol triphosphate (IP_3) and diacylglycerol (DAG) pathway, which result in an excitatory effect modulated by increases in intracellular calcium and decreased potassium conductance. They are present postsynaptically in the cerebral cortex, hippocampus, thalamus, striatum, raphe nucleus and the VTA. β receptors (β_1, β_2,

β_3) are coupled to G_s and stimulate the AC-cAMP-PKA cascade. They are also excitatory, promote repetitive discharge and facilitate LTP and are present in supratentorial structures, cerebellum and in astrocytes. α_2 (α_{2A}, α_{2B} and α_{2C}) receptors are $G_{i/o}$- coupled, inhibit AC, and result in inhibition of spontaneous firing and excitability and decreased neurotransmitter release (reviewed by Benarroch, 2009).

NE plays a very important role in arousal, emotional reactivity, memory, and stress, all which are processes involved in responses to drugs of abuse. In addition, cocaine and amphetamine are powerful NET blockers that increase extracellular NE levels. This convergence of neurobiological and biochemical data has led to the extensive study of the role of NE in behavioral responses to psychostimulants.

Norepinephrine and acute psychostimulant-induced locomotion

While psychostimulant-induced locomotion in rodents has been widely attributed to increases in dopaminergic transmission in the forebrain, recent studies suggest an important role of NE in this behavior. Noradrenergic denervation using DSP-4 lesions attenuates cocaine-induced locomotion in rats (Koiv et al., 2011), while selective activation and antagonism of each of the adrenergic receptor has revealed an important role of the α_1 receptor subtype. Systemically-administered α_1 antagonists inhibit acute locomotion to amphetamine and cocaine (Snoddy and Tessel, 1985; Dickinson et al., 1988; Berthold et al., 1992). Specifically, administration of an α_1 antagonist in the PFC decreases locomotion in response to an accumbal infusion of amphetamine (Blanc et al., 1994), while infusion of an α_1 antagonist in the NAc attenuates cocaine-induced locomotion (D. Mitrano, personal communication). Further studies suggest that the α_{1B}

receptor is responsible for the modulation of acute locomotion to psychostimulants. Pharmacological or genetic inhibition of α_{1B} receptors results in blunted cocaine- and amphetamine-induced locomotion (Darracq et al., 1998; Drouin et al., 2002a,b), while an α_{1A} receptor antagonist has no effect (Clifford et al., 2007). The individual roles of the α_2 and β receptor subtypes have not been thoroughly investigated, though both seem implicated in this behavior. Activation of α_2 receptors with administration of an agonist attenuates acute locomotion to cocaine in rats while an antagonist increases it (Jimenez-Rivera et al., 2006). Similarly, a β receptor antagonist increases cocaine-induced locomotion as well as increasing dopamine levels in the NAc (Harris et al., 1996).

Norepinephrine and locomotor behavioral sensitization to psychostimulants

Since NE was found to have a clear function in acute locomotion to psychostimulants, more recent studies have explored its role in the development of locomotor BS to this type of drugs. Similar to the acute drug effects described above, the α-adrenergic receptor subtypes seem to be responsible for this effect. α_1 receptor antagonists block the development and expression of BS to cocaine (Wellman et al., 2002; Jimenez-Rivera et al., 2006) and amphetamine (Vanderschuren et al., 2003). α_{1B} knockout mice have blunted behavioral sensitization to cocaine and amphetamine. While they do not differ from wild-type littermates in DA tissue levels, DA receptor number and DA reuptake sites, they do have impaired amphetamine-induced dopamine release (Drouin et al., 2002; Auclair et al., 2002). α_2 agonists inhibit BS to amphetamine (Vanderschuren et al., 2003), while an antagonist has no effect (Jimenez-Rivera, 2006). Only two studies have tested the effect of β antagonists on psychostimulant BS and their

results are conflicting. One group found that this manipulation enhanced locomotor BS to amphetamine (Vanderschuren et al., 2003) while another group found that systemic or intra-BNST administration of a β blocker prevented the initiation of locomotor BS to amphetamine (Colussi-Mas et al., 2005).

Norepinephrine and psychostimulant-induced stereotypy

Though stereotypic behaviors look drastically different from the horizontal ambulations described in the previous two sections, it is also a behavioral measure of motor activity and has similar neurological underpinnings, which include the involvement of NE. Unfortunately, there is a dearth of studies examining the specific effect of NE manipulations on stereotypic behaviors in response to psychostimulants. The first study, in 1978, found that ventral noradrenergic bundle lesions do not affect amphetamine-induced stereotypy (Jerlicz et al., 1978). Two other studies followed showing that amphetamine-induced stereotypy was unaffected by pretreatment with selective NE uptake inhibitors or NE depletion in the medial PFC (Carter and Pycock, 1980; Tyler and Tessel, 1980). Combined, these results suggested that the noradrenergic system may not be crucial for stereotypy. However, there is also evidence to the contrary.

Intra-ventricular NE inhibits licking and gnawing in response to amphetamine and this effect is mimicked by α_1 receptor agonists (Zebrowska-Lupina et al., 1975). Treatment with an α_2 antagonist, which enhances NE transmission by blocking inhibitory autoreceptor function, also decreases oral stereotypies. Conversely, dampening noradrenergic signaling with α_1 antagonists has the opposite effect, enhancing oral

stereotypic behaviors, such as gnawing, while decreasing excessive sniffing (Dickinson et al., 1988).

Norepinephrine and psychostimulant place conditioning

Similar to the role of DA, NE is also involved in processing motivationally-salient stimuli. For example, it modulates the reaction to reward-predicting stimuli; NE efflux in the medial PFC is increased during the presentation of a cue that was previously paired with a food reward (Mingote et al., 2004). The noradrenergic system is also engaged in processing aversive stimuli, as evidenced by a conditioned increase in NE following exposure to a foot-shock predicting stimulus (Feenstra et al., 1999; Dazzi et al., 2003) or to an aversive environmental stimulus (McQuade et al., 1999). Since administration of psychostimulants is a salient stimulus that can be both rewarding and aversive, depending on the dose or the context, the role of NE in psychostimulant-induced reward and aversion has been widely studied. The use of the place conditioning paradigm described earlier is ideal for testing the effect of genetic and pharmacological manipulations to the noradrenergic system on these parameters in response to psychostimulants. This procedure allows for the assessment of the acquisition of conditioned responses to the appetitive or aversive properties of stimuli paired with primary rewards and aversive events, providing a reliable measure of processes underlying the motivational salience attributed to these stimuli (Berridge and Robinson, 1998; Di Chiara et al., 2004)

Noradrenergic denervation via a DSP-4 lesion prevents place conditioning to cocaine (Koiv et al., 2011), while increasing noradrenergic transmission via NET knockout induces more pronounced preference for a cocaine-paired compartment (Xu et

al., 2000). The PFC seems to be crucial for this effect, as selective prefrontal NE depletion in mice is sufficient to abolish their conditioned place preference to a highly salient reward and conditioned place aversion to a highly salient aversive stimulus (Ventura et al., 2008). The same NE denervation procedure abolished amphetamine-induced place conditioning while reducing amphetamine-induced mesoaccumbens DA release (Ventura et al., 2003). Transmission via the α_1 receptor has been implicated in this effect, as mice lacking α_{1b} adrenergic receptors do not show a normal preference for cocaine solution (Drouin et al., 2002a), which is accompanied by a dampened effect of amphetamine on DA release in the NAc (Auclair et al., 2002). Combined, these studies highlight the importance of α_{1b} receptors in the PFC for place conditioning.

NE transmission in other brain regions is also important for psychostimulant place conditioning as well as signaling from other receptor subtypes such as the basolateral amygdala and β adrenergic receptors. Systemic administration of an α_1 or β2 antagonist following a place conditioning test attenuates the expression of cocaine place preference during a subsequent test, an effect mimicked by local administration of either antagonist directly into the basolateral amygdala (Bernardi et al., 2009). β adrenergic receptors are also involved in the reinstatement of place conditioning following extinction. A challenge injection of cocaine, exposure to a stressor or administration of the α_2 adrenergic receptor antagonist yohimbine are all are capable of reinstating previously-extinguished place conditioning to psychostimulants. Systemic administration of a β_2 but not a β_1 antagonist is able to block both yohimbine and stress-induced reinstatement of cocaine place conditioning. However, systemic injections of non-selective α_1, α_2 and β_1 adrenergic receptor antagonists do not prevent cocaine-induced reinstatement of cocaine place

conditioning. These findings suggest that β_2 and α_1 signaling in the basolateral amygdala are important for extinction of cocaine place conditioning and that stress-induced reinstatement of cocaine place preference requires signaling through β_2 adrenergic receptors.

Norepinephrine and psychostimulant self-administration

As previously discussed, DA is necessary for the acquisition and maintenance phase of psychostimulant self-administration. Conversely, NE signaling is dispensable for these two specific measures of drug reinforcement. For example, lesions of the dorsal and ventral noradrenergic bundles have little effect on acquisition and maintenance of cocaine self-administration (Roberts et al., 1977). Systemic administration of NE antagonists fails to have a task-specific effect on responding for cocaine or amphetamine (Yokel and Wise, 1975, 1976; Woolverton, 1987; Harris et al., 1996). Increased NE neurotransmission via administration of selective NET inhibitors does not alter psychostimulant self administration or do they support self administration themselves (Woolverton, 1987; Howell and Byrd, 1991; Skjoldager et al., 1993; Tella, 1995; Wee and Woolverton, 2004; Wee et al., 2006). To date, the only noradrenergic receptor agonist found to be self administered is clonidine (Shearman et al., 1981; Woolverton et al., 1982).While it is apparent that NE transmission is not necessary for the primary reinforcement effect of psychostimulants as assessed by acquisition and maintenance of self administration, it is in undoubtedly a critical mediator of reinstatement of drug seeking. In fact, NE is involved in modulating all three types of reinstatement: following

a drug-priming injection, the presentation of a drug-associated cue or exposure to a stressor (as reviewed by Weinshenker and Schroeder, 2007)

For example, increasing NE transmission by central injections of NE or systemic administration of yohimbine reinstates cocaine seeking (Lee et al., 2004, Brown et al., 2009, 2011). Conversely, blockade of NE synthesis using a DBH inhibitor or α_1 receptors using an antagonist attenuates drug-primed reinstatement of cocaine-seeking and a β-adrenergic receptor antagonist blocks stress-induced reinstatement of cocaine-seeking (Zhang and Kosten, 2005; Platt et al., 2007; Leri et al., 2002; Schroeder et al., 2010). Similarly, inhibiting NE activity by DBH inhibition or stimulating α_2 adrenergic autoreceptors attenuates stress- and cue-induced reinstatement of cocaine in rats (Erb et al., 2000; Highfield et al., 2001; Smith and Aston-Jones, 2011; J. Schroeder, personal communication).

1.4 Dopamine and norepinephrine interactions

As highlighted above, the individual contributions of the dopaminergic and noradrenergic systems to the expression of cocaine-induced behaviors are multifaceted. An additional layer of complexity lies in the interactions between both of these systems, which have a great degree of functional anatomical connections.

Noradrenergic cell groups in the brain, the locus coeruleus and the A1 and A2 brainstem nuclei, directly innervate regions of the dopaminergic mesocorticolimbic pathway such as the nucleus accumbens, VTA and PFC. These innervations provide excitatory drive to DA neurons and are therefore positive modulators of neuronal firing in the midbrain, an effect mediated by activation of α1 adrenergic receptors (reviewed by

Weinshenker and Schroeder, 2007). For example, the VTA receives noradrenergic projections from neurons from the locus coeruleus, A1 and A2 (Jones et al., 1977a,b; Simon et al., 1979; Liprando et al., 2004; Mejías-Aponte et al., 2009). Stimulating the LC leads to burst firing in DA-synthesizing neurons in the VTA, an effect that is blocked by local administration of α1 antagonists (Lategan et al., 1990; Grenhoff et al., 1993; Grenhoff and Svensson, 1993), while pharmacological stimulation of α1 receptors in the VTA increases these neurons' firing rates (Paladini and Williams, 2004).

Noradrenergic activation also has a facilitatory effect on dopamine neurons in the nucleus accumbens, as lesions of the locus coeruleus and the ascending noradrenergic bundles result in decreased neural activity and DA release in the NAc (Tassin et al., 1979; Russell et al., 1989; Lategan et al., 1990, 1992; Grenhoff et al., 1993). In turn, DA receptors enhance their sensitivity to compensate for the decreased level of neurotransmitter (Donaldson et al., 1976; Harro et al., 2000). The ventral noradrenergic bundle also has direct projections from the A1 and A2 nuclei to the accumbens (Berridge et al., 1997; Delfs et al., 1998; Tong et al., 2006).

The PFC receives dense direct projections from the LC (Swanson and Hartman, 1975; Morrison et al., 1981), and sends excitatory glutamatergic inputs to VTA DA neurons, potentially through a glutamatergic relay nucleus (Carr and Sesack, 2000a and b). This pathway is modulated by α_1 adrenergic receptors, as an α1 antagonist infused directly into the PFC blocks amphetamine-induced DA release in the NAc, presumably by inhibiting DA neuron firing in the VTA (Blanc et al., 1994; Darracq et al., 1998). Infusion of an α1 antagonist into the NAc also attenuates cocaine-induced DA release (P. Vezina, personal communication).

Given this extensive interplay between the DA and NE systems, it is likely that in addition to their individual contributions, their interactions may also modulate physiological and affective responses to psychostimulant drugs such as cocaine.

This dissertation will focus on the behavioral, molecular and cellular effects of cocaine that take place following inhibition of NE synthesis. The experiments discussed in the following chapters will confirm and extend the idea that NE plays an important role in the modulation of these effects to cocaine via the mesolimbic system, and will begin to identify the underlying neuroanatomical and molecular substrates.

1.5 DBH inhibition animal models

One way to study the interaction between the DA and NE systems is to use models in which the ratio of NE/DA is altered. Because the enzyme dopamine β-hydroxylase (DBH) converts DA to NE in noradrenergic neurons, genetic or pharmacological DBH inhibition leads to decreased synthesis of NE and intracellular accumulation of DA in the brain (Musacchio et al., 1966; Goldstein, 1966; Thomas et al., 1995, 1998; Bourdelat-Parks et al., 2005; Schroeder et al., 2010). In order to study the consequences of NE and DA manipulation on the behavioral and molecular effects of cocaine, the experiments described in this dissertation were performed using one or more of the following animal models of DBH inhibition.

The genetic approach involves use of DBH knockout (*Dbh* -/-) mice, which have a total and lifelong DBH inhibition and lack NE in their central and peripheral nervous systems from the time of birth. Because NE is essential for mouse fetal development, survival of homozygous embryos is dependent on exogenous administration of a NE

precursor that can be converted into NE by an enzyme other than DBH. This compound, dihydroxyphenylserine (DOPS), is given in the drinking water to pregnant dams until the day they give birth. Once the dams give birth, *Dbh -/-* mice are able to survive to adulthood in the absence of NE (Thomas et al., 1995, 1998).

While brain tissue levels of DA are elevated in these animals, the loss of noradrenergic drive onto dopaminergic neurons results in low basal extracellular DA levels as well as reduced psychostimulant-induced DA release in the striatum and PFC. Paradoxically, they show a behavioral hypersensitivity to psychostimulants as evidenced by their heightened behavioral activation in response to amphetamine, cocaine, and the D2 agonist quinpirole (Weinshenker et al., 2002; Schank et al., 2006). *Dbh -/-* mice also show altered responses to cocaine PC. Cocaine is able to induce a place preference in *Dbh -/-* mice at a low dose that does not support PC in control mice, while a higher dose of cocaine that does induce a place preference in control mice elicits a conditioned place aversion in *Dbh -/-* mice (Schank et al., 2006). These phenotypes suggest that chronic NE deficiency enhances the rewarding and aversive interoceptive effects of cocaine via increased D2 signaling, and make these mice a useful tool for the study of the role of catecholamines in cocaine-induced behaviors and the underlying downstream changes.

To complement this genetic inhibition model, two models of pharmacological DBH inhibition are used in the experiments in this dissertation. The compound nepicastat (NEPI) is a direct, competitive inhibitor of DBH with an IC_{50} of 9nM and is selective for DBH (Stanley et al., 1997). NEPI administration blocks cocaine-primed reinstatement of cocaine seeking in rats, but does not affect the maintenance phase of self-administration or food responding (Schroeder et al., 2010).

Disulfiram, also known as Antabuse, is the second DBH inhibitor used in these studies. The primary metabolite of disulfiram, diethyldithiocarbamate, is a copper chelator (Hald et al., 1952; Johnston, 1953), and disulfiram administration impairs the activity of enzymes that contain copper or require it as a cofactor. Because DBH is a copper-containing monooxygenase, disulfiram administration reduces NE production and increases DA intracellular levels in rodents and humans (Goldstein, 1966; Musacchio et al., 1966; Bourdelat-Parks et al., 2005, Schroeder et al., 2010). However, its utility as a DBH inhibitor is limited due to its lack of specificity. In fact, disulfiram impairs the function of enzymes such as aldehyde dehydrogenase, carboxylesterases and cholinesterases, which are involved in cocaine and catecholamine metabolism. Despite these limitations, it is of great interest to study the effects of disulfiram because it increases cocaine-induced paranoia and has shown promise for the treatment of cocaine dependence in humans (Hameedi et al., 1995; George et al., 2000; Petrakis et al., 2000; Carroll et al., 2004; R. Malison, personal communication). Comparing the effect of disulfiram against selective pharmacological and genetic DBH inhibition models allows us to test whether disulfiram's effect on cocaine-induced behaviors is due to its capacity to inhibit DBH activity.

1.6 Experimental design and rationale

The focus of this dissertation is to expand on the behavioral, molecular and cellular consequences of DBH inhibition, as they pertain to cocaine-induced behaviors. Genetic inhibition is modeled by *Dbh -/-* mice, while pharmacological inhibition is achieved by the administration of NEPI and disulfiram.

We assessed cocaine sensitization in control and *Dbh* -/- mice, with or without NEPI pretreatment. We predicted that pharmacological or genetic DBH inhibition would similarly confer behavioral hypersensitivity to cocaine sensitization (as measured by locomotor activity and stereotypy), and that NEPI is specific to DBH, and thus would have no effect in *Dbh* -/- mice. If both pharmacological and genetic DBH inhibition, in fact, enhance behavioral sensitivity to cocaine, then vehicle-treated *Dbh* -/- mice and NEPI-treated control mice should show an exacerbation of the behavioral effects of cocaine, compared to control mice. Furthermore, if pharmacological DBH inhibition (NEPI-treated control mice) mimics the effect of genetic DBH inhibition (vehicle-treated *Dbh-/-* mice), then the altered responses to psychostimulants in *Dbh* -/- mice can be attributed to a lack of DBH at the time of the test, as opposed to a developmental compensatory mechanism. Finally, if *Dbh* -/- mice are unaffected by NEPI treatment, then we can conclude that NEPI is specific in its effect, as *Dbh* -/- mice have no DBH enzyme for NEPI to inhibit.

In addition to their behavioral phenotype to psychostimulants, *Dbh* -/- mice are hypersensitive to a D2 agonist but insensitive to a D1 agonist (Weinshenker et al., 2002). Because *Dbh* -/- mice have normal D2 receptor densities (as measured by PET imaging and radioligand binding; Skinbjerg et al., 2010), changes in D2 receptor expression/distribution probably cannot account for these behavioral phenotypes. However, *Dbh* -/- mice do show increased striatal expression of two proteins involved in dopaminergic signaling, pERK and ΔfosB (Rommelfanger et al., 2007). We therefore sought to compare the levels of other proteins involved in DA receptor signaling in the NAc, CP and PFC of *Dbh* -/- and control mice by western blot analysis. Since *Dbh* -/-

mice have enhanced responses to dopaminergic compounds, particularly those that act on the D2 receptor, we hypothesized that levels of D2-related signaling proteins would be altered in these animals. Furthermore, any protein changes found would be confirmed in NEPI-treated control mice.

In our protein screening, we found that the protein β-arrestin2, a protein widely implicated in desensitization of the D2 receptor, was downregulated in the NAc of *Dbh -/-* mice and NEPI-treated control mice. Because receptor desensitization is linked to the expression of behavioral sensitization to psychostimulants (Nestler and Aghajanian, 1997; Nestler, 2001a, b), we decided to explore whether manipulation of β-arrestin2 levels alone can alter cocaine responses. We used viral constructs to overexpress β-arrestin2 levels in the NAc of *Dbh -/-* mice and knock down its expression in the NAc of control mice. If differential expression of β-arrestin2 is the primary molecular underpinning of locomotor behavioral sensitization to cocaine following DBH inhibition, then overexpression of β-arrestin2 in *Dbh -/-* mice should decrease their behavioral response to cocaine, while β-arrestin2 knockdown in control mice should phenocopy *Dbh -/-* mice and increase their behavioral response to cocaine.

In order to further elucidate the cellular mechanism underlying the behavioral sensitivity to D2 activation in *Dbh -/-* mice, we collaborated with Brandon Goertz and Carlos Paladini at UT-San Antonio, who used whole-cell electrophysiology to record from vehicle- or quinpirole-treated medium spiny neurons from the NAc core of *Dbh -/-*. The D2 receptor is a G_i-coupled receptor, and administration of an agonist like quinpirole typically results in suppression of neuronal firing. We hypothesized that because *Dbh -/-*

mice show behavioral hypersensitivity to D2 agonist, then their electrophysiological response to quinpirole would be similarly exacerbated.

The final set of experiments focused on the behavioral and molecular changes following disulfiram administration in control and *Dbh -/-* mice. There are currently no FDA-approved pharmacotherapies for the treatment of cocaine addiction. Disulfiram has become a promising alternative, but the mechanism of action underlying its clinical efficacy in decreasing cocaine intake in cocaine addicts is not well-understood. Because its primary metabolite is a copper chelator, disulfiram affects the activity of myriad enzymes that require copper as a co-factor. DBH is one such enzyme copper and its activity is decreased following disulfiram treatment. We suspect that DBH inhibition underlies the clinical efficacy of disulfiram because 1) DBH inhibition results in heightened responses to cocaine in mice, 2) low DBH levels in humans correlate with increased cocaine-induced paranoia and 3) disulfiram treatment enhances self-reports of anxiety, paranoia, and psychosis following cocaine administration in patients. To test these ideas, we administered disulfiram to *Dbh -/-* and control mice and assessed cocaine sensitization. We predicted that disulfiram pretreatment would increase behavioral responses to cocaine in control mice as measured by horizontal ambulations and/or the emergence of stereotypic behaviors, while having no effect in *Dbh -/-* mice.

Combined, the series of experiments outlined in this dissertation accomplishes the following objectives: 1) determines the influence of DBH inhibition on cocaine-induced behaviors, 2) provides a candidate molecular/cellular mechanism underlying these effects, and 3) suggests a mechanism of action for the ability of disulfiram to reduce

cocaine use in the clinic, and 4) validates NEPI as a more potent and selective DBH inhibitor for the treatment of cocaine dependence.

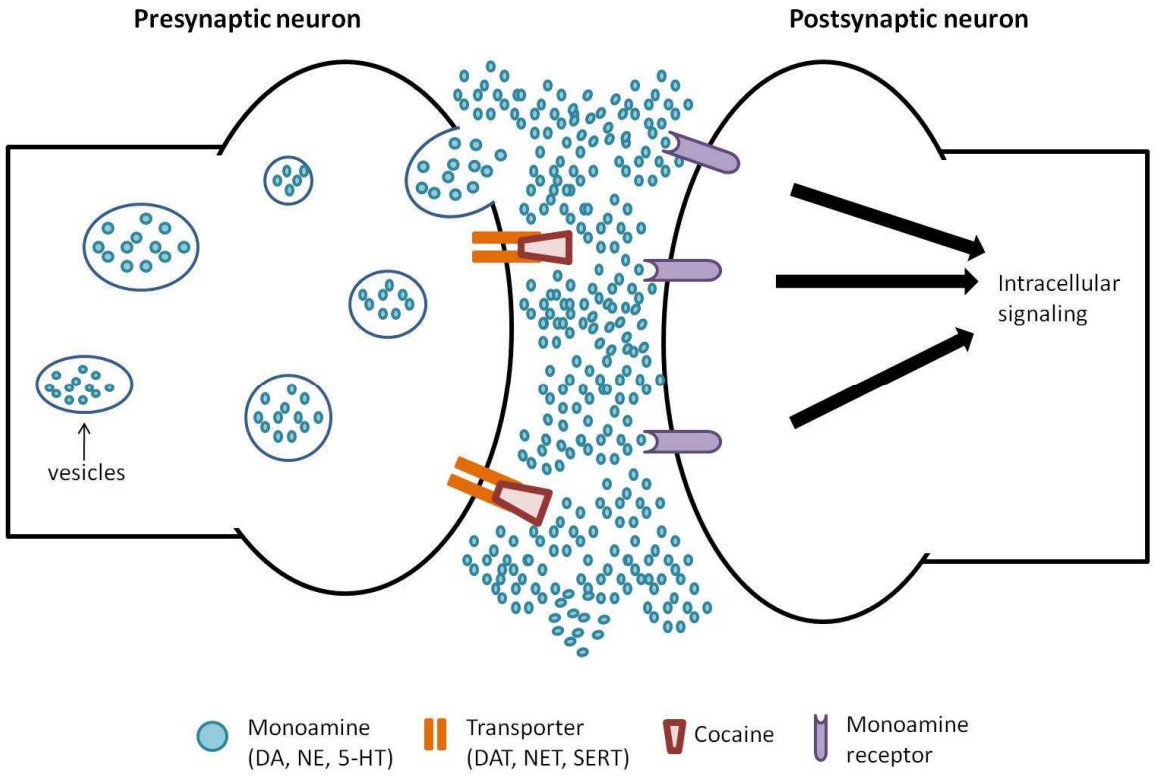


Figure 1.1 Mechanism of action of cocaine. Cocaine binds to and blocks monoamine transporters, preventing reuptake of DA, NE, and 5-HT from the synaptic cleft by presynaptic neurons. This leads to an increase in extracellular monoamine levels and results in an enhanced and prolonged postsynaptic effect of monoaminergic signaling.

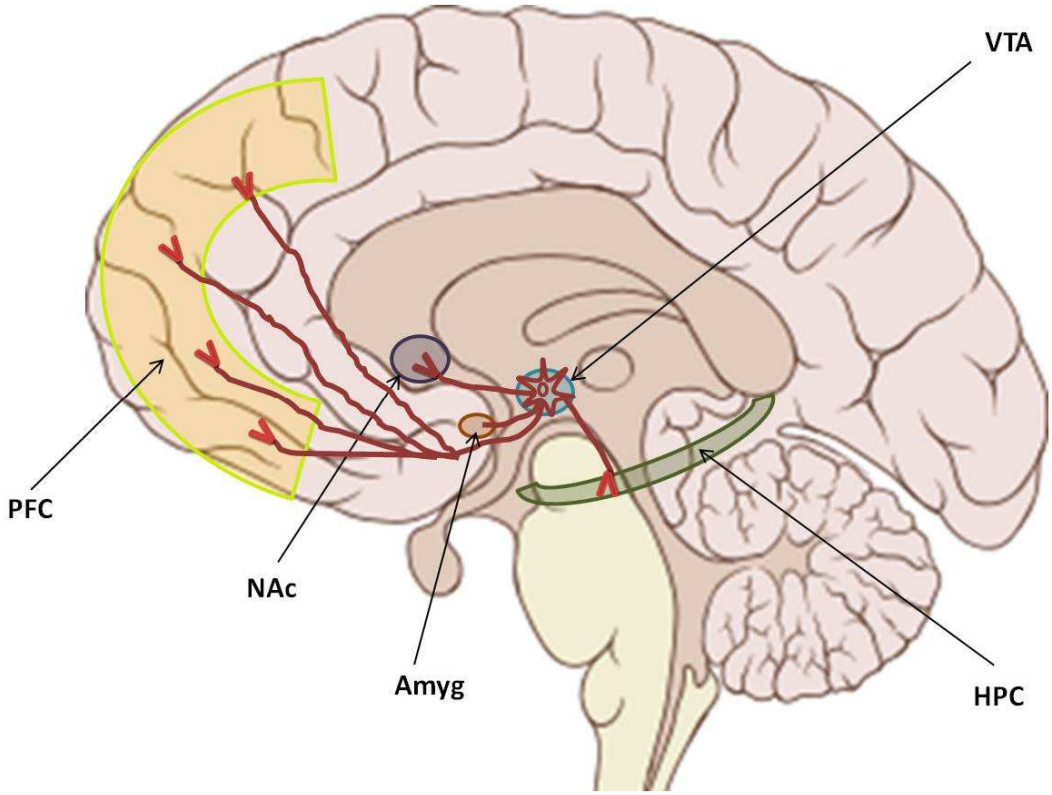


Figure 1.2 Mesocorticolimbic dopamine pathway. (PFC= prefrontal cortex; NAc= nucleus accumbens; Amyg= amygdala; VTA= ventral tegmental area; HPC= hippocampus)

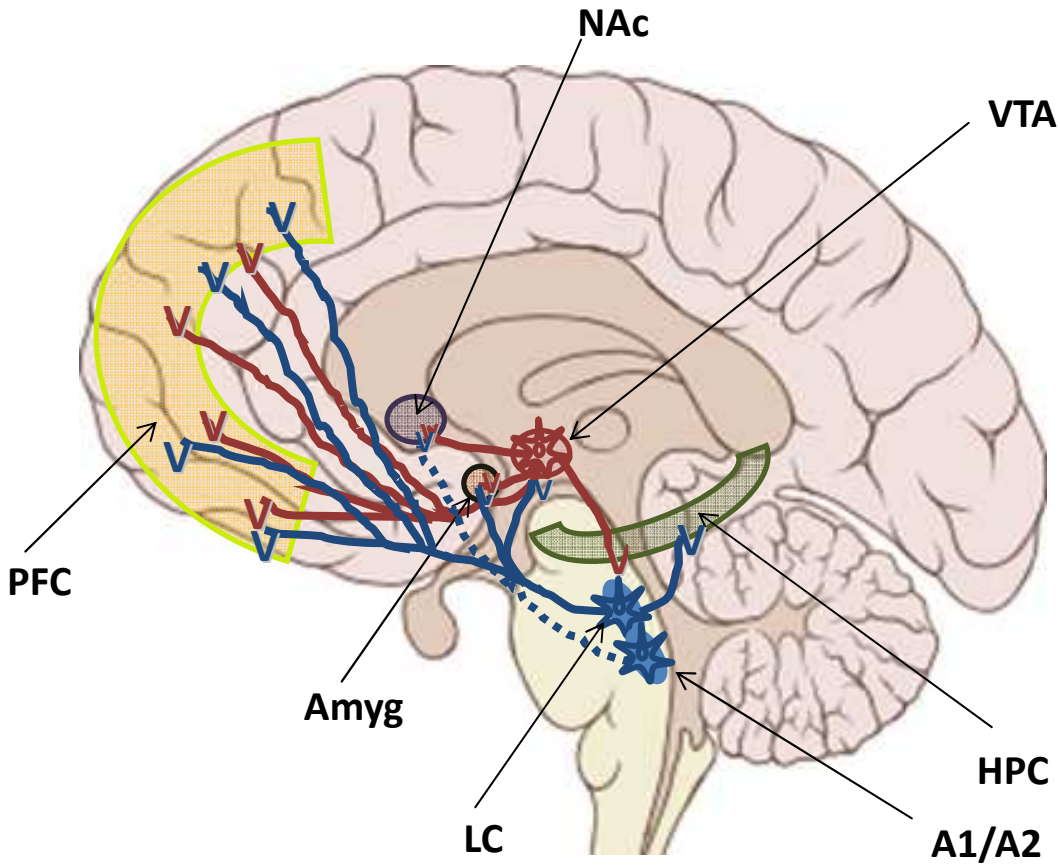


Figure 1.3 Noradrenergic projections onto the mesocorticolimbic dopamine pathway. (PFC= prefrontal cortex; NAc= nucleus accumbens; Amyg= amygdala; VTA= ventral tegmental area; HPC= hippocampus; LC= locus coeruleus)

CHAPTER II:

CHRONIC GENETIC OR PHARMACOLOGICAL REDUCTION OF NORADRENERGIC TONE LEADS TO BEHAVIORAL HYPERSENSITIVITY TO COCAINE

2.1 Abstract

Dopamine β–hydroxylase (DBH) is the enzyme responsible for the conversion of dopamine (DA) into norepinephrine (NE) in noradrenergic neurons. DBH knockout (*Dbh -/-*) mice completely lack NE from birth and show neurochemical and behavioral characteristics reminiscent of wild-type animals that have undergone psychostimulant sensitization, such as locomotor hypersensitivity to cocaine and D2 agonists. To determine whether chronic pharmacological DBH inhibition in an adult animal would recapitulate the knockout phenotype, we tested the effects nepicastat, a selective DBH inhibitor, on cocaine-, D1 agonist-, and D2 agonist-induced locomotion and stereotypic behaviors in adult control and *Dbh -/-* mice. While untreated *Dbh -/-* mice were hypersensitive to cocaine and were unaffected by nepicastat pretreatment, control mice treated chronically with nepicastat showed increased behavioral hypersensitivity to cocaine and a D2, but not a D1, agonist. These results indicate that the effects of nepicastat are mediated solely by DBH inhibition, and that selective genetic or pharmacological DBH inhibition enhances behavioral responses to cocaine, an effect likely mediated by alterations in D2 receptor signaling.

2.2 Introduction

Brainstem noradrenergic neurons project directly and indirectly onto midbrain DA neurons, regulating their firing patterns and thereby modulating DA release (Swanson and Hartman, 1975; Jones and Moore, 1977a and b; Grenhoff et al., 1993; Grenhoff and Svensson, 1993; Darracq et al., 1998; Ventura et al, 2003; Liprando et al., 2004). Dopamine β-hydroxylase (DBH) converts DA to NE in noradrenergic neurons, thus and controlling the NE/DA ratio. Because many drugs of abuse exert their effects through DA and NE, manipulations that alter the relative levels or activity of DBH are expected to have an effect on drug-induced behaviors, particularly psychostimulants. In addition, altering DBH function can serve as a tool to study the role of NE in these responses.

DBH inhibition, either genetic or pharmacologic, affects responses to psychostimulants in humans and animal models of addiction. Human addicts with a single-base polymorphism in the *Dbh* gene that confers low DBH activity report increased levels of cocaine-induced paranoia (Cubells et al., 2000). The DBH inhibitor disulfiram affects self-reported ratings of "high", anxiety, nervousness, paranoia, craving, and dysphoria following psychostimulant administration and has shown promise as a treatment for cocaine dependence in clinical settings (Hameedi et al., 1995; McCance-Katz et al., 1998a, b; Carroll et al., 1998, 2004; George et al., 2000; Petrakis et al., 2000; Baker et al, 2007; Kalayasiri, 2007; Sofuoglu et al., 2008; Mutschler et al., 2009). In rat models of relapse, disulfiram blocks cocaine-, yohimbine-, and cue-primed reinstatement of cocaine seeking (Schroeder et al., 2010; J. Schroeder, personal communication). *Dbh* -/- mice that lack NE completely also show altered responses to psychostimulants such as

behavioral hypersensitivity to amphetamine, cocaine, and a D2 agonist, and insensitivity to a D1 agonist (Weinshenker et al., 2002; Schank et al., 2006).

While disulfiram seems promising as a potential pharmacotherapy for the treatment of cocaine addiction, the mechanism of action underlying its clinical effect is not completely understood. Because its primary metabolite, diethyldithiocarbamate, is a copper chelator, disulfiram affects many enzymes other than DBH that also require copper as a cofactor, leading to a variety of unpleasant side effects and liver toxicity. Furthermore, the potency for disulfiram inhibition of DBH is relatively weak (low μM range). A drug that is more potent and selective for DBH would be a superior alternative for the treatment of psychostimulant addiction. Nepicastat (NEPI) is a selective, direct, competitive inhibitor of DBH that does not chelate copper and has 100-fold greater potency than disulfiram (IC50 of 9nM for NEPI, 1μM for disulfiram; Goldstein, 1966; Stanley et al., 1997). Because of its specificity and potency, NEPI is a potential treatment for psychostimulant addiction.

In this series of studies, we tested the effect of NEPI administration on drug-induced behaviors in control and *Dbh* -/- mice. We used a locomotor behavioral sensitization paradigm because it measures both the acute psychomotor effects of cocaine and the development of sensitization with repeated administration of the drug. In addition to increasing locomotor activity as measured by horizontal ambulations, high doses of cocaine administration result in stereotypy in mice (Schlussman, 1998). Stereotypic behaviors are intense, repetitive, persistent movements induced by high doses of psychostimulants that are accompanied by enhanced dopaminergic neurotransmission (Kelley, 2001) and require concurrent activation of D1 and D2 receptors in the striatum

(Delfs and Kelley, 1990). Given the enhanced behavioral responses of *Dbh -/-* mice to amphetamine and cocaine (Weinshenker et al., 2002; Schank et al., 2006), we predicted that chronic pharmacological DBH inhibition with NEPI pretreatment would increase behavioral responses to cocaine in control mice, while *Dbh -/-* mice would remain unaffected because they lack the primary NEPI target (DBH). Because *Dbh -/-* mice have increased D2 agonist-induced locomotion but are relatively insensitive to a D1 agonist, we also tested the effect of NEPI-pretreatment on D1- and D2-induced locomotor behavior, with the expectation that pharmacological DBH inhibition would phenocopy genetic DBH inhibition.

2.3 Materials and methods

Animals

Adult male and female control (*Dbh* +/−) and *Dbh* −/− mice (3-8 months) were group housed and food and water were available ad libitum throughout the course of the study. Because there were no detectable gender differences, data from male and female mice were combined. *Dbh -/-* mice were generated as described (Thomas et al., 1998) and maintained on a mixed C57Bl6/J and 129SvEv background. *Dbh* +/− mice were used as control mice because they have normal brain catecholamine levels and are behaviorally identical to wild-type (*Dbh* +/+) mice (Thomas et al., 1995; Thomas et al., 1998; Bourdelat-Parks, 2005).

All animals were treated in accordance with the National Institutes of Health (NIH) Intramural Animal Care and Use Program guidelines. The experiments described

in this article followed the Emory University Division of Animal Resources' Guide for the Care and Use of Laboratory Animals and were approved by the Emory Institutional Animal Care and Use Committee.

Quantification of norepinephrine levels

Mice were injected with NEPI (50 mg/kg, i.p.) or saline (1 ml/kg, i.p.) three times, each injection two hours apart. Two hours after the last injection, mice were euthanized by CO_2 asphyxiation, brains were removed, and the prefrontal cortex was dissected on ice and frozen. NE levels were determined using HPLC followed by coulometric detection. NE concentrations were normalized to wet tissue weight for each sample.

Analytical samples of saline and NEPI-treated mice were prepared by adding 70 µL of ice-cold 0.1 N perchloric acid and 0.04% sodium metabisulfite to the tissue, and then sonicating until completely homogenized. Samples were centrifuged at 15 rpm x 1000 for 10 min at 4□C. This supernatant was injected at a constant flow rate of 1 mL/min onto an Ultrasphere ODS 250 × 4.6 mm column, 5 µm (Beckman Coulter, Fullerton, CA, USA) with mobile phase (0.1 mM EDTA; 0.35mM sodium octyl sulfate; 0.6% phosphoric acid; 5% acetonitrile (pH 2.7)). A coulometric electrochemical array detector (Agilent Technologies; guard cell set at 600 mV and analytical cell at 300 mV) was used to visualize the peaks. The retention time, height, and area of NE peaks were compared with reference standard solutions (Sigma) and quantified by ChemStation chromatography software (Agilent Technologies).

Nepicastat pretreatment

Dbh +/- and -/- mice (n=4-13 per treatment and genotype group) were given injections of saline (1 ml/kg, i.p.) four times a day, each injection spaced two hours apart, for five days prior to the pretest day in order to habituate them to the total volume of the injections. On the sixth day, all mice were placed in locomotion (LM) recording chambers and allowed to habituate for 30 min before receiving a single injection of cocaine (15 mg/kg, i.p.), and their LM recorded for an additional two hours (Pretest day). LM was recorded as consecutive beam breaks in transparent plexiglass cages placed into a rack with 7 infrared photobeams spaced 5cm apart (San Diego Instruments Inc., La Jolla, CA). Mice were then assigned to treatment groups to balance cocaine-induced LM scores within each genotype (*Dbh -/-* mice are hypersensitive to cocaine, and thus LM could not be balanced between genotypes Schank et al., 2006; this study). Treatment groups consisted of daily pre-treatment with saline or NEPI (50 mg/kg) three times a day, each injection two hr apart, followed by saline or cocaine (15 mg/kg) treatment 2 hr after the last pretreatment injection. Thirty min before the treatment (saline or cocaine) injection, mice were placed in the LM chambers and activity was recorded for a total of two and a half hours. This treatment was repeated daily over the course of five days. The appearance of stereotypic behaviors in response to cocaine was noted qualitatively during these five days, but was only quantified on challenge day (see Figure 2.1 and below).

Cocaine challenge

Ten days after the last treatment injection, all mice were again placed in the LM chambers for 30 min, were given a cocaine injection (15 mg/kg, i.p.) and their activity

was recorded for an additional 2 hr ("Challenge day"). On this day, stereotypy was scored for 5 minutes, 5-20 minutes following the cocaine injection. Stereotypic behaviors were defined as circling, head-bobbing, nail biting and sniffing.

D1 and D2 agonist challenge

The next two days after the last treatment injection, all mice were again placed in the LM chambers for 30 min. Mice then received an injection of the D2 agonist quinpirole (2.5 mg/kg, i.p.) or the D1 agonist SKF81297 (5 mg/kg, i.p.), and their LM was recorded for an additional two hours. The order of quinpirole and SKF81297 administration was counterbalanced between the two days.

2.4 Results

Nepicastat inhibits DBH and decreases brain norepinephrine levels

DBH is the enzyme in the catecholamine biosynthetic pathway that converts DA to NE in noradrenergic neurons. To confirm that systemic NEPI administration results in DBH inhibition in the mouse brain, we measured NE in the prefrontal cortex following administration of saline or NEPI (50 mg/kg, i.p. x 3). We chose the prefrontal cortex because it contains moderately high levels of NE and is important for several drug-induced behaviors. This dosing regimen of NEPI decreased NE in the prefrontal cortex by approximately 75% (t_{23}=9.385; p=0.0001) (Figure 2.2).

Dbh -/- mice are hypersensitive to cocaine

As expected (Schank et al., 2006), *Dbh -/-* mice were hypersensitive to cocaine-induced LM (ambulations on Day 1 during the 2 hr test (repeated measures ANOVA $F_{(11,154)}= 6.831$, $p<0.0001$) (Figure 2.3A). As previously reported (Schuster et al, 1977), repeated cocaine administration in control mice led to an enhanced behavioral response over time that persisted following a ten-day period of abstinence (Figure 2.3B).

Nepicastat enhances behavioral sensitivity to cocaine in control mice

We used the selective DBH inhibitor NEPI to determine whether chronic pharmacological DBH inhibition in adult control mice would mimic the hypersensitivity to cocaine observed in *Dbh -/-* mice. NEPI pretreatment in control mice tended to decrease cocaine-induced LM compared to their saline-pretreated counterparts, but it did not reach significance due to high intra-group variability (Figure 2.4). Two-way repeated measures ANOVA revealed a significant effect of time ($F_{(5,130)=}6.38$; $p<0.0001$). Concurrent with this pronounced decrease in LM, NEPI pretreatment, either with or without daily pairing with cocaine, caused a dramatic increase in the incidence of stereotypic behaviors following cocaine on challenge day. All 18 NEPI-pretreated mice engaged in stereotypic behaviors, compared with only 1/18 saline-pretreated mice ($\chi^2=26.44$, $p<0.0001$), and all of these mice spent most of the 5-min scoring session engaged in stereotypy (Figure 2.5 and data not shown). Although the enhanced cocaine-induced LM seen in *Dbh -/-* mice and the enhanced cocaine-induced stereotypy seen in NEPI-treated control mice are qualitatively different behaviors, both represent increased sensitivity to the behavioral effects of cocaine. As expected, *Dbh -/-* mice were

unaffected by NEPI pretreatment, indicating that the effects of NEPI are likely mediated solely by DBH inhibition (Figure 2.6).

Effect of pharmacological DBH inhibition in response to a D1 and D2 agonist

We used the D2 agonist quinpirole and the D1 agonist SKF81297 to determine whether chronic pharmacological DBH inhibition in adult control mice would mimic the hypersensitivity to D2 agonists observed in *Dbh -/-* mice (Weinshenker et al., 2002). Combined NEPI + cocaine increased LM in response to quinpirole (2.5 mg/kg, i.p.; one way ANOVA, $F_{(2,21)}=4.189$; $p<0.05$), but had no effect on SKF81297-induced (5 mg/kg, i.p.) LM (one way ANOVA, $F_{(2,21)}=0.3844$; $p=0.6855$) (Figure 2.7). These results indicate that either chronic genetic or pharmacological DBH inhibition leads to alterations in the dopaminergic system, particularly affecting D2 signaling.

2.5 Discussion

Chronic DBH deficiency enhances cocaine locomotion

Our results confirm our previous report indicating that *Dbh -/-* mice are hypersensitive to cocaine-induced LM (Schank et al., 2006). We extended our analysis to show that daily pretreatment of control mice with the selective DBH inhibitor NEPI decreases cocaine-induced locomotion as measured by horizontal ambulations, but concomitantly increases stereotypy. This pattern or behavior (decreased LM and increased stereotypy) is typically observed with very high doses of cocaine, suggesting that chronic pharmacological DBH inhibition also leads to increased sensitivity to the

psychomotor effects of cocaine. Importantly, NEPI had no effect in *Dbh -/-* mice, indicating that its effects in control mice are mediated solely by DBH inhibition. Interestingly, *Dbh -/-* mice fail to show stereotypic behaviors following administration of this dose of cocaine, although they do show stereotypy in response to amphetamine (Weinshenker et al., 2002; Schank et al., 2006). We speculate that this qualitative difference in cocaine hypersensitivity between vehicle-pretreated *Dbh -/-* mice and NEPI-pretreated control mice is due to compensatory effects that result from a lifetime of complete DBH inhibition (*Dbh -/-*) compared with partial, 5-day DBH inhibition (NEPI-pretreated *Dbh +/-* mice). It is likely that a higher dose of cocaine would elicit stereotypical behaviors in *Dbh -/-* mice.

Pharmacological DBH inhibition confers hypersensitivity to quinpirole

Because changes in DA receptor signaling can alter responses to cocaine, and *Dbh -/-* mice are hypersensitive to a D2 agonist but insensitive to a D1 agonist, we tested the effect of the D2 agonist quinpirole and the D1 agonist SKF81297 following NEPI-cocaine treatment in control mice. Similar to the effect seen in *Dbh -/-* mice, NEPI-cocaine pretreatment significantly enhanced quinpirole-, but not SKF81297-, induced LM. These results indicate that sub-chronic NEPI + cocaine is sufficient to elicit D2 signaling hypersensitivity.

DBH inhibition and mesocorticolimbic dopamine transmission

Genetic or pharmacological DBH inhibition leads to decreased NE synthesis while increasing levels of DA in noradrenergic neurons. However, because noradrenergic

drive is necessary for efficient midbrain DA neuron burst firing (reviewed by Weinshenker and Schroeder, 2007), *Dbh -/-* mice and control mice treated with a DBH inhibitor have significantly reduced basal and stimulant-induced extracellular DA levels in the striatum (Schank et al., 2006; Weinshenker et al., 2008). This profound reduction in striatal DA availability likely results in compensatory upregulation of some aspect of postsynaptic DA receptor transmission, such as receptor number, availability or downstream signaling molecules. Although we originally reported that *Dbh -/-* mice have increased high-affinity state D2 receptors in the striatum (Schank et al., 2006), subsequent analysis failed to confirm this finding in vitro or in vivo (Skinbjerg et al., 2010). Thus, it seems likely that altered downstream DA receptor signaling underlies the observed hypersensitivity to stimulants and D2 agonists. DA signaling in the CP is thought to underlie stereotypic behaviors (Kelley et al., 2001; Makanjuola and Ashcroft, 1982), increases in extracellular DA in the NAc mediate the locomotor-activating properties of cocaine (Di Chiara and Imperato, 1988; Carboni et al., 1989; Broderick, 1991; Cass et al., 1992), and NE transmission in the PFC via α_1 adrenergic receptors is necessary for amphetamine-induced locomotion (Blanc et al., 1994). Therefore, these are three candidate brain regions for testing changes to DA receptor signaling following chronic DBH.

DBH and psychostimulant addiction

The increased sensitivity to cocaine conferred by DBH inhibition may seem counterintuitive when thinking about potential treatments for addiction. However, it is important to remember that cocaine has both rewarding (e.g. euphoria) and aversive (e.g.

anxiety, paranoia) subjective effects. *Dbh -/-* mice develop a conditioned place aversion to cocaine at doses that support a place preference in control mice, and individuals with genetically low DBH activity or treated with a DBH inhibitor report increased cocaine-induced paranoia (Cubells et al., 2000; Kalayasiri et al., 2007; R. Malison, personal communication). Hyperactivity and/or stereotypy in mice may represent an increase in the aversive effects of cocaine. The DBH inhibitor disulfiram, which reduces cocaine use in addicts (e.g. Carroll et al., 2004), facilitates the development of behavioral sensitization to cocaine in rats (Haile et al., 2003). Unfortunately, disulfiram lacks high specificity and potency for DBH, which causes unwanted side effects and reduces its efficacy and safety. Our results indicate that NEPI is a selective inhibitor of DBH that profoundly affects cocaine responses, making it a promising pharmacotherapy for the treatment of psychostimulant addiction.

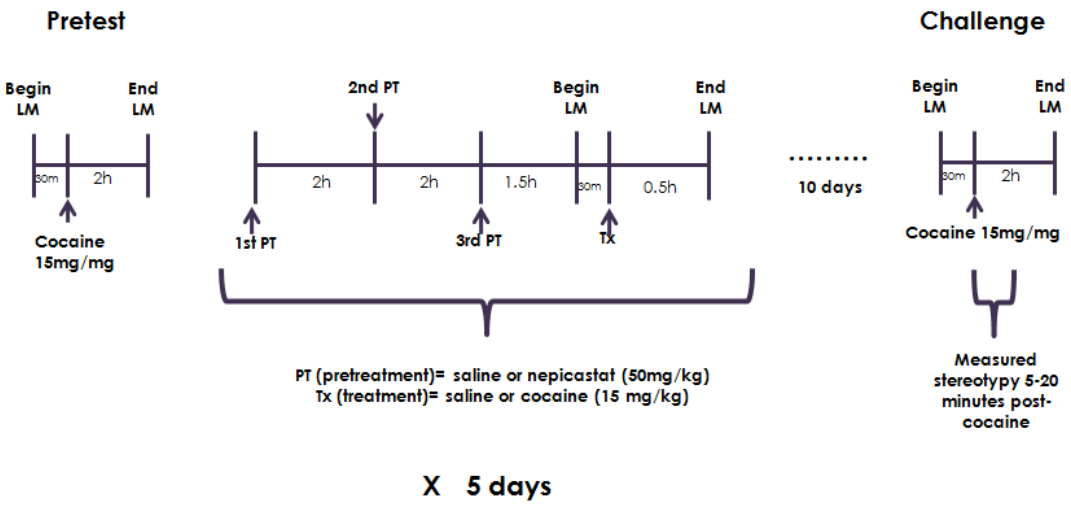


Figure 2.1 Behavioral sensitization paradigm timeline. On day 1, mice were injected with cocaine (15 mg/kg, i.p.) and were assigned to balanced treatment groups based on their cocaine-induced locomotor activity (LM).. On days 2-6, mice received 3 injections of saline or nepicastat (50 mg/kg, i.p.), each injection spaced 2 hr apart. Ninety min after the last pretreatment, mice were placed in locomotor chambers and LM measurements commenced. Thirty min later, mice were injected with saline or cocaine (15 mg/kg, i.p.) and their LM was recorded for an additional two hours. The mice then spent 10 days undisturbed in their home cage. On day 17, mice were placed in locomotor chambers for 30 min before receiving a cocaine injection (15 mg/kg, i.p.), and LM was recorded for 2 hr. Stereotypic behaviors were visually scored for 5 min, 5-20 min following cocaine injection.

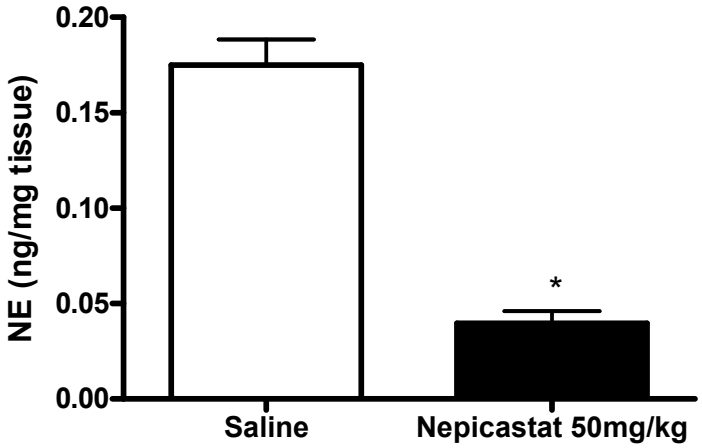
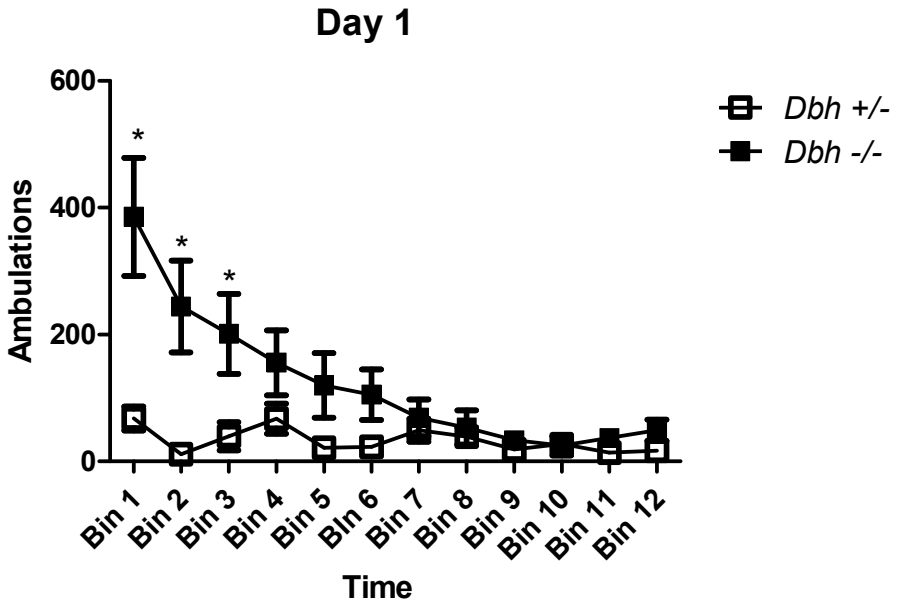


Figure 2.2. Nepicastat decreases norepinephrine in the prefrontal cortex. Mice received saline or nepicastat (3 injections of 50 mg/kg, i.p., each injection spaced 2 hr apart), and were euthanized 2 hr after the last injection. Prefrontal cortices were dissected out and NE levels were measured by HPLC. N=8 per genotype. *$p<0.0001$.

A



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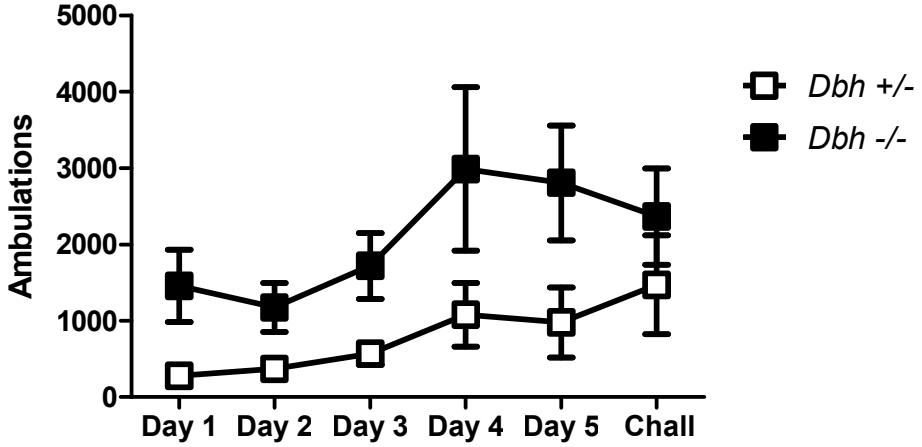


Figure 2.3 Cocaine-induced locomotion in control and *Dbh -/-* mice. A) B) *Dbh -/-* (n=8) and control mice (n=8) received a single injection of cocaine (15mg/kg) and their locomotion was recorded in for 2 hr, in 10 min bins. Shown is mean ± SEM ambulations

(consecutive beam breaks) for each 10 min bin, $p<0.05$. **B)** On days 1-5, *Dbh -/-* (n=8) and control mice (n=8) received saline pretreatments (3 injections, each spaced, 2 hr apart) followed by cocaine (15 mg/kg, i.p.) 2 hr after the last injection. Locomotion was recorded for 2 hr following the cocaine injection. Ten days later, all mice received a challenge (Chall) injection cocaine (15 mg/kg, i.p.) and their locomotion was recorded for 2 hr. Shown is mean $\pm$ SEM ambulations (consecutive beam breaks).

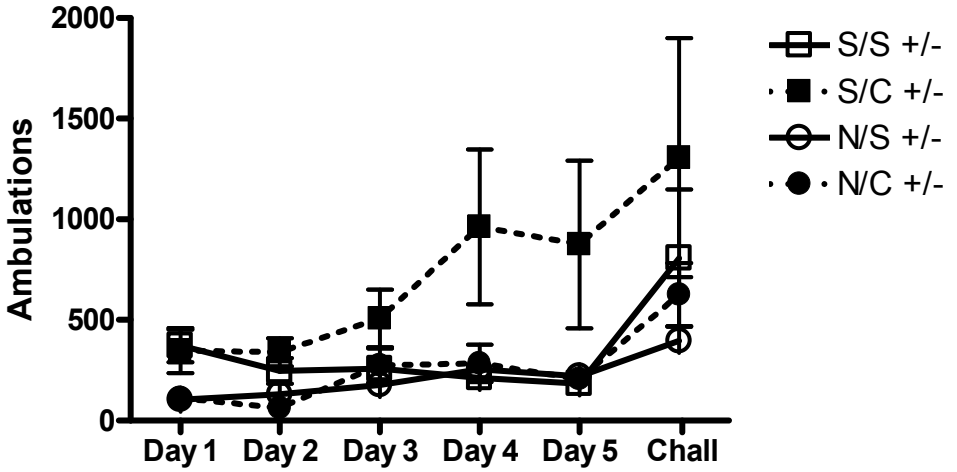


Figure 2.4 Effect of nepicastat treatment on locomotor activity in control mice. On days 1-5, control (*Dbh* +/-) mice were pretreated with saline or nepicastat (3 injections of 50 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Locomotor activity was recorded for the following 2 hr. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.) and their locomotor activity recorded for 2 hr. Shown is mean $\pm$ SEM ambulations (consecutive beam breaks). (S/S= saline/saline, n=7; S/C= saline/cocaine, n=9; N/S=nepicastat/saline, n=7; N/C= nepicastat/cocaine, n=7)

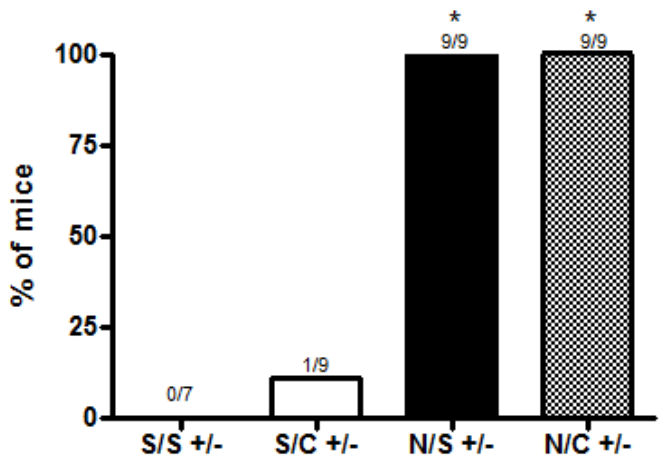


Figure 2.5 Effect of nepicastat treatment on stereotypic behavior in control and Dbh -/- mice. On days 1-5, control (Dbh +/-) and Dbh -/- mice were pretreated with saline or nepicastat (3 injections of 50 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.) and the presence of stereotypic behaviors was assessed for 5 min, 5-20 min following cocaine administration. Stereotypic behaviors were defined as circling, head-bobbing, nail biting and sniffing. *Dbh -/-* mice did not show stereotypic behaviors in response to cocaine. Shown is the percentage of mice engaged in stereotypy. * $p<0.0001$ compared to S/S group. (S/S= saline/saline; S/C= saline/cocaine; N/S=nepicastat/saline; N/C= nepicastat/cocaine)

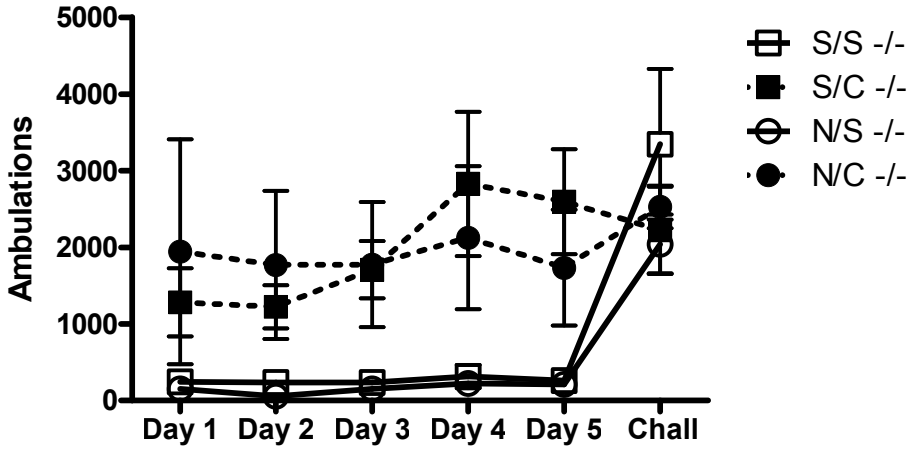


Figure 2.6 Effect of nepicastat treatment on locomotor activity in *Dbh -/-* mice.

On days 1-5, *Dbh -/-* mice were pretreated with saline or nepicastat (3 injections of 50 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Locomotor activity was recorded for the following 2 hr. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.) and their locomotor activity recorded for 2 hr. Shown is mean $\pm$ SEM ambulations (consecutive beam breaks). (S/S= saline/saline, n=7; S/C= saline/cocaine, n=7; N/S=nepicastat/saline, n=6; N/C= nepicastat/cocaine, n=4)

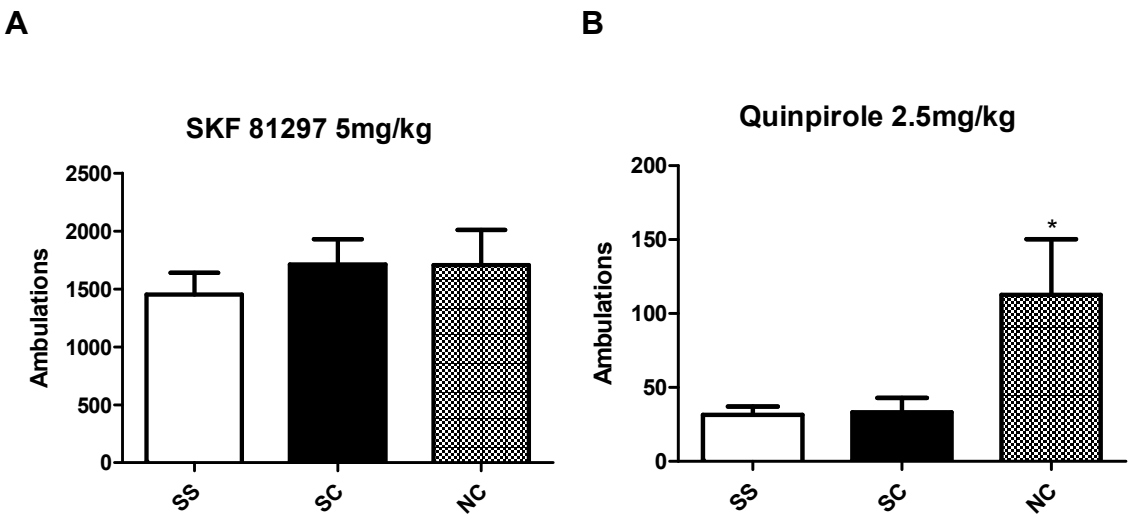


Figure 2.7 Effect of nepicastat on D1 and D2 receptor agonist-induced locomotor activity in control mice. On days 1-5, control (Dbh +/-) mice were pretreated with saline or nepicastat (3 injections of 50 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. On days 6 and 7, mice were injected with the D1 agonist SKF 81297 (5 mg/kg, i.p.)(shown in panel **A**) or the D2 agonist quinpirole (2.5 mg/kg, i.p.) (shown in panel **B**) and their locomotor activity was recorded for 2 hr. Shown is mean $\pm$ SEM ambulations (consecutive beam breaks). (S/S= saline/saline, n=8; S/C= saline/cocaine, n=8; N/C= nepicastat/cocaine, n=8). **p*=0.0294 compared to S/S group.

CHAPTER III:

MOLECULAR AND CELLULAR CHANGES FOLLOWING DBH INHIBITION

3.1 Abstract

Chronic inhibition of dopamine β–hydroxylase (DBH), the enzyme that converts dopamine to norepinephrine in noradrenergic neurons, has been shown to enhance the behavioral response to psychostimulants. For example, DBH knockout (*Dbh -/-*) mice are hypersensitive to locomotor activity induced by cocaine, amphetamine or the D2/D3 agonist quinpirole. Furthermore, chronic treatment with the selective DBH inhibitor nepicastat facilitates cocaine sensitization in control, but not Dbh -/- mice. To identify the mechanism(s) underlying this behavioral hypersensitivity, we compared the abundance of DA signaling proteins within the mesocorticolimbic pathway following genetic or pharmacological DBH inhibition. We found lower levels of β-arrestin2 in the nucleus accumbens (NAc) of *Dbh -/-* and nepicastat-treated control mice, while other D2 signaling proteins were unchanged. Using viral vectors to manipulate β-arrestin2 in the NAc, we found that knockdown of β-arrestin2 in control mice tended to increase cocaine-induced locomotor activity, while overexpression of β-arrestin2 in *Dbh -/-* mice attenuated their cocaine hypersensitivity. Finally, we compared electrophysiological responses to quinpirole in medium spiny neurons from the NAc of control and *Dbh -/-* mice. As previously reported, administration of quinpirole decreased evoked spikes following current injection in slices from control mice. By contrast, quinpirole increased spikes in *Dbh -/-* slices. These results indicate a profound alteration in accumbal D2 signaling following chronic loss of noradrenergic tone that are mediated, at least in part, by decreases in β-arrestin2.

3.2 Introduction

Dopamine β-hydroxylase (DBH) is the enzyme that converts DA into NE in noradrenergic neurons, thus controling NE production and the DA/NE ratio (reviewed by Weinshilboum, 1979). DBH knockout (*Dbh -/-*) mice completely lack NE from birth (Thomas et al., 1998). Despite the chronic absence of NE, the noradrenergic system is surprisingly unaffected in *Dbh -/-* mice, with normal abundance, distribution, and function of noradrenergic cells, projections, receptors and plasma membrane transporters (Weinshenker et al., 2002; Jin et al., 2004; Sanders et al., 2006; Paladini et al., 2007). Furthermore, most *Dbh -/-* phenotypes can be rescued by acute restoration of NE itself or adrenergic receptor agonists, suggesting that the noradrenergic system is fully functional other than the lack of NE (Thomas and Palmiter, 1997a and b; Thomas et al., 1998; Weinshenker et al., 1999; Weinshenker et al., 2001; Cryan et al., 2004; Murchison et al., 2004; Szot et al., 2004; Swoap et al., 2006; Paladini et al., 2007). By contrast, the mesocorticolimbic pathway, which is comprised of dopaminergic projections from the ventral tegmental area (VTA), to the nucleus accumbens (NAc) and the prefrontal cortex (PFC), is profoundly compromised in *Dbh -/-* mice. NE projections from the locus coeruleus, A1 and A2 innervate the VTA, the NAc, and the PFC, providing excitatory drive onto these DA neurons (Swanson and Hartman, 1975; Jones and Moore, 1977a and b; Simon et al., 1979; Morrison et al., 1981; Liprando et al., 2004; Berridge et al., 1997; Delf et al., 1998) (reviewed in Chapter 1). The loss of direct and indirect noradrenergic drive onto these DA neurons results in molecular and behavioral alterations in their DA system, such as hypersensitivity to psychostimulants and a D2 agonist, insensitivity to a

D1 agonist, and increased basal pERK and ΔFosB protein levels in the striatum (Weinshenker et al., 2002; Schank et al., 2006; Rommelfanger et al., 2007). Furthermore, normal mice treated chronically with the pharmacological DBH inhibitor nepicastat also show hypersensitivity to a psychostimulant and a D2 agonist (Chapter 2), indicating that loss of DBH leads to adaptive changes in the dopaminergic system.

It is of interest to study dysregulation of the DA system following chronic DBH inhibition because dopaminergic alterations underlie responses to drugs of abuse, such as psychostimulants. Specifically, dopaminergic transmission is essential for the development of behavioral sensitization to cocaine (Mattingly et al., 1996), a behavior enhanced by the loss of DBH (Chapter 2). Activation of DA receptors is necessary for this behavior. For example, rodents pretreated with DA receptor antagonists in the VTA fail to sensitize to cocaine or amphetamine (Reimer and Martin-Iverson; Tella, 1994; Vezina, 1996; White et al., 1998).

Dbh -/- mice and control mice treated chronically with nepicastat share many similarities with control mice that have undergone a sensitization regimen to psychostimulants, such as enhanced amphetamine- cocaine-, and quinpirole-induced locomotion and increased pERK and ΔFosB in the NAc (Weinshenker et al., 2002; Schank et al., 2006; Rommelfanger et al., 2007). The purpose of this study was to characterize DA signaling in *Dbh -/-* mice and to determine the mechanisms underlying D2 and psychostimulant hypersensitivity following chronic DBH inhibition. We previously reported that *Dbh -/-* mice have an increase in striatal high affinity-state D1 and D2 receptors, and speculated that this could underlie the behavioral hypersensitivity to psychostimulants (Schank et al., 2006). However, subsequent studies failed to confirm

this result (Skinbjerg et al., 2010), as did our experiments in the present study, suggesting that other components of the DA signaling pathway were altered and contribute to psychostimulant responses in *Dbh -/-* mice. Thus, we first compared the abundance of DA signaling proteins within the mesocorticolimbic pathway in *Dbh -/-* and control mice, and confirmed positive findings in mice treated chronically with the selective DBH inhibitor nepicastat. We next used viral knockdown and overexpression vectors to assess the contribution of β-arrestin2, which we found is decreased in the NAc of *Dbh -/-* and nepicastat-treated mice, to psychstimulant hypersensitivity. Finally, we compared electrophysiological responses to the D2 agonist quinpirole in medium spiny neurons from the NAc of control and *Dbh -/-* mice.

3.3 Materials and methods

Animals

Adult male and female control (*Dbh* +/−) and *Dbh* −/− mice were group housed and food and water were available ad libitum throughout the course of the study. Because there were no detectable gender differences, data from male and female mice were combined. *Dbh -/-* mice were generated as described (Thomas et al., 1998) and maintained on a mixed C57Bl6/J and 129SvEv background. The *Dbh* +/− mice were used as control mice because they have normal brain catecholamine levels and are behaviorally identical to wild-type (*Dbh* +/+) mice (Thomas et al., 1995; Thomas et al., 1998; Bourdelat-Parks, 2005; Mitchell et al., 2006).

All animals were treated in accordance with the National Institutes of Health (NIH) Intramural Animal Care and Use Program guidelines. The experiments described in this article followed the Emory University Division of Animal Resources' Guide for the Care and Use of Laboratory Animals and were approved by the Emory Institutional Animal Care and Use Committee.

Nepicastat pretreatment

The mice used for this study were those that previously underwent the nepicastat treatment described in Chapter 2. In summary, adult *Dbh* +/- and -/- mice (n=4-13 per treatment) received daily pre-treatment with saline or nepicastat (50 mg/kg, i.p.) three times a day, each injection two hours apart, followed by saline or cocaine (15 mg/kg, i.p.) two hours after the last pretreatment. This dosing schedule was repeated daily for five days, and mice were then left undisturbed in their home cage. Ten days after the last treatment injection, all mice were given a cocaine injection (15 mg/kg, i.p.). Twenty-four hours later, animals were sacrificed by CO_2 asphyxiation, their brains removed, and the striatum dissected on ice and stored at -80°C.

Competition binding assays

Striata from 3 naive *Dbh +/-* and *Dbh -/-* mice were homogenized and washed 3x in binding buffer (20mM Hepes; 0.1M NaCl; 5mL $MgCl_2$; 5mM KCl; 1.5mM $CaCl_2$; 0.25mM EDTA, 1 PI tablet). Each incubation tube received, in the following order, 0.7mL binding buffer, 30µL of tissue homogenate, 0.1mL of increasing concentrations of dopamine (15nM-500µM) or 0.5mM butaclamol; and 0.1mL of 0.5nM [^{3}H]spiperone.

The assay was done in duplicate. The tubes were incubated for 2 h at room temperature then filtered using a 12-well cell harvester and buffer-presoaked glass fiber filter mats. The filters were individually placed in scintillation minivials with 5mL each of scintillant overnight and the radioactivity measured by scintillation spectrometer. The competition data were analyzed by non-linear least-squares regression and the goodness of fit was judged with one-site fit or two-site fit (Prism 4.0 software; GraphPad, San Diego, CA).

Western blotting

Mouse brain tissue was homogenized in 500µL harvest buffer (1M HEPES, 1M NaCl, 250mM EDTA, pH 7.4, supplemented with protease inhibitors) using a sonicator. 6x loading dye was added to samples after measuring protein concentrations with a BCA Assay (ThermoScientific, Rockford, IL). Samples were resolved by SDS-PAGE on 4-20% Tris-Glycine precast gels followed by transfer to nitrocellulose membranes. Following transfer, membranes were incubated with Ponceau staining in order to assess even protein loading, then rinsed with distilled water. Membranes were then incubated in blocking buffer [(1M Hepes, 1M NaCl, 1% Tween-20, 2% dry milk, pH 7.4, for most antibodies; (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk, for pAKT, GSK3β and pGSK3β)] for 30 minutes and then incubated with primary antibody overnight at 4°C. The primary incubation buffer is the same as blocking buffer for most antibodies except pAKT, GSK3β and pGSK3β. For these, the primary incubation buffer was 1X TBS, 0.1% Tween-20 with 5% BSA. The membranes were washed three times in blocking buffer and incubated with either a fluorescent (1:10000) or HRP-conjugated secondary (1:4000) antibody (Invitrogen, Carlsbad, CA) for 30 minutes, washed three

more times, and then visualized using either the Odyssey imaging system (Li-Cor) or via ECL reagent (Thermoscientific, Rockford, IL) followed by exposure to film. Membranes were stripped for 20 minutes at 37°C and 10 minutes at room-temperature with stripping buffer and re-probed for α-actin to confirm equal loading of samples. Blots were analyzed by densitometry using Image J Software.

Antibody information

The antibodies used and their working dilutions were the following: β-arrestin2 (anti-rabbit; 1:2500; Cell Signaling Technology (Danvers, MA), CS3857); MAPK (ERK) (anti-rabbit; 1:2000; Cell Signaling, CS9102); pMAPK-Thr202/Tyr204(pERK) (anti-mouse; 1:1000; Cell Signaling, CS9106); ΔFosB (anti-rabbit; 1:1000; Cell Signaling, CS2551); and α-actin (anti-mouse; 1:1000; Santa Cruz Biotechnology (Santa Cruz, CA), SC5671).

β-arrestin2 viral vectors

Five different Barr2 shRNA viral particles (Sigma Aldrich) were tested in Hek293 cells for their ability to decrease β-arrestin2 protein expression. The construct with the greatest knockdown efficiency, along with a control construct with a scrambled sequence not found on the genome, were made into bacterial glycerol stocks (Sigma Aldrich). These glycerol stocks were purified then inserted into a pLV-CMV-Cre lentiviral vector by the Emory University Viral Vector Core with a titer of approximately $6x10^8$ viral particles/µl. Viral generation of the β-arrestin2 overexpressing viral constructs and GFP control constructs was performed by the Duke Neurotransgenic Laboratory. Each

construct was inserted into adenovirus vectors, and viruses were harvested with a titer of $2x10^{12}$/ µL (β-arrestin2) and $5x10^{9}$µL (GFP control).

β-arrestin2 viral infusions

Mice (n=8 for each treatment group: β-arrestin2 overexpression adenovirus, GFP adenovirus, β-arrestin2 shRNA knockdown lentivirus, and "scramble" lentivirus) were anesthetized using isofluorane and placed in a stereotaxic frame with a nose bar. The animal's scalp was opened and bregma and lambda aligned to flat-skull position. The stereotaxic arm was then lowered to the NAc core. The core subregion was chosen because it has been implicated in cocaine-induced locomotion and behavioral sensitization to cocaine (Ikemoto and Witkin, 2003; Li et al., 2004) . The anteroposterior (AP) and mediolateral (ML) coordinates of the NAc core in relation to bregma (AP= 1.6mm; ML= ±0.6mm) and a small hole was drilled in the skull at these coordinates. Then, a 5μL Hamilton microsyringe was lowered to target the NAc core (dorsoventral coordinate= -3.6mm). The Hamilton's needle 26-gauge beveled tip was precoated with 1% anti-bovine serum (BSA) prior to loading the virus to prevent molecular interactions between the syringe and the viral vectors. Animals received 1.0μL viral injections bilaterally at a rate of 0.2μL/minute**.** In order to avoid virus diffusion, the needle remained in place for five minutes after the injection and removed slowly. The skin was glued together using Vetbond tissue glue. All animals received meloxicam (0.5 mg/kg) for post-operative pain and water/liquid ibuprofen at a dose of 0.1mg/mL placed in the cage floor, and allowed at least 10 days to recover.

Two weeks after the infusion of β-arrestin2 knockdown and scramble viral vectors, mice were placed in locomotor chambers and their basal locomotor activity was recorded for 30 min before receiving an injection of cocaine (15 mg/kg i.p.), and locomotion was recorded for an additional 2 hrs. Mice were anesthetized,transcardially perfused with saline and 4% paraformaldehyde 24-48 hours later, their brains removed, stored in 4% paraformaldehyde for 4 days, then transferred to 30% sucrose.

Ten days after the infusion of β-arrestin2 overexpression and control vectors, all mice were placed in locomotor chambers and their basal locomotion recorded for 30 min before receiving an injection of cocaine (15 mg/kg, i.p.), and cocaine-induced locomotion was recorded for 2 hrs. Immediately following the end of locomotor activity recording, Mice were anesthetized,transcardially perfused with saline and 4% paraformaldehyde 24-48 hours later, their brains removed, stored in 4% paraformaldehyde for 4 days, then transferred to 30% sucrose.

Electrophysiological recordings of nucleus accumbens neurons

Control (n=13) and *Dbh -/-* (n=9) mice were anesthetized and rapidly decapitated. Dissected brains were transferred to ice-cold ACSF containing the following (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1 ascorbic acid (saturated with 95% O_2 and 5% CO_2). Horizontal brain slices containing the nucleus accumbens (200–300 μm) were cut with a vibratome (VT1200, Leica) and prepared as described previously (Torrecilla et al., 2002). The experimenter was blind to the genotype of the animal until after the experiments were completed. Horizontal slices were placed in a chamber (0.5 ml) superfused with physiological saline (35°C) at a rate of 1.5 ml/min.

The solution was equilibrated with 95% O25% CO2 (pH 7.4) and contained 126 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl2, 2.4 mM CaCl2, 1.4 mM NaH2PO4, 25 mM NaHCO3, and 11 mM D-glucose. The internal solution used for whole-cell recordings contained 115 mM K-methyl sulfate, 20 mM KCl, 1 mM MgCl2, 10 mM Hepes, 10 mM BAPTA, 2 mM ATP, 0.3 mM GTP, and 10 mM creatine phosphate. Patch recordings were made by using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Medium spiny neurons in the nucleus accumbens core were visually identified and, using a current clamp configuration, current was injected for 200ms at 100mA step intervals (100-500mA) with 20s between each pulse, until the cell was depolarized and spikes were evoked. An input/output curve was obtained under control conditions before superfusing a 10mL of a 5μM solution of quinpirole for approximately 5 min. All experiments were done in the presence of: NBQX (AMPA antagonist), D-APV (NMDA antagonist), picrotoxin (GABAa antagonist), and SCH 23390 (D1 antagonist).

Values are presented as mean ± SEM. For all experiments, $p < 0.05$ was considered as a significant difference. The change produced by a drug was calculated as the mean holding evoked current amplitude 30 s after equilibrium had been reached relative to the holding current before drug superfusion. Unpaired comparisons between two groups were made with a Mann–Whitney U test, whereas paired comparisons were made by using a Wilcoxon signed-rank test. Firing data for each genotype was subtracted (post-pre quinpirole) and then fit with a 3rd order curve. A sum of squares F test was performed using Prism (GraphPad Software, La Jolla, CA).

3.4 Results

Dbh -/- mice have normal D2 receptor density ($D2_{High}$) in the striatum

D2 receptors exist in two affinity states for the agonist DA, high and low affinity, and receptors can rapidly change between the two states. DA binds primarily to the high-affinity state of the D2 receptor ($D2_{High}$), making this the most functionally-relevant state. An increase in the proportion of these receptors in the striatum is associated with behavioral sensitivity to psychostimulants (even if they produce no change or even a decrease in the total number of D2 receptors Seeman et al., 2004, 2005, 2007). We previously reported that *Dbh -/-* mice have an increase in striatal high affinity-state D1 and D2 receptors, and speculated that this could underlie the behavioral hypersensitivity to psychostimulants (Schank et al., 2006). However, subsequent studies failed to confirm this result (Skinbjerg et al., 2010). We measured D2high sites using two-site competition analysis of spiperone radioligand binding assays, and found no difference in $D2_{High}$ receptors between control and *Dbh -/-* mice. The percentage of high affinity receptors was similar in control (29.3%) and *Dbh -/-* mice (35.7%) (Figure 3.1). The Bmax for control mice was 70.31 and 67.70 for *Dbh -/-* mice ($p=0.7521$), and the IC_{50} (24.67μM), and the K_D values (4.11μM) were the same for both of the curves. These results indicate that differences in the total number of high-affinity state D2 receptors are unlikely to underlie the behavioral hypersensitivity to D2 agonists observed following chronic DBH inhibition.

Dbh -/- mice have decreased β-arrestin2 in the nucleus accumbens

Because the proportion of $D2_{High}$ receptors is similar between genotypes, we hypothesized that differences downstream of the D2 receptor underlie the behavioral hypersensitivity to D2 agonists and psychostimulants in *Dbh -/-* mice. In support of this hypothesis, we have previously reported that *Dbh -/-* mice have increased pERK and ΔFosB in the striatum (Rommelfanger et al., 2007). Therefore, we measured levels of other DA signaling proteins, including β-arrestin2, protein phosphatase 1 (PP1), phosphorylated extracellular signal-regulated kinase (pERK), dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32), G-protein-coupled receptor kinase (GRK), and phosphorylated and total isoforms of protein kinase B (Akt) and glycogen synthase kinase 3 (GSK3β) in the NAc, PFC and CP of control and *Dbh -/-* mice. While no differences were detected in any brain region for most of these proteins (Figures 3.2, 3.3 and 3.4) *Dbh -/-* mice had significantly less β-arrestin2 in the NAc (Unpaired t-test, p=0.0129) (Figure 3.5A). There was also a trend for higher pERK in the NAc and CP (Figure 3.3 and 3.4)(t_6=1.306, p=0.2393 for the NAc; t_6=2.193, p=0.0708 for the CP), although it did not reach significance in this study as it did in our previous publication (Rommelfanger et al., 2007).

Nepicastat-treated control mice have decreased β-arrestin2 and increased ΔFosB in the nucleus accumbens

Because *Dbh -/-* mice have decreased β-arrestin2 in the NAc and increased pERK and ΔFosB in the striatum, we measured the relative levels of these proteins in the NAc of control (*Dbh +/-*) mice following chronic saline/saline (S/S), saline/cocaine (S/C), nepicastat/saline (N/S), and nepicastat/cocaine (N/C) treatment. One-way ANOVA

showed a main effect of treatment ($F_{(3,28)}$= 5.873; p<0.005). Post-hoc comparisons revealed that β-arrestin2 was significantly decreased, ΔFosB levels were increased, and pERK levels were unchanged following nepicastat treatment, either with or without cocaine (Figure 3.5). Combined, these results indicate that chronic genetic or pharmacological DBH inhibition results in decreased β-arrestin2 and increased ΔFosB in the NAc.

β-arrestin2 in the nucleus accumbens core modulates cocaine-induced locomotion

Both *Dbh -/-* mice and nepicastat-treated control mice have decreased β-arrestin2 in the NAc and behavioral hypersensitivity to cocaine. To test whether the cocaine hypersensitivity is mediated by a reduction in β-arrestin2, we manipulated β-arrestin2 protein levels *in vivo* in the NAc core, an sub-area of the NAc implicated in cocaine-induced locomotion (Ikemoto and Witkin, 2003; Li et al., 2004). Knockdown of β-arrestin2 expression in the NAc core of control mice tended to enhance cocaine-induced locomotion, though the trend did not reach statistical significance. Two way repeated measures ANOVA showed a main effect of time ($F_{(11,348)}$=4.756, p<0.0001) (Figure 3.6). Conversely, overexpression of β-arrestin2 expression in the NAc core of *Dbh -/-* mice decreased cocaine-induced locomotion. Two way repeated measures ANOVA showed a main effect of treatment ($F_{(1,11)}$= 5.362, p<0.05) and time ($F_{(11,121)}$= 5.242, p<0.0001) and a time x treatment interaction ($F_{(1,121)}$= 1.979, p<0.05)(Figure 3.7).

Dopamine D2 receptor activation in the nucleus accumbens core of Dbh -/- mice facilitates neuronal firing

D2-like receptor signaling is mediated by $G_{\alpha i/o}$ proteins, and activation of the receptor results in neuron hyperpolarization and the reduction of cell excitability (Kurose et al., 1983; Bokoch et al., 1983; Surmeier et al., 1993). To determine whether a change in cellular responses to a D2 agonist underlie the D2 agonist hypersensitivity observed in *Dbh -/-* mice, electrophysiological recordings were made from medium spiny neurons in the NAc core. In control mice, quinpirole suppressed neuronal firing, as previously described (Surmeier et al., 1993; Yan et al., 1997; Page et al., 1997; Herlite et al., 1997; Zamponi and Snutch, 1998; Hernandez-Lopez et al., 2000). By contrast, quinpirole administration has the opposite effect in *Dbh -/-* mice, increasing neuronal firing as measured by the number of evoked spikes and the spiking frequency ($p<0.0001$)(Figure 3.8). Input resistance was unchanged between treatments (Figure 3.9), while membrane potential was slightly lower following quinpirole treatment in control, but not *Dbh -/-* mice ($p<0.005$). These results indicate that chronic loss of DBH promotes a switch in D2 signaling from inhibitory to excitatory in NAc medium spiny neurons.

3.5 Discussion

Dbh -/- mice are hypersensitive to locomotor activity induced by psychostimulants and the D2/D3 agonist quinpirole. Chronic treatment with the selective DBH inhibitor nepicastat facilitates cocaine sensitization in control, but not Dbh -/- mice and hypersensitivity to quinpirole. To identify the molecular underpinning of this behavioral hypersensitivity, we compared relative levels of DA signaling proteins within the mesocorticolimbic pathway following genetic or pharmacological DBH inhibition.

We found lower levels of β-arrestin2 in the NAc of *Dbh -/-* and nepicastat-treated control mice.. We then found that knockdown of β-arrestin2 in the NAc core of control mice tended to increase cocaine-induced locomotor activity, while overexpression of β-arrestin2 in this region in *Dbh -/-* mice attenuated their cocaine hypersensitivity. Finally, we compared electrophysiological responses to quinpirole in medium spiny neurons from the NAc core of control and *Dbh -/-* mice. Quinpirole administration decreased evoked spikes following current injection in slices from control mice but increased spikes in *Dbh -/-* slices. These results indicate a profound alteration in D2 signaling in the NAc following DBH inhibition that is modulated, at least in part, by decreases in β-arrestin2.

Dbh -/- mice have normal densities of D2 dopamine receptors in the high-affinity state

In 2006, Schank et al., reported that while *Dbh -/-* and control mice have similar number of total striatal DA receptors, *Dbh -/-* mice showed an increased proportion of $D2_{High}$ in the NAc and CP, as measured by saturation binding of the D2 receptor antagonist raclopride in the presence and absence of guanine nucleotides (GN). A more recent study sought to test this in vivo by scanning *Dbh -/-* and control mice with the agonist PET radioligand [^{11}C]MNPA, thought to bind preferentially to the high affinity state of the D_2 receptor. They also performed *in vitro* binding experiments on striatal homogenates with the D2 antagonist [^{3}H]methylspiperone. Both of these assays showed that the percentages of D_2 receptors in the high affinity state were not significantly different between these two groups (Skinbjerg et al., 2010). Our results support this most recent report, as in vitro binding experiments on striatal homogenates with [^{3}H]spiperone, which is thought to bind preferentially to $D2_{High}$ receptors, did not reveal any difference

in Bmax values, KD, or percentages of D2 receptors in the high-affinity state between control and *Dbh -/-* mice. Therefore, additional downstream mechanisms must contribute to the behavioral sensitivity to psychostimulants *Dbh -/-* mice.

Selective DBH inhibition alters the abundance of dopamine signaling molecules in the nucleus accumbens

Both *Dbh -/-* mice and control mice treated with nepicastat have enhanced behavioral sensitivity to cocaine (Schank et al., 2006; Chapter 2), and our results show this is accompanied by decreased β-arrestin2 in the NAc. Knockdown of this protein in the core of the NAc of control mice tended to decrease their locomotion to cocaine, while overexpression in *Dbh -/-* mice reduced their cocaine-induced locomotion, suggesting that decreased β-arrestin2 mediates, at least in part, the behavioral sensitivity to cocaine seen in mice with genetic or pharmacologic DBH inhibition. Interestingly, a previous study by Bohn et al., (2003) found that β-arrestin2 knockout mice show enhanced morphine-induced striatal DA release, enhanced place conditioning to morphine and attenuated tolerance to the same drug (Bohn et al., 1999, 2000, 2002). This morphine phenotype support the general principle that the inactivation of components that mediate desensitization, such as arrestins, can lead to enhanced receptor signaling, and thereby, enhanced behavioral responses. Specifically, the behavioral effects of morphine on β-arrestin2 -/- is thought to be due to the impairment of β-arrestin2- dependent μ-opioid receptor resensitization following chronic morphine treatment (Dang et al., 2011). Paradoxically, β-arrestin2 knockout mice have slightly decreased cocaine-induced locomotion and no difference in the acquisition or expression of place conditioning to

cocaine. Several key methodological differences may contribute to the decreased cocaine-induced locomotion in the Bohn study vs. the increase we observed. Importantly, β-arrestin2 knockout mice have a complete and lifelong lack of β-arrestin2 throughout the brain and body, while our virus-delivered shRNA approach produced a partial reduction of β-arrestin2 specifically in the NAc.

Downstream of DA receptor activation, we found increased ΔFosB in the NAc of nepicastat-treated control mice (N/S and N/C), suggesting that DBH inhibition is sufficient to induce this change. Chronic cocaine administration increases ΔFosB following cocaine administration (Hope et al., 1994; Marttila et al., 2007), and we found a similar trend in this study, although the difference between the S/C and S/S treatment group did not reach statistical significance. An increase in ΔFosB is expected to contribute to cocaine hypersensitivity as ΔFosB overexpression in the NAc increases locomotor sensitization to cocaine (Kelz et al., 1999).

By contrast, we did not find any difference in pERK levels across treatment groups. This result is difficult to interpret because pERK levels are elevated in *Dbh -/-* mice (Rommelfanger et al., 2007) and typically increase in control mice following repeated cocaine treatment (Shin et al., 2007; Janes et al., 2009). For this study, subjects were sacrificed 24 hr after the last cocaine injection. If the increase in pERK following cocaine is transient, we would not have detected it. A transient increase in pERK in immediate response to the challenge injection could still contribute to cocaine hypersensitivity, as it would be able to alter expression of immediate-early genes that involved in synaptic plasticity, such as cAMP response element-binding protein (CREB) (reviewed by Nestler, 2004).

D2 receptor stimulation in the nucleus accumbens core of Dbh -/- mice facilitates neuronal firing

Electrophysiological recordings from NAc core neurons revealed that D2 receptor activation in enhanced neuronal firing in *Dbh -/-* mice, compared to the suppression seen in control mice. As a Gi-coupled receptor, D2 activation typically inhibits evoked action potentials, reducing neuronal firing and spike frequency (Hu and Wang 1988; Gulledge and Jaffe 1998; Cepeda et al. 2001; West and Grace 2002; Tseng and O'Donnell 2004; control mice in this study). By contrast, Gs-coupled receptors, such as D1, have a facilitatory effect on neuronal responses. The excitatory effect of quinpirole in *Dbh -/-* NAc neurons suggests to us that the D2 receptors are coupled to Gs instead of Gi. There are precedents for similar Gi-to-Gs switches, as is the case of the μ-opioid receptor and the CB1 cannabinoid receptor following repeated treatment with low dose of a receptor agonist (Wang e al., 2005; Paquette et al., 2007), but would be the first description of this phenomenon in a DA receptor to our knowledge.

As expected, we did not find any changes in input resistance following quinpirole treatment (Perez et al., 2006). However, we did find a small but significant reduction in membrane potential following quinpirole in neurons from control mice, which is indicative of hyperpolarization. Quinpirole had no effect on membrane potential in *Dbh -/-* neurons, which may contribute to the facilitatory response. Because the inhibitory effect of D2 signaling has been related to increases in potassium conductance (Momiyama et al., 1993; Greif et al. 1995; Congar et al. 2002; Ljungstrom et al. 2003), this suggests that the altered response to quinpirole in *Dbh -/-* mice is mediated by changes in the function of potassium channels.

To test these hypotheses, we are currently using selective G-protein inhibitors in slice preparations. If a Gi-to-Gs switch is occurring, then a Gi inhibitor should block quinpirole-induced inhibition in control mice, while a Gs inhibitor should block quinpirole-induced excitation in *Dbh* -/- mice. Another possibility is the activation of Gβγ following D2 agonist binding. The $G_{\beta\gamma}$ subunit is capable of activating AC, leading to the mobilization of intracellular calcium stores and depolarization of the cell (Bertorello et al. 1990; Hopf et al. 2003). If this is the case, a Gβγ inhibitor should block quinpirole-induced excitation in *Dbh* -/- mice. Finally, if changes in potassium currents contribute to the *Dbh* -/- phenotype, blockade of voltage-sensitive, slowly inactivating A-type potassium currents should reverse the effects of quinpirole.

Working model of molecular and cellular changes underlying DBH inhibition

Combined, our present results provide strong evidence for the cellular and molecular modulators of behavioral sensitivity to cocaine following chronic DBH inhibition. We propose that genetic or pharmacologic DBH inhibition leads to loss of noradrenergic drive onto midbrain DA neurons, decreasing their DA release. As a result, compensatory alterations in the dopaminergic system ensue, including a decrease in β-arrestin2 levels in the NAc. Concurrently, D2 receptors in this region become uncoupled from Gi and couple to either Gs or Gβγ, resulting in enhanced neuronal firing following activation of the D2 receptor and behavioral hypersensitivity to psychostimulants and D2 agonists. Whether a decrease in β-arrestin2 leads directly to a G protein switch remains to be determined.

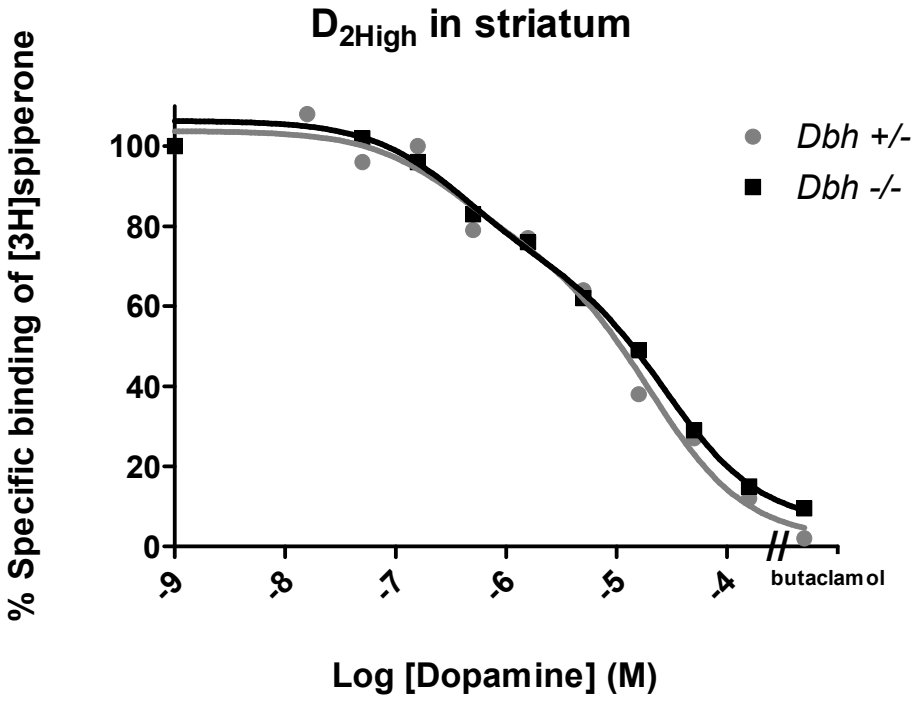


Figure 3.1 *Dbh -/-*mice have normal density of striatal high-affinity state dopamine D2 receptors. Competition binding experiments on control and *Dbh -/-* striatal membrane homogenates with [^{3}H] spiperone and dopamine. N=3 per genotype.

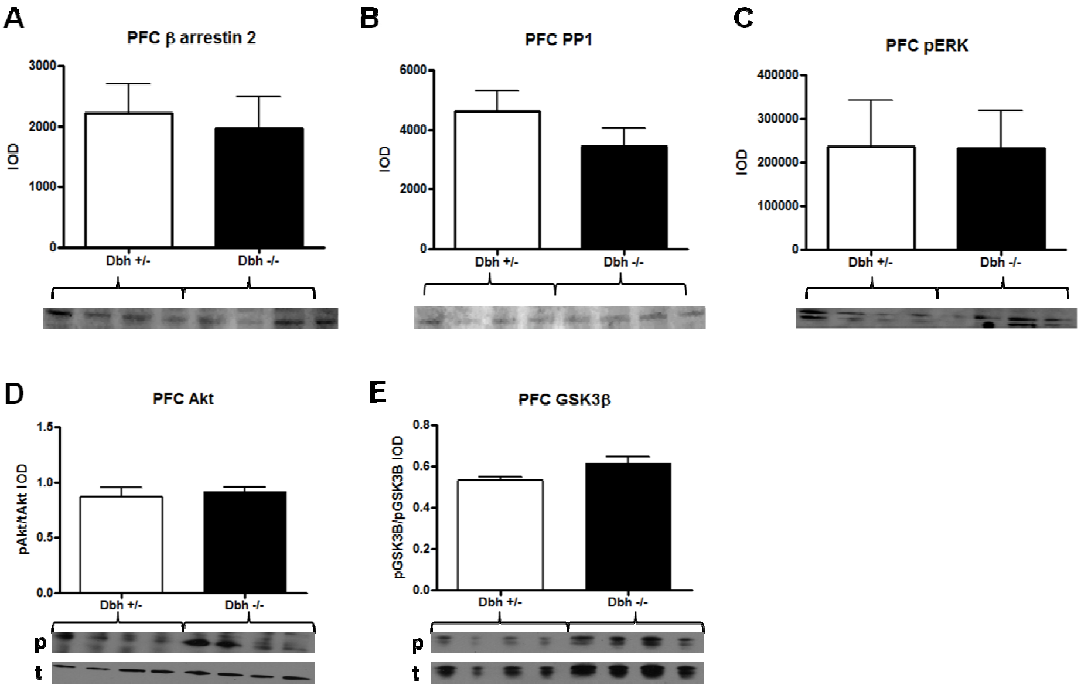


Figure 3.2 Relative levels of dopamine-related signaling proteins in the prefrontal cortex of control and *Dbh -/-* mice. Naïve control and *Dbh -/-* mice (n=8 per genotype) were euthanized, and the prefrontal cortex was dissected and processed for western blot analysis of β-arrestin2 (A), PP1 (B), pERK (C), phosphor (p) and total (t) Akt (D) and phospho (p) and total (t) GSK3β (E). Shown is the mean ± SEM integrated optical density of densitometry analysis.

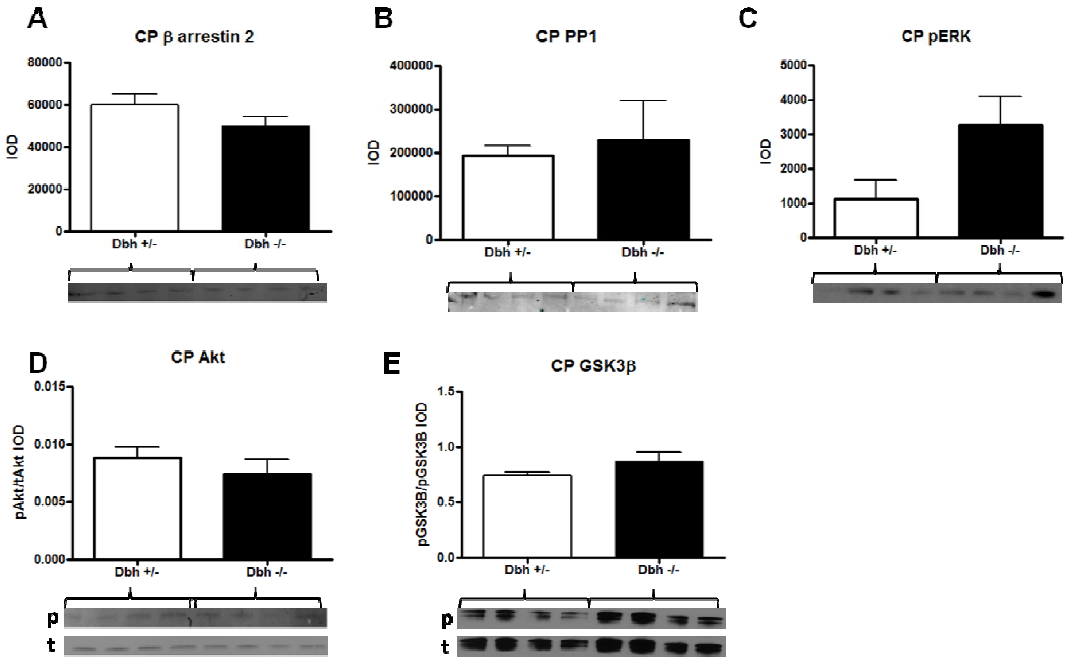


Figure 3.3 Relative levels of dopamine-related signaling proteins in the caudate putamen of control and *Dbh -/-* mice. Naïve control and *Dbh* -/- mice (n=8 per genotype) were euthanized and the caudate putamen were dissected and processed for western blot analysis of β-arrestin2 (A), PP1 (B), pERK (C), phosphor (p) and total (t) Akt (D) and phospho (p) and total (t) GSK3β (E). Shown is the mean ± SEM integrated optical density of densitometry analysis.

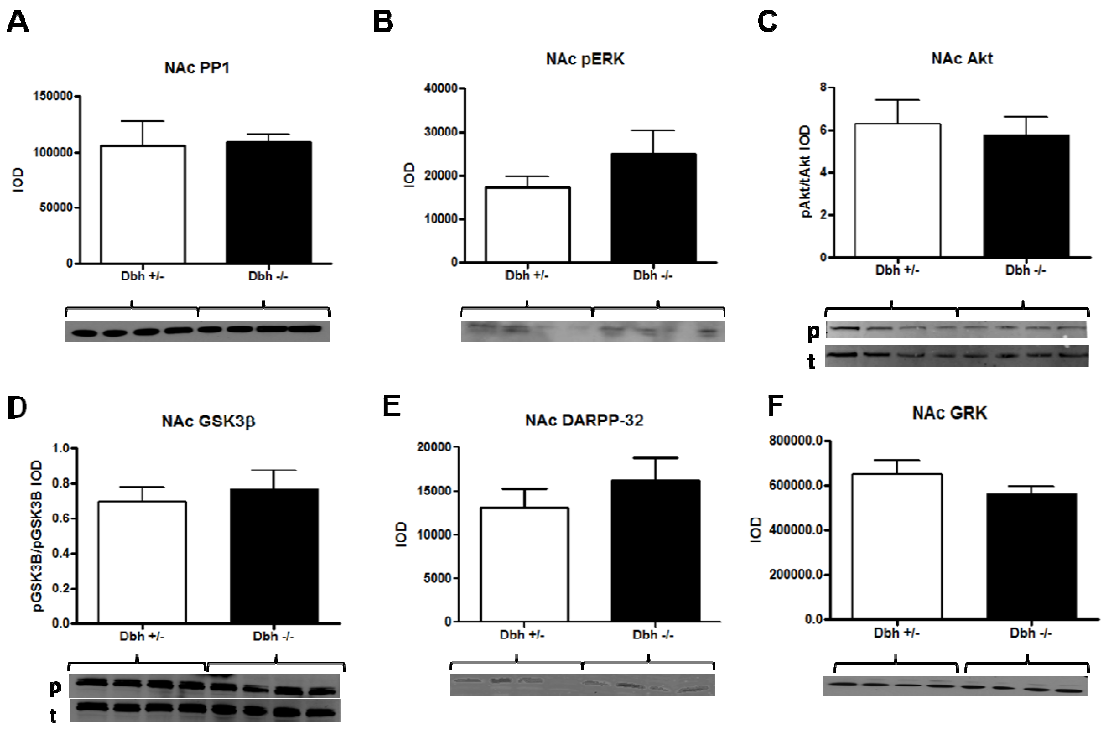


Figure 3.4 Relative levels of dopamine-related signaling proteins in the nucleus accumbens of control and *Dbh -/-* mice. Naïve control and *Dbh -/-* mice (n=8 per genotype) were euthanized and the nucleus accumbens was dissected and processed for western blot analysis of PP1 (A), pERK (B), phosphor (p) and total (t) Akt (C) and phospho (p) and total (t) GSK3β (D), DARPP-32 (E) and GRK (F). Shown is the mean ± SEM integrated optical density of densitometry analysis.

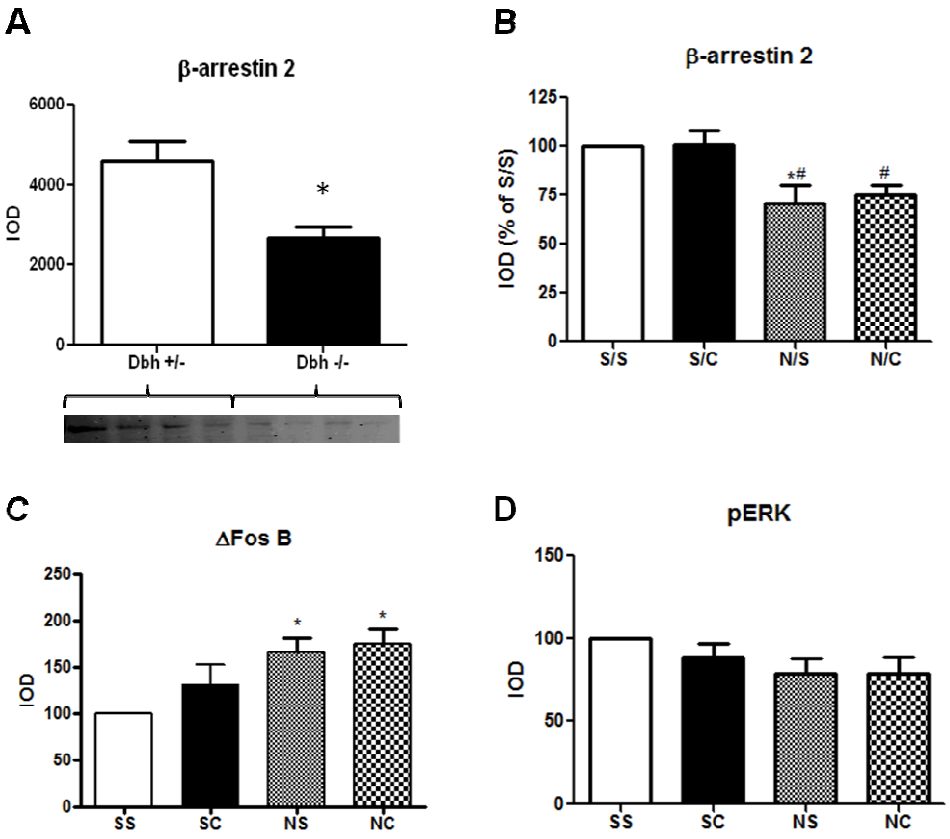


Figure 3.5 Dopamine signaling proteins are altered in the nucleus accumbens of *Dbh -/-* mice and control mice treated with chronic nepicastat. (A) β-arrestin2 levels are decreased in the nucleus accumbens of *Dbh -/-* mice. Naïve control and *Dbh -/-* mice (n=8 per genotype) were euthanized, and the nucleus accumbens was dissected and processed for western blot analysis. Shown is the mean ± SEM integrated optical density of densitometry analysis. *p<0.05. (B-D) Chronic nepicastat treatment decreases β-arrestin2 and increases ΔFosB levels but has no effect on pERK in the nucleus accumbens of control mice. *Dbh* +/- mice were pretreated with saline or nepicastat (3 injections of 50 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment for 5 days. Ten days later, all mice received a

challenge injection of cocaine (15 mg/kg, i.p.). 24 hr later, mice were euthanized and the nucleus accumbens was dissected and processed for western blot analysis of β-arrestin2. Shown is the mean ± SEM integrated optical density of densitometry analysis. n=8 per treatment group. (S/S= saline/saline; S/C= saline/cocaine; D/S= disulfiram/saline; D/C= disulfiram/cocaine), $p<0.01$; *compared to S/S; $^{\#}$ compared to S/C

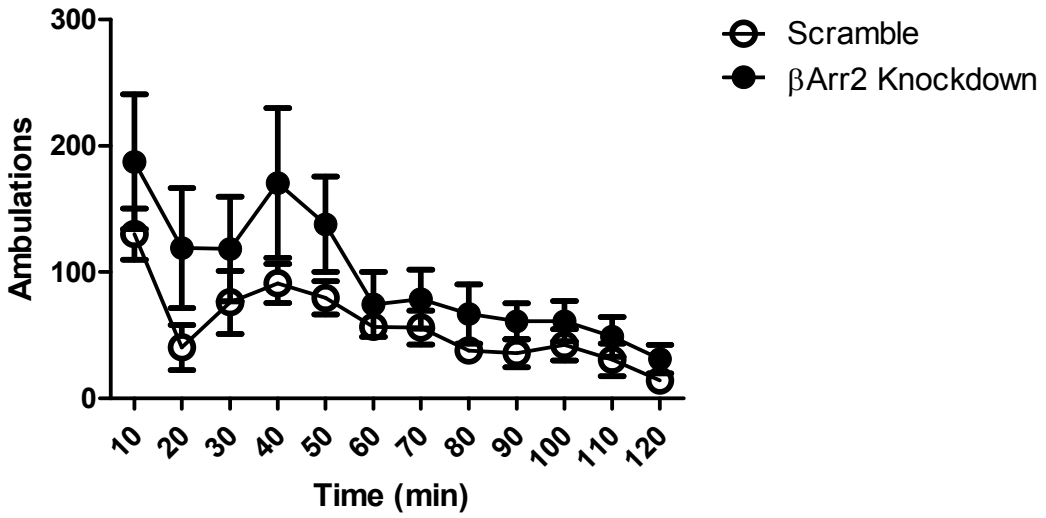


Figure 3.6 β-arrestin2 knockdown in the nucleus accumbens increases cocaine-induced locomotion in control mice. Control mice received bilateral infusions of lentiviral vectors with either β-arrestin2 shRNA (n=15) or scrambled RNA (n=16) in the nucleus accumbens core. Two weeks later, mice were placed in locomotor chambers for 30 min, then injected with cocaine (15mg/kg, i.p.), and their locomotion was recorded for 2 hr in 10 min bins. Shown are mean ± SEM ambulations.

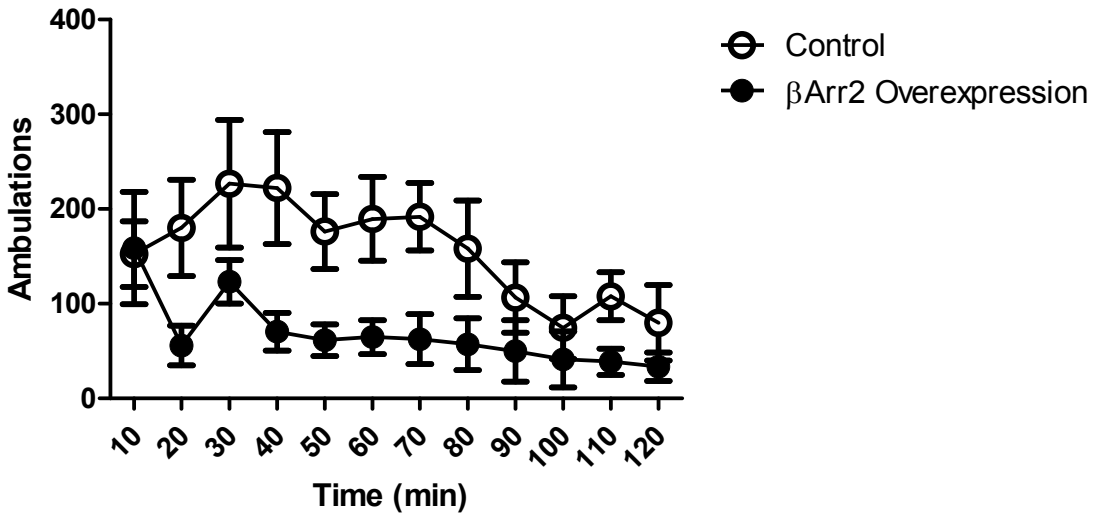


Figure 3.7 β-arrestin2 overexpression in the nucleus accumbens reverses hypersensitivity to cocaine-induced locomotion in *Dbh -/-* mice. *Dbh -/-* mice received bilateral infusions of adenovirus vectors that drive expression of β-arrestin2 (n=7) or GFP (n=5). in the nucleus accumbens core Ten days later, mice were placed in locomotor chambers for 30 min, then injected with cocaine (15 mg/kg, i.p.) and their locomotion was recorded for 2 hr in 10 min bins. Shown are mean ± SEM ambulations.

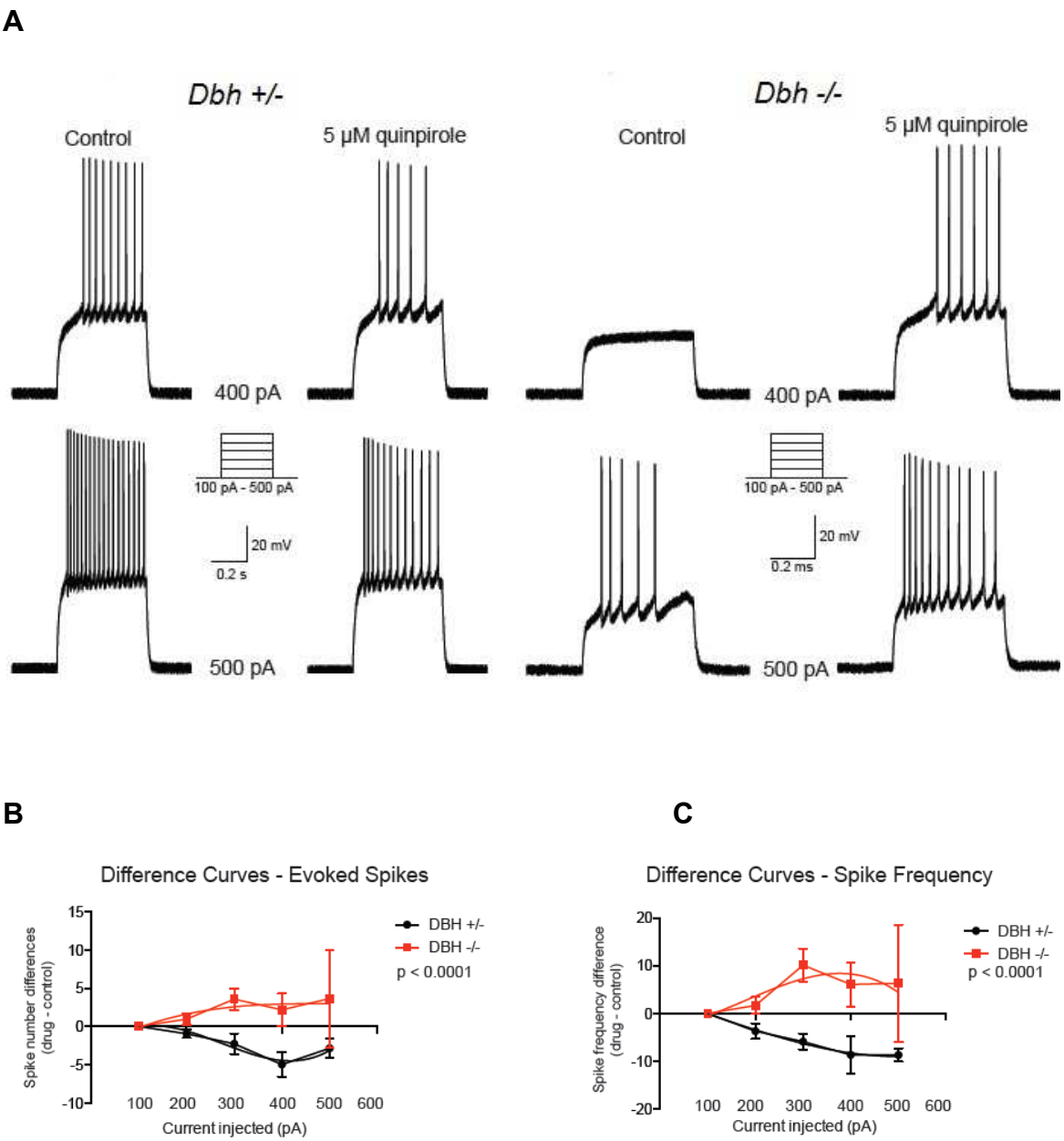


Figure 3.8 The D2 agonist quinpirole suppresses medium spiny neuron firing in control mice, but activates firing in *Dbh -/-* mice. Whole-cell recordings were performed on visually-identified medium spiny neurons located in the nucleus accumbens core from control and *Dbh -/-* mice. Cells were patched using a current clamp and current steps (100-500pA) were injected. After obtaining an input/output curve under

control conditions, quinpirole (5 μM) was bath applied. All experiments were performed in the presence of AMPA, NMDA, GABA and D1 receptor antagonists. Shown are (A) representative electrophysiological traces of control and *Dbh* -/- neurons before and after treatment with quinpirole, (B) number of evoked spikes (mean ± SEM), and (C) spike frequency in neurons from control (n=13) and *Dbh* -/- (n=9) mice following quinpirole application for each current step (mean ± SEM). $p<0.0001$.

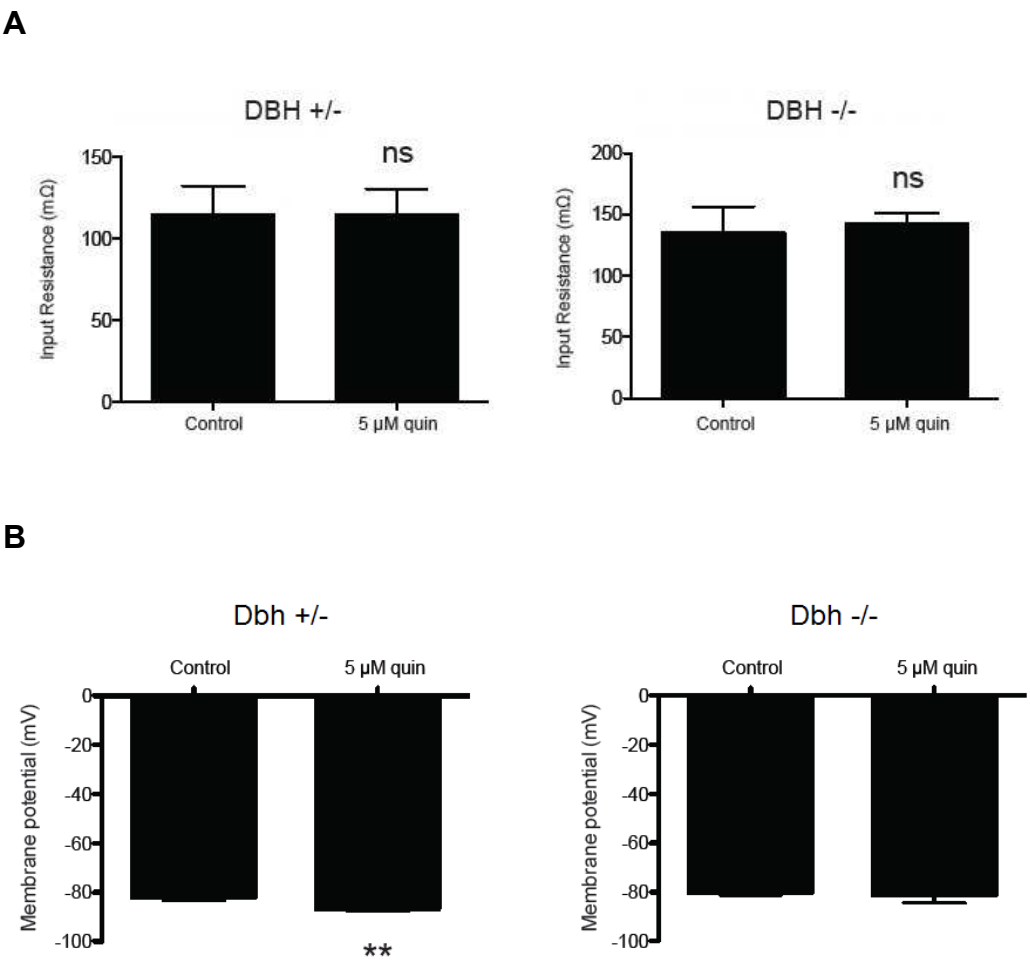


Figure 3.9 Effect of quinpirole on input resistance and membrane potential of accumbens medium spiny neurons in control and *Dbh -/-* mice. Whole-cell recordings were performed on visually-identified medium spiny neurons located in the nucleus accumbens core from control (n=13) and *Dbh -/-* mice (n=9). Cells were patched using a current clamp and current steps (100-500pA) were injected. After obtaining an input/output curve under control conditions, quinpirole (5 µM) was bath-applied for 5 min. All experiments were performed in the presence of AMPA, NMDA, GABA and D1 receptor antagonists. Shown are (A) input resistance and (B) membrane potential (mean ± SEM**).** **$p<0.001$ compared to control for that genotype.

CHAPTER IV:

THE ROLE OF DBH INHIBITION IN THE MECHANISM OF ACTION OF DISULFIRAM

Parts of this chapter are adapted from: Gaval-Cruz M and Weinshenker D. Mechanisms of disulfiram-induced abstinence: Antabuse and cocaine relapse. Mol Interv. 2009; 9(4):175-187.

4.1 Abstract

Disulfiram reduces alcohol intake in alcoholics by inhibiting aldehyde dehydrogenase (ALDH) and inducing an aversive reaction following alcohol consumption. Recently, disulfiram has been shown to decrease cocaine intake regardless of concurrent alcohol consumption, but the mechanisms underlying this efficacy are unknown. In addition to its effects on ALDH, disulfiram also inhibits dopamine β-hydroxylase (DBH), which converts dopamine (DA) to norepinephrine (NE) in noradrenergic neurons. Because cocaine acts primarily via monoamines, a shift in NE and DA levels could alter its subjective properties and underlie the efficacy of disulfiram in treating cocaine dependence. DBH knockout (*Dbh -/-*) mice are hypersensitive to the behavioral effects of amphetamine and cocaine. Similarly, disulfiram enhances cocaine sensitization in rats. To test whether disulfiram induces behavioral hypersensitivity to cocaine via DBH inhibition, we tested the effects of chronic pretreatment with disulfiram on cocaine-induced locomotor activity and stereotypic behaviors in control and *Dbh -/-* mice. Disulfiram-treated control mice showed an accelerated rate of behavioral hypersensitivity to cocaine as measured by locomotor response and/or increases in cocaine-induced stereotypy. Naïve *Dbh -/-* mice were hypersensitive to cocaine and were unaffected by disulfiram treatment, indicating that the effects of disulfiram are mediated, at least in part, by DBH inhibition. Because both genetic (*Dbh -/-* mice) and selective pharmacological (nepicastat) DBH inhibition results in decreased levels of β-arrestin2 and increased levels of phosphorylated extracellular signal-regulated kinase (pERK) and deltaFosB (ΔFosB) in the nucleus accumbens, we measured accumbal levels of these proteins in saline- and disulfiram-treated control mice. We found increased pERK and ΔFosB in mice pretreated

with disulfiram, while no differences in β-arestin2 levels were detected. These results indicate that disulfiram-induced behavioral hypersensitivity is due, at least in part, to DBH inhibition, and may involve changes in the DA pathway signaling proteins pERK and ΔFosB. We speculate that inhibition of DBH contributes to the ability of disulfiram to reduce cocaine use in a clinical setting.

4.2 Introduction

4.2.1 Cocaine addiction

The most potent stimulant of natural origin, cocaine, is also the most abused illicit stimulant in America. The Office of National Drug Control Policy estimates that 3.6 million Americans meet the criteria for chronic cocaine dependence. In 2009, approximately 11.3 percent of those seeking treatment for an addiction disorder in publicly-funded facilities did so for their addiction to cocaine. In the same year, out of almost one million visits to the emergency room involving an illicit drug, 422,896 of them involved problems associated with cocaine use (SAHMSA Treatment Episode Data). Given its abuse liability and the debilitating nature of its addiction, research has been committed to understanding its mechanism of action as well as the neurobiological underpinning of its abuse. Due to the mechanism of action of cocaine and the circuitry underpinning reward and reinforcement, potential cocaine addiction pharmacotherapies mainly focusing on modulators of the DA, glutamate and GABA systems and how they impact relapse prevention.

While some treatment strategies have shown promise, none are widely accepted and to date, there are still no FDA-approved pharmacotherapies for the treatment of cocaine addiction. Below, we discuss the compound disulfiram as an alternative for the treatment of cocaine addiction. Originally used as a pharmacotherapy for alcohol abuse, it has more recently shown promise in decreasing cocaine intake in addicts.

4.2.2 Disulfiram as a pharmacotherapy for alcohol and cocaine addiction

Disulfiram and ethanol metabolism

Disulfiram (Antabuse) first received pharmacological interest in the 1930s, after workers in a rubber factory, where the compound was used as an antioxidant, became ill. In particular, workers who consumed alcohol after having been exposed to disulfiram experienced flushing of the face, nausea, vertigo, headache, and hypotension. This series of unpleasant symptoms are now known as the "disulfiram-ethanol reaction" (Kitson 1977). These eventually led, in 1951, to the approval of disulfiram, under the name Antabuse, by the Food and Drug Administration for the treatment of alcoholism. The drug's efficacy relies on aversive conditioning; simply put, alcoholics who are prescribed Antabuse learn to avoid alcohol in order to avoid the negative consequences of the disulfiram-alcohol reaction.

The mechanism by which disulfiram is inducing this response is well-characterized and widely accepted. Ethanol is converted to acetaldehyde by the enzyme alcohol dehydrogenase, and acetaldehyde is further metabolized to acetate by aldehyde dehydrogenase (Deitrich and Erwin, 1971). Disulfiram is an inhibitor of aldehyde dehydrogenase, which is directly relevant to its role in curbing alcohol consumption. The high levels of acetaldehyde that accumulate following alcohol ingestion in patients taking disulfiram cause the mild to moderate levels of facial flushing, weakness, throbbing headache, nausea, vomiting, sweating, vertigo, hypotension, and other unpleasant symptoms that typify the disulfiram-ethanol reaction (also known as the Antabuse reaction) (Johansson, 1992; Hald and Jacobsen, 1948). The direct association of this

aversive reaction with alcohol consumption establishes a psychological deterrent in alcoholic patients who abide by the dosing regimen.

To date, there have been eight supervised clinical trials, ranging from 56 to 270 days in duration, which assess oral disulfiram for the treatment of alcoholics (Sofuoglu et al., 2006). The percentage of disulfiram-treated patients who completed these trials is higher for those populations in which drug administration was supervised by clinic staff or a family member. Indeed, adherence to treatment must be recognized as a confounding factor in interpreting the effectiveness of disulfiram from clinical trial data. Problems with adherence pose the main clinical challenge in using disulfiram to treat alcoholism. Other limitations of its use are side effects (Wilson, 1962; Frisoni and Di Monda, 1989; Kristenson, 1995) and hepatic toxicity in alcoholics with compromised liver function (Torronen and Marselos, 1978; Depuy et al., 1995), both of which are a result of disulfiram's multiple enzymatic targets and lead to the underuse of the drug as a pharmacotherapy for alcoholism.

Disulfiram and cocaine dependence

On the theory that diminishing alcohol consumption in cocaine- and alcohol-dependent individuals might lead to a decrease in cocaine use, two research groups examined the use of disulfiram in this patient population in 1993. In the first study, although disulfiram treatment reduced both alcohol and cocaine use, the effect on cocaine use was attributed to a course of behavioral therapy that had been implemented in the patient population (Higgins et al., 1993). The second study, a randomized twelve-week pilot trial, compared the effects of disulfiram administration to the effects of naltrexone,

an opioid antagonist that may prevent drug craving; both treatments were accompanied by cognitive behavioral therapy. Disulfiram proved more effective than naltrexone at lowering the frequency of cocaine use (McCance-Katz et al., 1998). A larger clinical trial ensued, in which disulfiram treatment improved abstinence from cocaine as compared to no treatment. Disulfiram treatment in this larger trial appeared more effective for those outpatients who also received cognitive behavioral therapy (Carroll et al., 1998). The beneficial effect of disulfiram was still evident a year later in a follow-up study (Carroll et al, 2000), and the efficacy of disulfiram therapy in diminishing cocaine use as observed earlier in comorbid addicts (McCance-Katz et al., 1998) was corroborated.

The notion that the mechanism of disulfiram-induced cocaine abstinence might not be related to the disulfiram-alcohol reaction emerged from the results of two studies published in 2000. In these trials, the effects of disulfiram on cocaine use were assessed in patients who were addicted to both cocaine and opiates, and who were maintained on methadone (Petrakis et al., 2000) or buprenorphine (George et al., 2000). In agreement with previous studies, the addicts treated with disulfiram were better able, relative to those addicts not receiving disulfiram, to reduce their intake of alcohol, cocaine, and opiates (Petrakis et al., 2000). In addition, disulfiram shortened the time necessary for patients to reach continuous cocaine, but not heroin, abstinence (George et al., 2000). Throughout these trials, alcohol consumption was minimal for all subjects, regardless of medication group, and baseline alcohol use did not predict responses to disulfiram. It was therefore something of a conceptual breakthrough when, in 2004, a randomized, placebo-controlled trial not only confirmed the effectiveness of disulfiram in treating cocaine dependence, but moreover revealed that the drug's effectiveness in this regard

could be differentiated from its role in curbing alcohol abuse (Carroll et al., 2004). Specifically, the groundbreaking trial addressed cocaine use both with and without comorbidity for alcohol abuse, showing that the benefits of disulfiram therapy were most pronounced in patients who either were not alcohol dependent at baseline or who fully abstained from alcohol during treatment. These observations directly suggest that disulfiram undermines cocaine addiction in a manner independent of its action in inhibiting alcohol intake. Several other double-blind, randomized, placebo-controlled trials have confirmed the efficacy of disulfiram on cocaine intake (Grassi et al., 2007; Pettinati et al., 2008), with a potentially greater effect in males (Nich et al., 2004), but none have been designed to investigate the mechanisms of disulfiram-induced cocaine abstinence.

Unfortunately, there are too few animal studies of the effect of disulfiram in cocaine-induced behaviors in laboratory animals. Early studies showed that disulfiram pretreatment suppresses amphetamine-induced (Maj et al., 1968) and cocaine-induced (Maj and Przegalinski, 1967) locomotor activity in mice and rats. More recent studies indicate that disulfiram has minimal effects on baseline activity levels, but repeated administration prior to cocaine facilitates the development of behavioral sensitization to cocaine in rats (Haile et al., 2003). Disulfiram pretreatment also enhances cocaine-induced seizures in mice (Gaval-Cruz et al., 2008).

DBH inhibition as the putative mechanism of action of disulfiram

Because the primary metabolite of disulfiram- N,N-diethyldithiocarbamate, is a copper chelator, disulfiram can impair the function of any enzyme which requires copper

as a cofactor. One of these is the enzyme dopamine β-hydroxylase (DBH), which converts DA into NE in noradrenergic neurons and therefore controls the NE/DA ratio in the brain. Disulfiram inhibits DBH and ALDH with similar potency (IC_{50} in the low μM range for both enzymes; Green et al., 1964; Mays et al., 1998), and it inhibits the production of NE in rodents and humans (Musacchio et al., 1966; Lake et al., 1977; Schroeder et al., 2010). Because DA and NE are both critical for the addictive properties of cocaine and DBH controls their relative levels in the brain, DBH inhibition is an obvious candidate for mediating the effect of disulfiram on cocaine use. Therefore, models of genetic DBH inhibition are a valuable tool to study the mechanism of action of disulfiram in psychostimulant responses.

Dbh –/– mice essentially have total and lifelong DBH inhibition (Thomas et al., 1995; 1998). Due to the loss of noradrenergic excitatory drive onto midbrain DA neurons, these mice have low basal and stimulant-evoked DA release, accompanied by a paradoxical behavioral hypersensitivity to psychostimulants and a D2 DA receptor agonist, manifested in heightened locomotor activity and related stereotypical behavior (Schank et al., 2006; Weinshenker 2002). Furthermore, a low dose of cocaine that does not support a conditioned place preference in control mice does induce a conditioned place preference in *Dbh -/-* mice, while a higher dose of cocaine that supports a conditioned place preference in control mice results in conditioned place aversion in *Dbh–/–* mice (Schank et al., 2006).

The molecular mechanisms underlying behavioral hypersensitivity to cocaine following DBH inhibition are not fully understood. As described in Chapter 2, *Dbh -/-* and nepicastat-treated control mice have decreased levels of β-arrestin2 in the nucleus

accumbens (NAc). β-arrestin2 is a crucial mediator of desensitization and trafficking of G-protein-coupled receptors (reviewed by Shenoy and Kefkowitz, 2011), including the DA D2 receptor (Kim et al., 2001). In addition, *Dbh -/-* mice have increased levels of phosphorylated extracellular signal-regulated kinase (pERK) and the transcription factor ΔFosB levels in the NAc (Rommelfanger et al., 2006). In control mice, pERK and ΔFosB are increased following cocaine administration and regulate long-lasting cocaine-induced behavioral plasticity, including behavioral sensitization (Hope et al., 1994; Marttila et al., 2007; Shin et al., 2007; Janes et al., 2009; DiRocco et al., 2009).

The hypersensitivity of *Dbh -/-* mice to the behavioral effects of psychostimulants and quinpirole suggests that chronic NE deficiency augments the interoceptive effects of cocaine via a mechanism involving D2 receptors, β-arrestin2, pERK and ΔFosB. We reasoned that if disulfiram is acting via DBH inhibition, then chronic disulfiram treatment in control mice should recapitulate the phenotypes observed in *Dbh -/-* mice, while disulfiram should have no further effect in *Dbh -/-* mice. To test this hypothesis, we assessed the following: 1) the effect of disulfiram pretreatment in control and *Dbh-/-* mice in a cocaine behavioral sensitization paradigm; 2) β-arrestin2, ΔFosB and pERK levels in the NAc of disulfiram-treated control mice and; 3) the effect of D1 and D2 agonist administration following disulfiram-cocaine pretreatment in control mice. In the behavioral sensitization paradigm, we examined both horizontal ambulations and the emergence of stereotypic behaviors in response to cocaine. An increase in either of these responses to cocaine can be interpreted as behavioral hypersensitivity to the drug.

4.3 Materials and methods

Animals

Adult male and female *Dbh* +/− and *Dbh* −/− mice were group housed and food and water were available ad libitum throughout the course of the study. Because there were no detectable gender differences, data from male and female mice were combined. Dopamine β-hydroxylase mice were generated as described (Thomas et al., 1998) and maintained on a mixed C57Bl6/J and 129SvEv background.
The *Dbh* +/− mice were used as control mice because they have
normal brain catecholamine levels and are behaviorally identical to wild-type (*Dbh* +/+) mice (Thomas et al., 1995; Thomas et al., 1998; Bourdelat-Parks, 2005).

All animals were treated in accordance with the National Institutes of Health (NIH) Intramural Animal Care and Use Program guidelines. The experiments described in this article followed the Emory University Division of Animal Resources' Guide for the Care and Use of Laboratory Animals and were approved by the Emory Institutional Animal Care and Use Committee.

Disulfiram pretreatment

Adult *Dbh* +/- and -/- mice (n=4-13 per treatment) were given injections of saline (1mL/kg) in their home cage 4 times per day, each injection 2 hr apart, for five days prior to the pretest day in order to habituate them to the total volume of the injections. On the sixth day, all mice were placed in locomotion (LM) recording chambers and allowed to habituate for 30 min before receiving a single injection of cocaine, (15 mg/kg, i.p.), and

their LM recorded for an additional 2 hr (Pretest day). LM was recorded as consecutive beam breaks in transparent plexiglass cages placed into a rack with 7 infrared photobeams spaced 5cm apart (San Diego Instruments Inc., La Jolla, CA).

Mice were then assigned to treatment groups with similar cocaine-induced LM scores. Treatment groups consisted of daily pre-treatment with saline or disulfiram (100 mg/kg, i.p.) 3 injections per day, each injection 2 hr apart, followed by saline or cocaine (15mg/kg, i.p.) 2 hr after the last pretreatment. Thirty min before the saline/cocaine injection, mice were placed in the LM chambers and activity was recorded for a total of 2.5 hr. This paradigm was repeated for 5 days. The appearance of stereotypic behaviors in response to cocaine was noted qualitatively during these five days, but was only quantified on Challenge day (See Figure 5.1 for pictorial representation of paradigm and below for description of Challenge day).

Cocaine challenge

Following the last injection on the 5th day of treatment, animals were placed back in their home cage and left undisturbed for ten days. Ten days after the last treatment injection, all mice were placed in the LM chambers, and 30 min later given an injection of cocaine (15 mg/kg, i.p.), and their LC was recorded for an additional 2 hr (Challenge day). Stereotypy was scored for 5 min, 20-40 min following the cocaine injection. Stereotypic behaviors were defined as circling, head-bobbing, nail biting and sniffing. Total time engaged doing these behaviors was also recorded.

D1 and D2 agonist challenge

A separate group of animals underwent the 5-day saline/disulfiram pretreatment described above. The next 2 days after the last treatment injection, all mice were again placed in the LM chambers and thirty minutes later given an injection of quinpirole (2.5mg/kg) or SKF81297 (5mg/kg), i.p. and their activity recorded for an additional two hours. Administration of quinpirole and SKF81297 was counterbalanced between the two days. LM was recorded as consecutive beam breaks in transparent plexiglass cages placed into a rack with 7 infrared photobeams spaced 5cm apart (San Diego Instruments Inc., La Jolla, CA).

Western blotting

Mouse brain tissue was homogenized in 500µL harvest buffer (1M HEPES, 1M NaCl, 250 mM EDTA, pH 7.4, supplemented with protease inhibitors) using a sonicator. 6x loading dye was added to samples after measuring protein concentrations with a BCA Assay (ThermoScientific, Rockford, IL). Samples were resolved by SDS-PAGE on 4-20% Tris-Glycine precast gels followed by transfer to nitrocellulose membranes. Following transfer, membranes were incubated with Ponceau staining in order to assess even protein loading, then rinsed with distilled water. Membranes were then incubated in blocking buffer [(1M Hepes, 1M NaCl, 1% Tween-20, 2% dry milk, pH 7.4] for 30 minutes and then incubated with primary antibody overnight at 4°C. The primary incubation buffer is the same as blocking buffer. For these, the primary incubation buffer was 1X TBS, 0.1% Tween-20 with 5% BSA. The membranes were washed three times in blocking buffer and incubated with either a fluorescent (1:10000) or HRP-conjugated secondary (1:4000) antibody (Invitrogen, Carlsbad, CA) for 30 minutes, washed three

more times, and then visualized using either the Odyssey imaging system (Li-Cor) or via ECL reagent (Thermoscientific, Rockford, IL) followed by exposure to film. Membranes were stripped for 20 minutes at 37°C and 10 minutes at room-temperature with stripping buffer and re-probed for α-actin to confirm equal loading of samples. Blots were analyzed by densitometry using Image J Software.

Antibody information

The antibodies used and their working dilutions were the following: β-arrestin2 (anti-rabbit; 1:2500; Cell Signaling Technology (Danvers, MA), CS3857); MAPK (ERK) (anti-rabbit; 1:2000; Cell Signaling, CS9102); pMAPK-Thr202/Tyr204(pERK) (anti-mouse; 1:1000; Cell Signaling, CS9106); ΔFosB (anti-rabbit; 1:1000; Cell Signaling, CS2551); and α-actin (anti-mouse; 1:1000; Santa Cruz Biotechnology (Santa Cruz, CA), SC5671).

4.4 Results

Disulfiram inhibits DBH and decreases brain norepinephrine levels

In order to confirm that systemic disulfiram administration results in DBH inhibition in the mouse brain, we measured NE in the prefrontal cortex following administration of saline or disulfiram (3 x 100 mg/kg, i.p. each injection spaced 2 hr apart, prefrontal cortex isolated 2 hr after the last injection). Disulfiram reduced NE levels in the prefrontal cortex by ~ 50% (Unpaired t test, F=1.038; p=0.0002) (Figure 5.2)

as previously described (Bourdelát-Parks, et al., 2005). This result confirms that our disulfiram dosing paradigm inhibits DBH in the mouse brain.

Dbh -/- mice are hypersensitive to cocaine

As expected (Schank et al., 2006), *Dbh -/-* mice were hypersensitive to cocaine-induced LM (ambulations on Day 1 during the 2 hr test (repeated measures ANOVA $F_{(11,154)}$= 6.831, $p<0.0001$) (Figure 4.3A). As previously reported (Schuster et al, 1977), repeated cocaine administration in control mice led to an enhanced behavioral response over time that persisted following a ten-day period of abstinence (Figure 4.3B).

Disulfiram facilitates cocaine sensitization in control, but not Dbh -/- mice

As previously reported (Schuster et al., 1977), repeated cocaine administration in control mice gradually enhanced behavioral response over time, which persisted following a ten-day period of abstinence (repeated measures ANOVA $F_{(5,47)}$=6.71, $p<0.05$) (Figure 5.3). *Dbh -/-* mice showed a somewhat different pattern; their LM increased over the first few days, then returned closer to baseline levels by Challenge day (repeated measures ANOVA $F_{(5,41)}$=3.13, $p<0.05$). The "sensitized" LM response of *Dbh +/-* mice on Challenge day was similar to the initial cocaine-induced LM response of *Dbh +/-* mice on Day 1 (Figure 5.3), indicating that naïve *Dbh -/-* mice behave as if they were "presensitized", as we have previously described for amphetamine (Weinshenker et al., 2002).

Disulfiram/cocaine (D/C) treatment resulted in either enhanced locomotor activity or in the appearance of stereotypic behaviors in response to cocaine in control mice. D/C-

treated mice that did not exhibit stereotypic behaviors showed an initial increase in locomotor response to cocaine compared to their saline/cocaine-treated (S/C) counterparts that remained stable over treatment days (Figure 5.4). Two-way repeated measures ANOVA showed a significant effect of time ($F=3.447$; $p=0.05$ and treatment ($F_{(3,120)}=9.862$; $p=0.0002$). Bonferroni post-hoc tests revealed a significant difference in locomotor activity between S/C- and D/C-treated control mice on days 1 and 3 ($p<0.01$). Saline/Saline (S/S) and disulfiram/saline pretreatment (D/S) did not affect the LM of control mice during the 5 treatment days or on Challenge day (Figure 5.4). D/C pretreatment also significantly increased the probability of developing cocaine-induced stereotypy (Figure 5.5A). On Challenge day, only 1/9 control mice pretreated with S/C showed stereotypy in response to cocaine, compared to 8/13 D/C-treated ($\chi^2=5.594$; $p=0.018$), and all of these mice spent most of the 5-min scoring session engaged in stereotypy (Figure 5.5 and data not shown). By contrast, disulfiram pretreatment, alone or accompanied by cocaine, had no effect on cocaine-induced LM in *Dbh -/-* mice (Figure 5.6). None of the *Dbh -/-* mice exhibited stereotypic behaviors following any of the treatments. Combined, these results indicate that disulfiram enhances the behavioral effects of cocaine in a DBH-dependent manner.

Disulfiram pretreatment does not have an effect on D1 or D2-induced locomotion

Given that *Dbh -/-* mice show hypersensitivity to a D2 agonist but not a D1 agonist (Weinshenker et al., 2002) we sought to examine the effect of pharmacological DBH inhibition with disulfiram on locomotor activity in response to the D1 agonist SKF81297 and the D2 agonist quinpirole. One way ANOVAs showed that, in control mice, neither

S/C nor D/C pretreatment affected SKF81297- ($F=1.74$, $p=0.1999$) or quinpirole- ($F=0.1457$; $p=0.8653$) induced LM (Figure 5.7).

Chronic disulfiram alters the abundance of dopamine signaling proteins in the nucleus accumbens of control mice

Dbh -/- mice have decreased levels of β-arrestin2 in the NAc (Chapter 2 of this dissertation) and elevated levels of ΔFosB and pERK in the striatum (Rommelfanger et al., 2007). While disulfiram had no effect on β-arrestin2 levels in the NAc of control mice (Figure 5.8) ($F=1.619$; $p=0.2072$) it significantly increased ΔFosB ($F=3.676$, $p<0.05$; Figure 5.9) and pERK ($F=30.97$, $p<0.0001$; Figure 5.10) whether it was paired with saline or cocaine. These results indicate that pharmacological DBH inhibition alone can increase the abundance of these DA signaling proteins, regardless of whether the inhibition results in behavioral hypersensitivity to cocaine (i.e. D/S does not facilitate cocaine sensitization but increases ΔFosB and pERK. Furthermore, increases in pERK and ΔFosB do not require changes in β-arrestin2 levels.

4.5 Discussion

DBH inhibition and behavioral sensitivity to cocaine

In addition to its effects on ALDH, disulfiram inhibits DBH, altering brain catecholamine concentrations. Because cocaine acts primarily via monoamines, a shift in NE and DA levels could alter its properties and underlie the efficacy of disulfiram in treating cocaine dependence. In support of this idea, we have shown that genetic and

selective pharmacological DBH inhibition results in behavioral hypersensitivity to psychostimulants and a D2 agonist (Weinshenker et al., 2002; Schank et al., 2006; Chapter 2) and that genetic DBH inhibition is associated with increases in DA signaling proteins such as pERK and ΔFosB in the striatum (Rommelfanger et al., 2007). Similarly, disulfiram treatment also increases sensitivity to cocaine-induced locomotion in rats (Haile et al., 2003). Furthermore, humans with genetically low DBH activity and those taking disulfiram experience dysphoria following cocaine administration and are particularly prone to cocaine-induced paranoia (Cubells et al., 2000; Dr. Robert Malison, personal communication). Because of the clinical efficacy of disulfiram and the similarities between disulfiram treatment and DBH inhibition, we decided to test whether disulfiram administration augments the psychomotor effects of cocaine on mice. We predicted that if disulfiram was active via DBH, then disulfiram-treated control mice would phenocopy *Dbh -/-* mice, and disulfiram would have no further effect on *Dbh -/-* mice. We further characterized the effect of disulfiram on the DA system by measuring levels of β-arrestin2, pERK and ΔFosB in the striatum.

Daily disulfiram-cocaine pretreatment enhanced cocaine-induced locomotion and stereotypy in control mice but had no effect on *Dbh -/-* mice. These result confirm previous reports that disulfiram enhances the behavioral effects of cocaine (Haile et al., 2003), and identify DBH inhibition as the mechanism of action. The cocaine responses of control mice pretreated with disulfiram-cocaine and vehicle-treated *Dbh -/-* mice are not identical (e.g. cocaine only elicits stereotypy in disulfiram-cocaine control mice at the dose tested, only *Dbh -/-* mice are hypersensitive to quinpirole). There could be several explanations for this. First, disulfiram pretreatment paired with saline did not affect

cocaine-induced locomotion after a challenge injection of cocaine, indicating that co-treatment with cocaine is required during the dosing regimen for disulfiram to exert its effect at a later timepoint. In addition, *Dbh -/-* mice have a complete and lifelong ablation of DBH, while disulfiram-treated control mice only experience several days of partial DBH inhibition. Finally, *Dbh -/-* mice have a specific deletion of DBH, while disulfiram inhibits the activity of many enzymes, some of which could impact cocaine-induced behaviors.

Neurochemical mechanisms of disulfiram action

Although we have not yet tested the effect of disulfiram on mesocorticolimbic signaling directly, we have shown that acute pharmacological (using the non-selective DBH inhibitor fusaric acid) or genetic DBH inhibition (*Dbh -/-* mice) results in decreased basal extracellular DA concentrations in the NAc and CP, while amphetamine-induced DA release is impaired in the NAc, CP, and PFC (Schank et al., 2006; Weinshenker et al., 2008). This reduction in DA transmission is likely due to the loss of NE production and noradrenergic drive onto midbrain dopaminergic neurons (reviewed by Weinshenker and Schroeder, 2007). ALDH also has an important role in DA metabolism, and its inhibition results in decreased DA synthesis and dampened dopaminergic transmission (Yao et al., 2010). Such a decrease in DA neurotransmission caused by disulfiram could result in blunted euphoric and stimulant effects of cocaine. However, patients in some laboratory studies report just the opposite, namely, augmentation of the “high” elicited from cocaine (McCance-Katz et al., 1998) and dextroamphetamine (Sofuoglu et al., 2008), and clearly the behavioral phenotypes in mice indicate DA hypersensitivity, not insensitivity.

However, it is important to note that chronic reduction in DA availability typically results in upregulation of DA receptor signaling in terminal regions (Arnt, 1985; Kim et al., 2000), which we believe underlies the behavioral hypersensitivity in *Dbh -/-* mice and disulfiram-cocaine treated control mice.

Molecular mechanisms of disulfiram action

β-arrestin2 is a protein involved in DA receptor desensitization (Kim et al., 2001), a phenomenon which is important for the modulation of DA-induced behaviors such as locomotion. ΔFosB overexpression enhances sensitivity to psychostimulants, augmenting locomotor responses to cocaine-induced locomotion, conditioned place preference and self-administration (Kelz et al., 1999; Colby et al., 2003). Similarly, phosphorylation of ERK, which occurs downstream of D1 signaling and cAMP accumulation, also increases following cocaine (Berhow et al., 1996), and administration of an ERK inhibitor attenuates cocaine-induced pERK accumulation and hyperlocomotion (Valjent et al., 2000). Finally, *Dbh -/-* mice, which are hypersensitive to a D2 agonist and psychostimulants, have reduced levels of β-arrestin2 levels in the NAc (Chapter 3) and increased pERK and ΔFosB in the striatum (Rommelfanger et al., 2007). Thus, we assessed the effect of disulfiram on the relative abundance of these proteins in the NAc of control mice.

Disulfiram pretreatment did not affect β-arrestin2 levels but did result in increased pERK and ΔFosB in the NAc compared to saline-saline pretreatment. Interestingly, disulfiram-saline and disulfiram-cocaine treatment had similar effects, but only the disulfiram-cocaine combination elicited behavioral hypersensitivity to cocaine. These

results suggest that reduction of β-arrrestin2 is not required for the observed changes in pERK and ΔFosB, and that changes in these 3 proteins are not required for behavioral cocaine hypersensitivity. One caveat to this interpretation is the non-specificity of disulfiram for DBH. Although the behavioral hypersensitivity conferred by chronic disulfiram + cocaine treatment appear to be mediated by DBH, as this effect is abolished in *Dbh -/-* mice, it is difficult to ascertain whether DBH inhibition contributes to the disulfiram-induced changes in DA signaling proteins. It is possible that the effect of disulfiram on other enzymes may result in molecular changes that counteract those due to specific DBH inhibition.

Disulfiram as an addiction pharmacotherapy

In the clinic, DBH inhibition by disulfiram may be contributing to abstinence of drug-taking in several ways. Dampening of noradrenergic transmission may decrease the ability of stress and environmental factors to induce relapse. Chronic intake of disulfiram may also enhance the interoceptive effects of cocaine, which include aversive effects such as paranoia, psychosis, anxiety and dysphoria. Other than some preliminary data suggesting that disulfiram increases cocaine-induced paranoia (R. Malison, personal communication), there is little clinical evidence to support these ideas, as most clinical trials have measured current cocaine use and used "frequency of drug use" as the point-prevalent variable. Unfortunately, these studies have not been consistently accompanied by thorough interviews and biochemical measures that can validate the mechanism underlying the effect of treatment. Future clinical studies should include interviews with participants with self-report measures in order to distinguish between abstinence due to

altered subjective drug effects versus healthier responses to environmental triggers. The knowledge acquired by studying disulfiram could be translated into safer and more effective pharmacotherapies for the treatment of cocaine dependence. Because disulfiram use is limited by its non-specificity, side effects and toxicity, the development and testing of selective DBH inhibitors will be essential to mechanistic studies as well as to improved therapeutics.

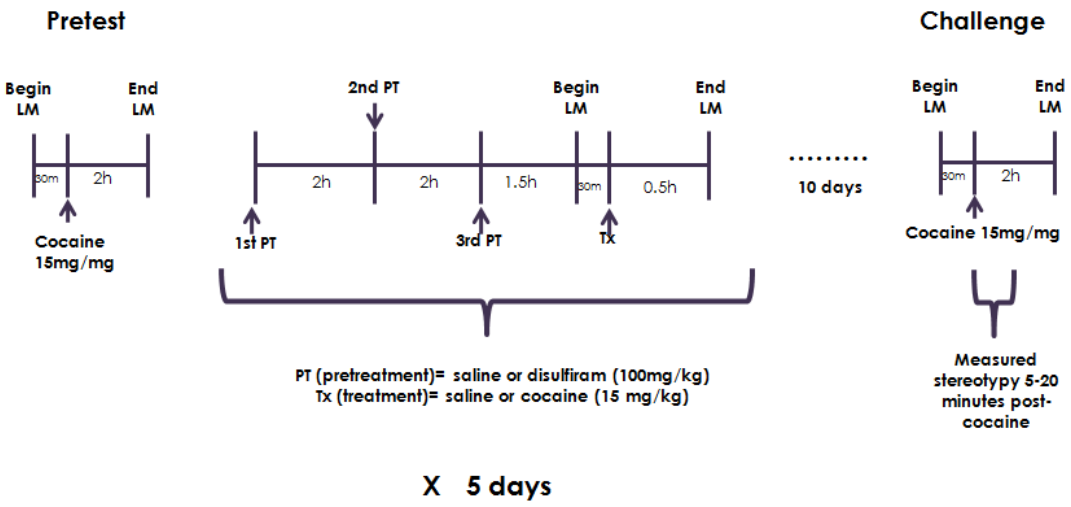


Figure 4.1 Behavioral sensitization paradigm timeline. On day 1, mice were injected with cocaine (15 mg/kg, i.p.) and were assigned to balanced treatment groups based on their cocaine-induced locomotor activity (LM).. On days 2-6, mice received 3 injections of saline or disulfiram (100 mg/kg, i.p.), each injection spaced 2 hr apart. Ninety min after the last pretreatment, mice were placed in locomotor chambers and LM measurements commenced. Thirty min later, mice were injected with saline or cocaine (15 mg/kg, i.p.) and their LM was recorded for an additional two hours. The mice then spent 10 days undisturbed in their home cage. On day 17, mice were placed in locomotor chambers for 30 min before receiving a cocaine injection (15 mg/kg, i.p.), and LM was recorded for 2 hr. Stereotypic behaviors were visually scored for 5 min, 5-20 min following cocaine injection.

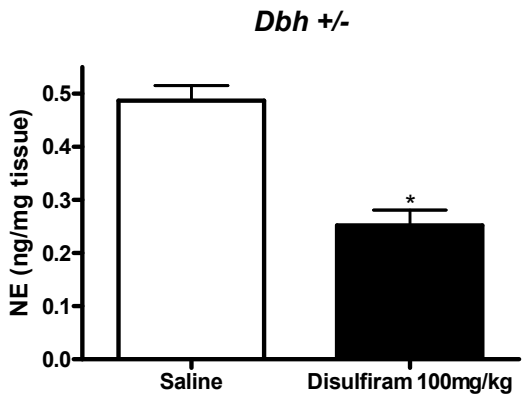
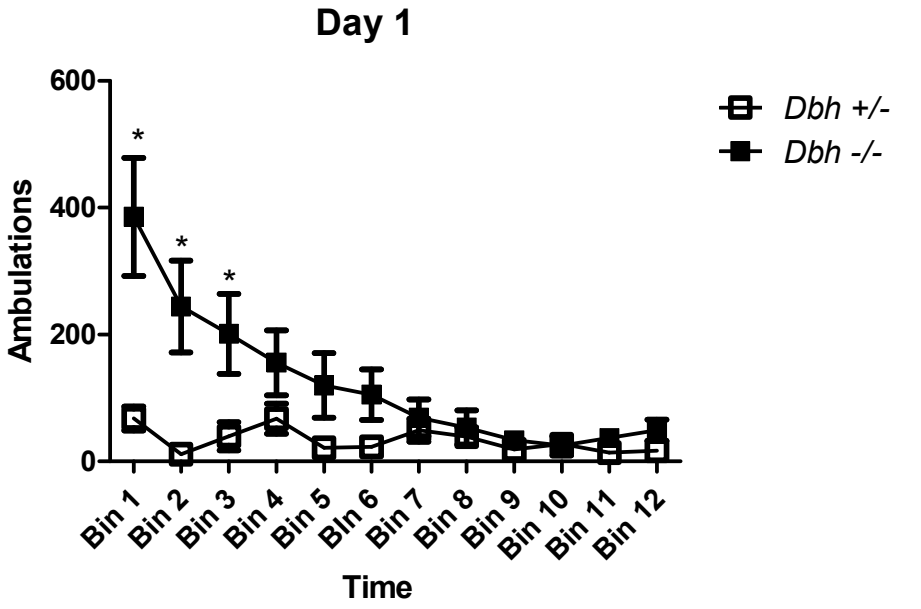


Figure 4.2. Disulfiram decreases norepinephrine in the prefrontal cortex. Mice received saline or disulfiram (3 injections of 100mg/kg, i.p., each injection spaced 2hr apart), and were euthanized 2 hr after the last injection. Prefrontal cortices were dissected out and NE levels were measured by HPLC. N=8 per genotype. *$p<0.001$.

A



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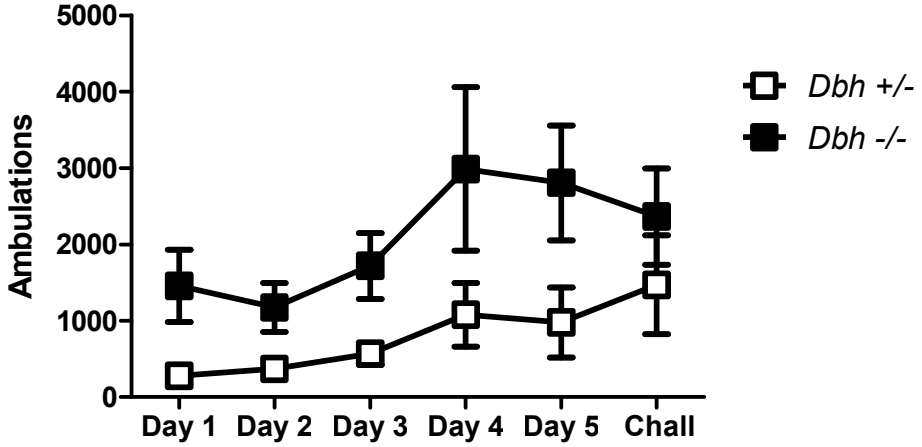

Figure 4.3 Cocaine-induced locomotion in control and *Dbh -/-* mice. A) B) *Dbh -/-* (n=8) and control mice (n=8) received a single injection of cocaine (15mg/kg) and their locomotion was recorded in for 2 hr, in 10 min bins. Shown is mean ± SEM ambulations

(consecutive beam breaks) for each 10 min bin, $p<0.05$. **B)** On days 1-5, *Dbh -/-* (n=8) and control mice (n=8) received saline pretreatments (3 injections, each spaced, 2 hr apart) followed by cocaine (15 mg/kg, i.p.) 2 hr after the last injection. Locomotion was recorded for 2 hr following the cocaine injection. Ten days later, all mice received a challenge (Chall) injection cocaine (15 mg/kg, i.p.) and their locomotion was recorded for 2 hr. Shown is mean $\pm$ SEM ambulations (consecutive beam breaks).

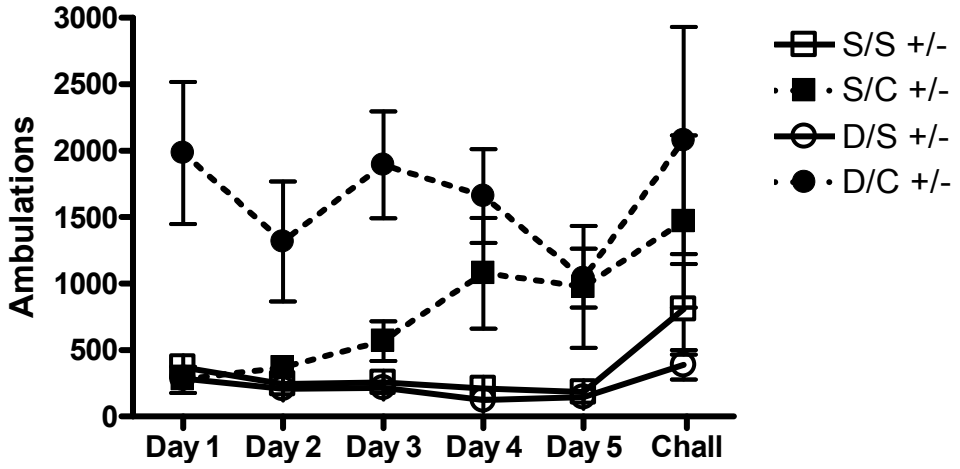

Figure 4.4. **Effect of disulfiram on locomotor activity in control mice.**

On days 1-5, control (*Dbh* +/-) mice were pretreated with saline or disulfiram (3 injections of 100 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Locomotor activity was recorded for the following 2 hr. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.) and their locomotor activity recorded for 2 hr. Shown is mean $\pm$ SEM ambulations (consecutive beam breaks). (S/S= saline/saline, n=7; S/C= saline/cocaine, n=9; D/S=disulfiram/saline, n=8; D/C= disulfiram/cocaine, n=5)

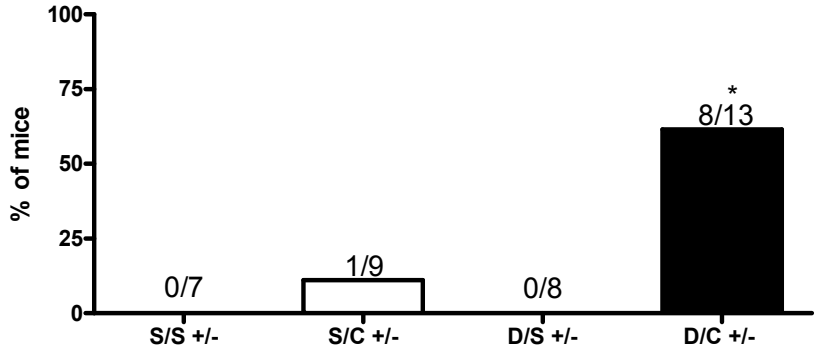

Figure 4.5. Effect of disulfiram treatment in stereotypic behavior in control and *Dbh* -/- mice. On days 1-5, control (Dbh +/-) and Dbh -/- mice were pretreated with saline or disulfiram (3 injections of 100 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.) and the presence of stereotypic behaviors was assessed for 5 min, 5-20 min following cocaine administration. Stereotypic behaviors were defined as circling, head-bobbing, nail biting and sniffing. *Dbh -/-* mice did not show stereotypic behaviors in response to cocaine. Shown is the percentage of mice engaged in stereotypy. * $p<0.05$ compared to S/S group. (S/S= saline/saline; S/C= saline/cocaine; D/S=disulfiram/saline; D/C= disulfiram/cocaine)

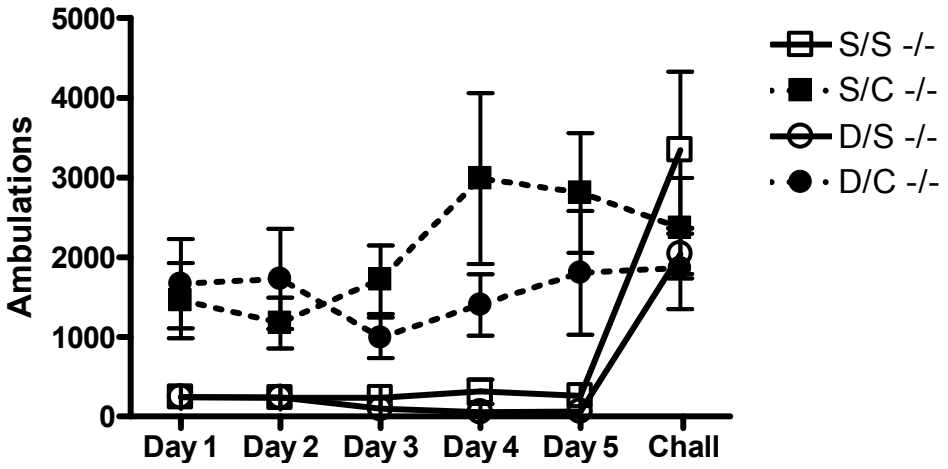

Figure 4.6. Effect of disulfiram treatment on locomotor activity in *Dbh -/-* mice.

On days 1-5, *Dbh* -/- mice were pretreated with saline or disulfiram (3 injections of 100 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Locomotor activity was recorded for the following 2 hr. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.) and their locomotor activity recorded for 2 hr. Shown is mean $\pm$ SEM ambulations (consecutive beam breaks). (S/S= saline/saline, n=7; S/C= saline/cocaine, n=7; D/S= disulfiram/saline, n=7; D/C= disulfiram/cocaine, n=7)

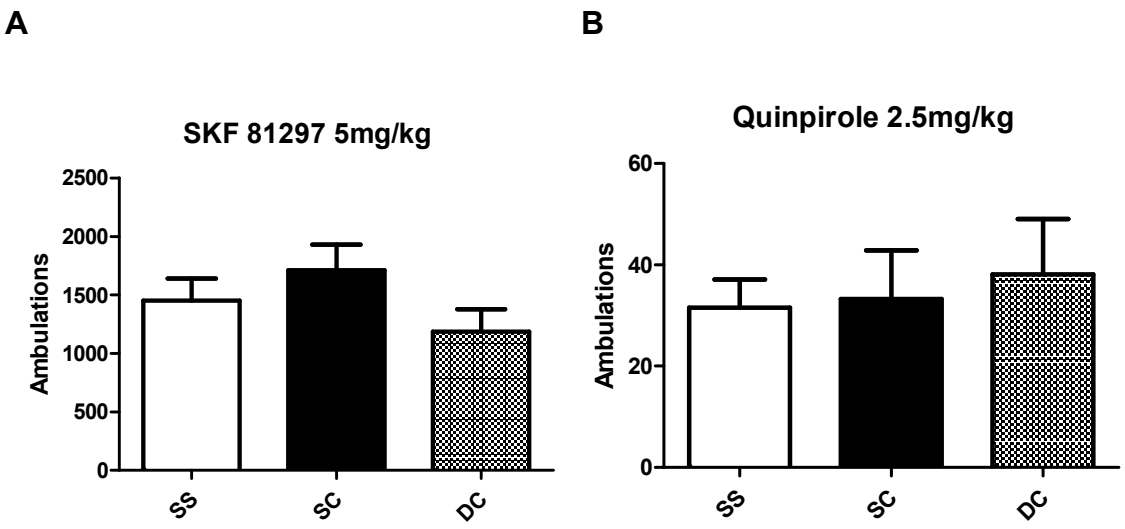

Figure 4.7 Effect of D1 and D2 receptor agonists on locomotor activity in control mice pretreated with saline and disulfiram. On days 1-5, control (Dbh +/-) mice were pretreated with saline or disulfiram (3 injections of 100 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. On days 6 and 7, mice were injected with the D1 agonist SKF 81297 (5 mg/kg, i.p.)(shown in panel **A**) or the D2 agonist quinpirole (2.5 mg/kg, i.p.) (shown in panel **B**) and their locomotor activity was recorded for 2 hr. Shown is mean ± SEM ambulations (consecutive beam breaks). (S/S= saline/saline, n=8; S/C= saline/cocaine, n=8; D/C= disulfiram/cocaine, n=8)

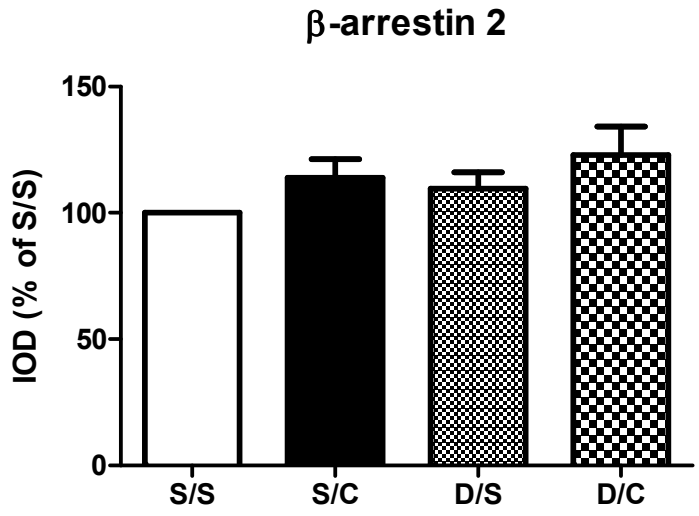

Figure 4.8 β-arrestin2 expression in the nucleus accumbens of control mice pretreated with disulfiram. For 5 days, *Dbh* -/- mice were pretreated with saline or disulfiram (3 injections of 100 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.). 24 hr later, mice were euthanized and the nucleus accumbens was dissected and processed for western blot analysis. Shown is integrated optical density ± SEM of densitometry analysis. N=8 per treatment. (S/S= saline/saline; S/C= saline/cocaine; D/S= disulfiram/saline; D/C= disulfiram/cocaine)

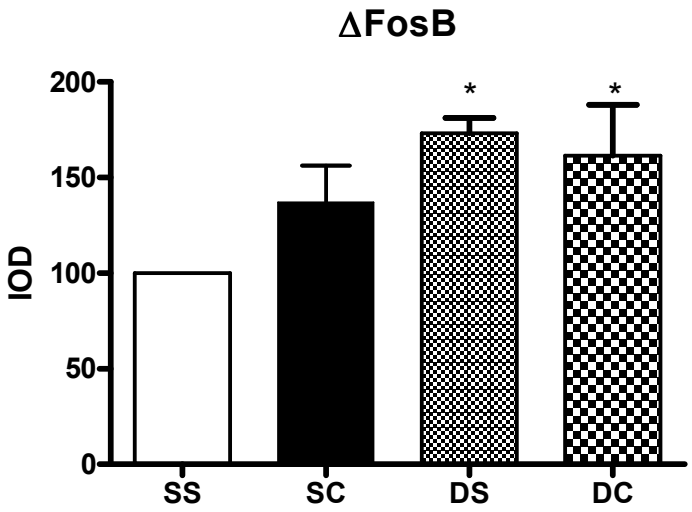

Figure 4.9 Effect of disulfiram pretreatment on accumbal ΔFosB levels in control mice. For 5 days, *Dbh* -/- mice were pretreated with saline or disulfiram (3 injections of 100 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.). 24 hr later, mice were euthanized and the nucleus accumbens was dissected and processed for western blot analysis. Shown is integrated optical density $\pm$ SEM of densitometry analysis. N=8 per treatment. (S/S= saline/saline; S/C= saline/cocaine; D/S= disulfiram/saline; D/C= disulfiram/cocaine), * $p<0.05$.

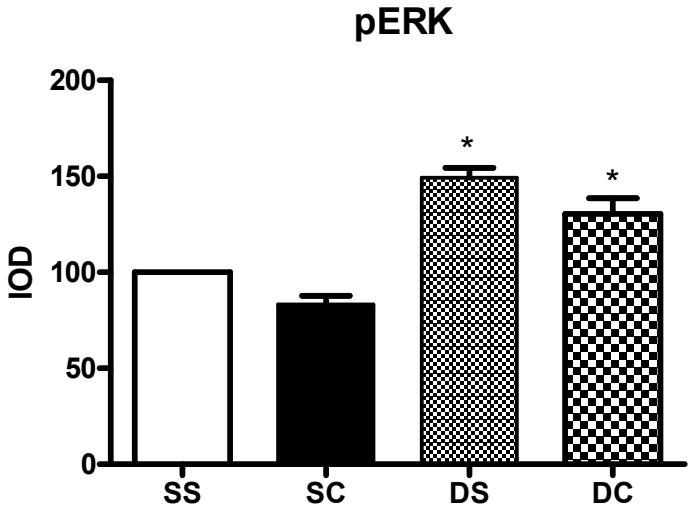

Figure 4.10 Effect of disulfiram pretreatment on accumbal pERK levels in control mice. For 5 days, *Dbh* -/- mice were pretreated with saline or disulfiram (3 injections of 100 mg/kg, i.p., each injection 2 hr apart) followed by saline or cocaine (15 mg/kg, i.p.) 2 hr after the last pretreatment. Ten days later, all mice received a challenge (Chall) injection of cocaine (15 mg/kg, i.p.). 24 hr later, mice were euthanized and the nucleus accumbens was dissected and processed for western blot analysis. Shown is integrated optical density $\pm$ SEM of densitometry analysis. N=8 per treatment. (S/S= saline/saline; S/C= saline/cocaine; D/S= disulfiram/saline; D/C= disulfiram/cocaine), * $p<0.0001$.

CHAPTER V:

DISCUSSION

5.1 Introduction

The experiments described in this dissertation have shown that genetic (*Dbh -/-* mice) or chronic selective pharmacological (nepicastat treatment) DBH inhibition enhances behavioral sensitivity to cocaine. In *Dbh -/-* mice, this behavior is accompanied by decreased β-arrestin2 and increased pERK and ΔFosB in the NAc. Nepicastat-treated control mice have decreased β-arrestin2 and increased ΔFosB in the NAc, but no change in pERK. Co-administration of cocaine and disulfiram (DS), a non-selective DBH inhibitor that has shown promise for the treatment of cocaine addiction, also enhances cocaine-induced behavioral sensitivity. DS treatment, alone or paired with cocaine, increases pERK and ΔFosB in the NAc, but does not alter β-arrestin2 in this region.

While increased pERK and ΔFosB are known to enhance cocaine responses, very little is known about the role of β-arrestin2 in mediating cocaine-induced behaviors. Because β-arrestin2 is directly downstream of DA receptor activation but upstream of pERK and ΔFosB, it is a likely candidate for the modulation of signaling proteins in response to dopaminergic drugs such as psychostimulants. To test whether β-arrestin2 is mediating the cocaine hypersensitivity conferred by DBH reduction, we performed *in vivo* manipulations of β-arrestin2 using viruses, knocking down its expression in the NAc core of control mice (to levels similar to those of *Dbh -/-* mice) and overexpressing it the same region in *Dbh -/-* mice (to bring their levels close to those of control mice). β-arrestin2 knockdown tended to increase cocaine-induced locomotion in control mice, while β-arrestin2 overexpression decreased it in *Dbh -/-* mice. These results represent the first evidence, to our knowledge, that manipulation of β-arrestin2 in the NAc core is necessary and sufficient to modulate cocaine-induced behaviors.

Dbh -/- mice and nepicastat-treated control mice have increased locomotion in response to the D2/3 agonist quinpirole but not the D1 agonist SKF81297, implicating enhanced D2 signaling in the behavioral hypersensitivity to cocaine. In order to establish the cellular underpinning of altered D2 signaling, we tested the quinpirole-induced electrophysiological responses of NAc core medium spiny neurons in control and *Dbh -/-* mice. We found that while quinpirole suppressed neuronal firing in neurons from control mice, as expected and previously reported, it has the opposite effect in neurons from *Dbh - /-* mice, facilitating neuron firing and increasing spiking frequency. This suggests that chronic loss of DBH produces a fundamental change in accumbal D2 signaling that underlies psychostimulant hypersensitivity.

Combined, these data confirm and extend the important role of DBH and NE in cocaine-induced behaviors and in DA signaling. The findings presented here provide insight into the functional interactions between the catecholamine systems, the role of receptor signaling protein alterations in DA system plasticity, and molecular pathways controlling psychostimulant responses. The implications of this work include defining a new player in cocaine sensitization (β-arrestin2), discovery of a potentially novel signaling pathway for DA D2 receptors in vivo, and promoting DBH inhibition as a strategy for cocaine addiction pharmacotherapy.

5.2 Dopamine β-Hydroxylase inhibition and cocaine-induced behaviors

We have previously shown that *Dbh -/-* mice are hypersensitive to acute cocaine-induced locomotion (Schank et al., 2006) as well as amphetamine-induced locomotion and stereotypy (Weinshenker et al., 2002). This behavioral profile is reminiscent of wild-

type animals that have undergone psychostimulant sensitization. Here, we show that chronic selective pharmacological DBH inhibition with nepicastat in adult animals closely phenocopies the *Dbh -/-* phenotypes, producing cocaine hypersensitivity expressed as increased cocaine-induced stereotypy that lasts at least 10 days following the last nepicastat injection.

While enhanced locomotion and stereotypy are not direct measures of cocaine reward, *Dbh -/-* mice are also hypersensitive to both the rewarding and aversive effects of cocaine, as measured by a place conditioning paradigm (Schank et al., 2006). Furthermore, cocaine abusers with low-activity *DBH* haplotypes have increased sensitivity to cocaine-induced paranoia (Cubells et al., 2000; Kalayasiri et al., 2007) and euphoria (R. Malison, personal communication). This enhancement of both modalities indicates that DBH inhibition may increasing both the "positive", rewarding effects of cocaine such as euphoria, and the "negative", aversive effects such as paranoia and anxiety. Testing the effect of pharmacological DBH inhibition on cocaine place conditioning would address whether the increase of the locomotor-activating effects of cocaine correlates with enhanced interoceptive effects of the drug. Drug discrimination is another possibility for examining how DBH reduction influences the subjective effects of cocaine.

Another way to test the reinforcing properties of cocaine is using an operant self-administration model. We have shown that acute administration of nepicastat has no effect on cocaine responding on an FR1 schedule (Schroeder et al., 2010), but significantly reduces the breakpoint for cocaine responding in a progressive ratio schedule (J. Schroeder, personal communication). This reduction in breakpoint is

indicative of an enhancement of the interoceptive effects of cocaine. The hypersensitivity to cocaine (which extends to cocaine aversion; Schank et al., 2006) only occurs following chronic DBH inhibition (*Dbh -/-* mice or 5 day administration of DBH inhibitors). In order to fully address the question of whether DBH inhibition enhances the rewarding or aversive effects of cocaine, the effect of DBH inhibitors should be tested on a dose-response curve of cocaine, as well administered using a chronic dosing regimen before testing its effect on cocaine responding.

Schroeder et al., also showed that acute administration of nepicastat blocks cocaine-primed reinstatement only at doses that significantly inhibit DBH (Schroeder et al., 2010). Nepicastat can also attenuate reinstatement elicited by stress or cocaine-associated cues (J. Schroeder, personal communication).These results suggest that acute DBH inhibition can interfere with the ability of different classes of stimuli to trigger relapse-like behavior, and is consistent with previous data showing that NE signaling via α_1 and β adrenergic receptors is required for different forms of reinstatement of drug-seeking (Leri et al., 2002; Lee et al., 2004; Zhang and Kosten, 2005; Platt et al., 2007; Brown et al., 2009, 2011; Schroeder et al., 2010; Smith and Aston-Jones, 2011). We speculate that acute DBH inhibitor administration is reducing NE production and signaling, thus lowering the likelihood of relapse in the face of environmental influences and stressors. By combining a reduction of NE with a hypersensitive DA system, we propose that chronic DBH inhibition decreases cocaine intake by simultaneously reducing the risk of relapse and, if patients do relapse, increasing cocaine aversion.

5.3 Dopamine receptor signaling

Because we failed to detect any differences in D2 receptor abundance, we hypothesized that behavioral hypersensitivity to psychostimulants and D2 agonists following genetic or pharmacological DBH inhibition was due to alterations in DA signaling proteins downstream of the receptor at the cell surface. In order to lay the foundation for our next set of experiments and model of how chronic DBH inhibition leads to dopaminergic hypersensitivity, the DA receptor signaling cascades will be summarized in the following paragraphs.

Following binding of an agonist, DA neurotransmission is modulated by two classes of G-protein coupled receptors, D1-like (D1, D5) and the D2-like (D2, D3 and D4). The two DA receptor families typically have opposing effects, with activation of D1-like receptors promoting neuronal depolarization and excitation, and activation of D2-like receptors promoting neuronal hyperpolarization and inhibition.

The D1-like receptors signal via the heterotrimeric G proteins $G_{\alpha s}$ and $G_{\alpha olf}$, which activate adenylate cyclase (AC) (Jones and Reed, 1989). AC catalyzes the conversion of ATP into cyclic AMP (cAMP), which binds to protein kinase A (PKA), disinhibiting its catalytic subunits. PKA, in turn, phosphorylates many proteins involved in signal transduction and regulation of gene expression (reviewed by Neve et al., 2004), such as dopamine and cyclic AMP-regulated phosphoprotein, 32kDA (DARPP-32). DARPP-32, in turn, phosphorylates and inactivates protein phosphatase 1 (PP1). PP1 inhibition prevents it from dephosphorylating other DARPP-32 targets. Therefore, its inactivation serves a signal amplification role for DARPP-32 (reviewed by Neve et al., 2004). PPI inhibition, along with PKA/DARPP-32 activation, leads to the phosphorylation and

opening of several voltage and ligand-gated cation channels, resulting in depolarization of the cell membrane (Figure 5.1). D1 receptor stimulation also activates the extracellular signal regulated kinase (ERK), which modulates the phosphorylation and expression of transcription factors such as the cAMP response element binding protein (CREB) and ΔFosB. Both CREB and ΔFosB-mediated changes in gene expression are important in synaptic plasticity and are likely involved in synaptic rearrangements that underlie behavioral sensitization to psychostimulants (Konradi et al., 1994; Liu and Graybiel, 1996; Berke and Hyman, 2000; Nestler et al., 2001; Zhang et al., 2002; Muller and Unterwald 2004, 2005).

D2-like receptor signaling, on the other hand, is mediated by $G_{\alpha i/o}$. D2 activation inhibits AC and the formation of cAMP, and decreases PKA-stimulated phosphorylation of DARPP-32. $G_{\alpha i/o}$ signaling increases potassium currents and decreases the activity of N-and L-type channels, thus hyperpolarizing neurons and reducing cell excitability (Surmeier et al., 1993; Yan et al., 1997; Herlitze et al., 1997; Zamponi and Snutch, 1998; Hernandez-Lopez et al., 2000) . In addition to its effect on PKA, D2 also signals via Gβγ subunits that are released upon the activation of $G_{\alpha i/o}$ proteins (reviewed by Neve et al., 2004). Activation of D2 receptors also stimulates mitogen-activating protein kinases (MAPK), including ERK, via $G_{\alpha i/o}$, Gβγ, and the MAPK MEK (Alblas et al, 1993; Luo et al., 1998; Welsh et al., 1998; Choi et al., 1999; Yan et al., 1999; Cussac et al., 1999; Ghahremani et al., 2000; Cai et al., 2000; Oak et al., 2001; Wang et al., 2004)(Figure 5.2). This D2 pathway stimulates DNA synthesis and is involved in cell survival, synaptic plasticity, and acute beavioral responses to dopamine receptor stimulation (Fukunaga and Miyamoto, 1998; Otani et al., 1999; Impey et al., 1999; Cai et al., 2000). On its own,

Gβγ can stimulate AC in heterologous systems and possibly in neurons (Hopf et al., 2003).

Dopamine receptor desensitization: role of arrestins

Following binding of an agonist, GPCRs such as D1 and D2 receptors undergo receptor desensitization. The scaffolding proteins β-arrestin1 and β-arrestin2 have been traditionally associated with the termination of GPCR signaling and receptor internalization (Attramadal et al., 1992; Ferguson et al., 1996; Lefkowitz, 1998; Pitcher et al., 1998). After agonist binding, GPCRs are phosphorylated by G-protein-coupled receptor kinases (GRKs), which then signal the recruitment of β-arrestins. β-arrestin binding blocks further G protein activation by sterically hindering access to the receptor binding domains, causing desensitization of G-protein signaling (reviewed by Shenoy and Lefkowiz, 2011). β-arrestins are also involved in receptor trafficking by mediating receptor endocytosis via clathrin-coated pits (Zhang et al., 1996; Goodman et al., 1996). For example, overexpression of β-arrestins enhances agonist-stimulated β-adrenergic receptor internalization (Ferguson et al., 1996; Kohout et al., 2001; Ahn et al., 2003) and β-arrestin2 knockout mice (*βArr2 -/-*) are incapable of internalizing D2 receptors (Skinbjerg et al., 2009).

In addition to its their role in GPCR desensitization and internalization, the recruitment of β-arrestin2 to D1 and D2 receptors can activate cellular signaling in a G protein-independent manner. For example, following prolonged stimulation of the D2 receptor, β-arrestin2 acts as a scaffold for signaling complexes including Akt, GSK3β, and protein phosphatase 2A (PP2A) in the striatum (Beaulieu et al., 2005; Urs et al.,

2011). DA-associated behaviors, such as tolerance to morphine is attenuated in βArr2 -/- mice, indicating that, in addition to mediating GPCR desensitization, β-arrestin2 also promotes some positive modalities of DA receptor signaling (Beaulieu et al., 2005).

5.3.1 Changes in dopamine signaling proteins following selective dopamine β-hydroxylase inhibition

In our current study, we found that while most DA signaling proteins were unchanged in *Dbh* -/- mice, they did have decreased β-arrestin2 and increased ΔFosB and pERK in the NAc. These alterations are indicative of enhanced dopaminergic activity, and nepicastat treatment recapitulated the decrease in β-arrestin2 and increase in ΔFosB. To determine whether the signaling capabilities of β-arrestin2 via Akt and GSK3β contribute to the *Dbh* -/- phenotype, we assessed abundance of these proteins but did not detect any differences compared to control mice. These results suggest that direct β-arrestin2 signaling is not important for the dopaminergic hypersensitivity in *Dbh* -/- mice, and point to a primary role for changes in β-arrestin2-DA receptor interactions.

A causal relationship between decreased β-arrestin2 and the upregulation of ΔFosB and pERK has not been established. To determine the molecular underpinnings of β-arrestin2 in DA-agonist induced behaviors, we can test how β-arrestin2 affects DA signaling proteins. Using western blotting and immunohistochemistry, we can assess the abundance of D1 and D2 signaling proteins such as ΔFosB and pERK in the NAc of mice with β-arrestin2 knockdown or overexpression.

In addition to inducing alterations in DA signaling, β-arrestin2 can also affect affect DA receptor trafficking. In order to study whether β-arrestin2 affects DA receptor

trafficking in vivo, we can perform live cell imaging in primary cultures from *Barr2 -/-* mice or mice with knockdown or overexpressed β-arrestin2. This assay will measure the 'turnover rates' of membrane-bound DA receptors following treatment with DA agonists.

5.3.2 β-arrestin2 and dopamine-dependent behaviors

In addition to decreasing β-arrestin2 in the NAc, selective pharmacological DBH inhibition leads to an enhancement in behavioral hypersensitivity to cocaine. To test whether β-arrestin2 modulates cocaine-induced behaviors, we measured locomotor activity in response to cocaine following in vivo manipulations of β-arrestin2 in the NAc core and found that β-arrestin2 is a modulator of this behavior. While *β-arr2 -/-* do not show changes in cocaine-induced locomotion or place conditioning (Bohn et al., 2003), this indicates the importance of this protein in the NAc core in the modulation of locomotion to cocaine. Given the regional specificity of this result, it would be telling to test the effect of β-arrestin2 manipulations in other specific brain regions on other cocaine-induced behaviors, such as the NAc shell and place conditioning.

Selective DBH inhibition and decreased β-arrestin2 also correlate with increased quinpirole-induced locomotion. To determine the role of β-arrestin2 in the modulation of responses to DA agonists, we can test whether knockdown and overexpression of β-arrestin2 in the NAc result in changes in locomotion to dopaminergic drugs. Measuring D1- an D2-agonist-induced locomotion following knockdown or overexpression of β-arrestin2 can be used clarify D1 vs. D2 interactions with β-arrestin2.

5.3.3 Changes in nucleus accumbens cellular responses following selective dopamine β-hydroxylase inhibition

If low β-arrestin2 levels following DBH inhibition can affect the length of time of time a DA receptor is able to signal, it is possible that compensatory mechanisms would take effect to counteract this. We have shown that NAc core neurons of *Dbh -/-* mice have increased neuronal firing in response to the D2 agonist quinpirole. Because D2 receptors are typically G_i-coupled and their activation inhibits neuronal activity, this effect may be due to a switch in G-protein coupling from G_i to G_s. Cannabinoid CB1 receptors and μ-opioid receptors, both G_i-coupled in the striatum, are able to switch from G_i to G_s coupling following chronic treatment with an agonist (Wang et al., 2005; Paquette et al., 2007) . Therefore, it is possible that enhanced dopaminergic signaling as a result of decreased β-arrestin2 would lead to a similar switch in G-protein coupling in D2 receptors. This argument is strengthened by the fact that phenotypically, naïve *Dbh -/-* mice resemble mice that have undergone a sensitization regimen with cocaine and that genetic and pharmacological DBH inhibition confers behavioral sensitivity to a D2 agonist, but not a D1 agonist. To confirm that DBH inhibition is responsible for this effect, we can perform similar experiments in slices from mice treated with nepicastat. To test the G_i to G_s switch hypothesis, G_i/G_s pharmacological inhibitors may be co-applied with quinpirole in slices from *Dbh -/-* and control mice. If such a switch underlies the increased neuronal activity following D2 agonist treatment, then a Gi inhibitor would block quinpirole-induced inhibition in control mice but have no effect in *Dbh -/-* mice, while a G_s inhibitor would block quinpirole-induced excitation in *Dbh -/-* mice but have no effect on control mice. In order to elucidate whether quinpirole-induced excitation in

Dbh -/- mice is due to changes in sodium, calcium (inducing cell depolarization) or potassium (reducing cell hyperpolarization) currents, various ion channel blockers could be systematically tested.

There are two additional alternatives to the G_i to G_s switch hypothesis. It is possible that following D2 activation, the Gβγ subunit is preferentially activated and interacts with a different subtype of AC, inducing cAMP production and cell depolarization further downstream. To test this, a pharmacological Gβγ inhibitor can be testes in slices from *Dbh -/-* and control mice. Alternatively, $G_{\alpha q}$ signaling, usually seen in response to activation of D1-D2 heterodimers, may be activated. If *Dbh -/-* mice have an increased proportion of these DA heterodimers in the NAc core, $G_{\alpha q}$ may be the most prevalent signaling G-protein and increase cell excitability. Treatment of slices with a DA agonist selective for D1-D2 heterodimers can test whether activation of this particular G protein is responsible for the enhanced firing rate following quinpirole in *Dbh -/-* mice.

5.3.4 β-arrestin2 and nucleus accumbens cellular responses

Because *Dbh -/-* mice have decreased β-arrestin2 in the NAc, a decrease in the availability of this protein in this region may underlie the facilitatory effect of quinpirole in *Dbh -/-* mice. To test the role of β-arrestin2 in cellular responses, we can perform electrophysiological recordings from neurons from *βarr2 -/-* mice or mice with knockdown or overexpessed β-arrestin2 in response to selective D1 and D2 agonists. In addition, we can test for changes in membrane properties such as threshold of action potential, spike amplitude, action potential duration, resting membraine potential and

input resistance. Specifically, if we wish to learn whether β-arrestin2 affects G_i or G_s DA receptor coupling and therefore signaling, striatal homogenate can be treated with DA agonists in the presence of radioactively-labeled GTPγS followed by co-immunoprecipitation of G_α particles and probing for G_i or G_s subunits. Quantification of the incorporated radioctivity into the G_α particles will give a measure of preferential G-protein coupling.

5.4 Putative mechanism of action of disulfiram

Disulfiram and cocaine-induced behaviors

Chronic treatment with the non-selective DBH inhibitor DS accelerates cocaine sensitization (locomotion and stereotypy), but unlike nepicastat, it only does so when paired with cocaine. These results are in agreement with a previous report of DS enhancing behavioral sensitization to cocaine in rats (Haile et al., 2003). *Dbh -/-* mice are unaffected by either nepicastat or DS treatment, suggesting that the effects of these drugs are mediated, at least in part, by DBH. In support of this Schroeder et al., showed that acute administration of DS mimics the effect of nepicastat and blocks cocaine-primed reinstatement (Schroeder et al., 2010). Given the similarities between nepicastat and DS treatment, nepicastat or another selective DBH inhibitor would be a safer alternative to DS in the clinic. To test this, clinical trials both DS and nepicastat should be accompanied by thorough interviews in which participants give self-reported measures of the subjective effects of cocaine throughout the study, before and after periods of abstinence.

Dopamine neurotransmission following disulfiram treatment

Because noradrenergic neurons project both directly and indirectly to midbrain dopamine (DA) neurons, where they regulate firing patterns and DA release (Swanson and Hartman, 1975; Jones and Moore, 1977; Grenhoff et al, 1993; Grenhoff and Svensson, 1993; Darracq et al, 1998; Ventura et al, 2003; Liprando et al, 2004), loss of NE is expected to result in decreased DA release from midbrain neurons. In fact, NE depletion by locus coeruleus lesions decrease DA availability in the striatum (Russell et al, 1989; Lategan et al, 1990, 1992) and *Dbh -/-* mice have decreased basal and amphetamine-induced extracellular DA levels in the NAc and CP (Schank et al., 2006). A decrease in DA release typically results in supersensitivity of striatal DA receptors, as is the case following locus coeruleus lesions (Donaldson et al, 1976; Lategan et al, 1989; Harro et al, 2000).

Because genetic or pharmacological DBH inhibition decreases basal, amphetamine- and methamphetamine-evoked DA release in the striatum (Schank et al., 2006; Weinshenker et al., 2008), we always assumed that cocaine-induced DA release would similarly be attenuated. However, Devoto et al. (2011) recently found that DS *increased* basal DA in every region tested (medial prefrontal cortex, occipital cortex, NAc and CP) and cocaine-induced DA in the medial prefrontal cortex. Both basal and cocaine-induced DA release was prevented by systemic administration of the α2agonist clonidine, suggesting that DS removes NE-mediated inhibitory control, inducing DA release from those noradrenergic terminals. The lack of effect of DS on cocaine-induced DA in the NAc and CP was attributed to allogenic DA clouded by DA released from

dopaminergic terminals. These results are difficult to reconcile with those of *Dbh -/-* mice and the authors suggest that the difference is due to compensation phenomena in the mice as a consequence of genetic DBH ablation. However, we have shown that pharmacological DBH inhibition results in phenotypes similar to those seen in *Dbh-/-* mice. In this dissertation, we have also provided evidence that genetic or pharmacological DBH inhibition result in similar changes in DA signaling molecules. In addition, the authors conclude that increased DA release by DS may act as a replacement therapy for cocaine addiction. However, they do not address the large body of evidence indicating that activation of the dopamine system by D1 receptors increases cocaine-seeking behavior in animal models of addiction (Sánchez et al., 2003; Capriles et al., 2003; McFarland et al., 2004; Sun and Rebec, 2005). It will be important for another group to repeat these experiments and confirm the results, and a similar study testing the effect of nepicastat would also be valuable.

Dopamine-signaling proteins following disulfiram treatment

DS treatment did not fully recapitulate the changes in DA signaling proteins seen following selective DBH inhibition with nepicastat or in naïve *Dbh -/-* mice. DS treatment did increase pERK and ΔFosB, but did so regardless of whether it induced behavioral hypersensitivity to cocaine. That is, DS/S treatment was sufficient to increase relative levels of these proteins but did not result in enhanced locomotion or stereotypy in response to cocaine. Moreover, these changes in DA-signaling proteins were not accompanied by a change in β-arrestin2. Together, this implies that behavioral hypersensitivity to cocaine is not solely mediated by changes in these proteins. It is likely

that multiple combinations of factors are able to elicit this phenotype, including changes in proteins that we have yet to test. This is supported by our data showing β-arrestin2 knockdown does not fully reproduce the *Dbh -/-* phenotype and that nepicastat treatment enhances the behavioral effects of cocaine without increasing pERK levels. Each of the DA signaling elements discussed in this dissertation likely provide individual contributions to this behavior, modulating its expression, but do not completely mediate the effects of cocaine.

5.5 Conclusion

Together, our data suggests the following model: Selective, chronic *Dbh -/-* inhibition decreases NE synthesis. Loss of noradrenergic tone leads to decreased DA release from midbrain dopaminergic neurons. As a consequence of this reduction in DA release, post-synaptic DA signaling becomes enhanced in the NAc. This enhancement in DA signal is partially due to decreases in D2-associated β-arrestin2 in this region. Chronic reduction of β-arrestin2 leads to a molecular switch in D2-coupled G proteins, from G_i to G_s. Recruitment of G_s following agonist binding to the D2 receptor leads to the activation of DA-associated G_s signaling proteins (those normally activated by D1 agonist binding), which may include pERK and ΔFosB. Activation of these targets results in the transcription of genes that mediate synaptic plasticity in DA-dependent behaviors such as behavioral responses to psychostimulants (Figure 5.3).

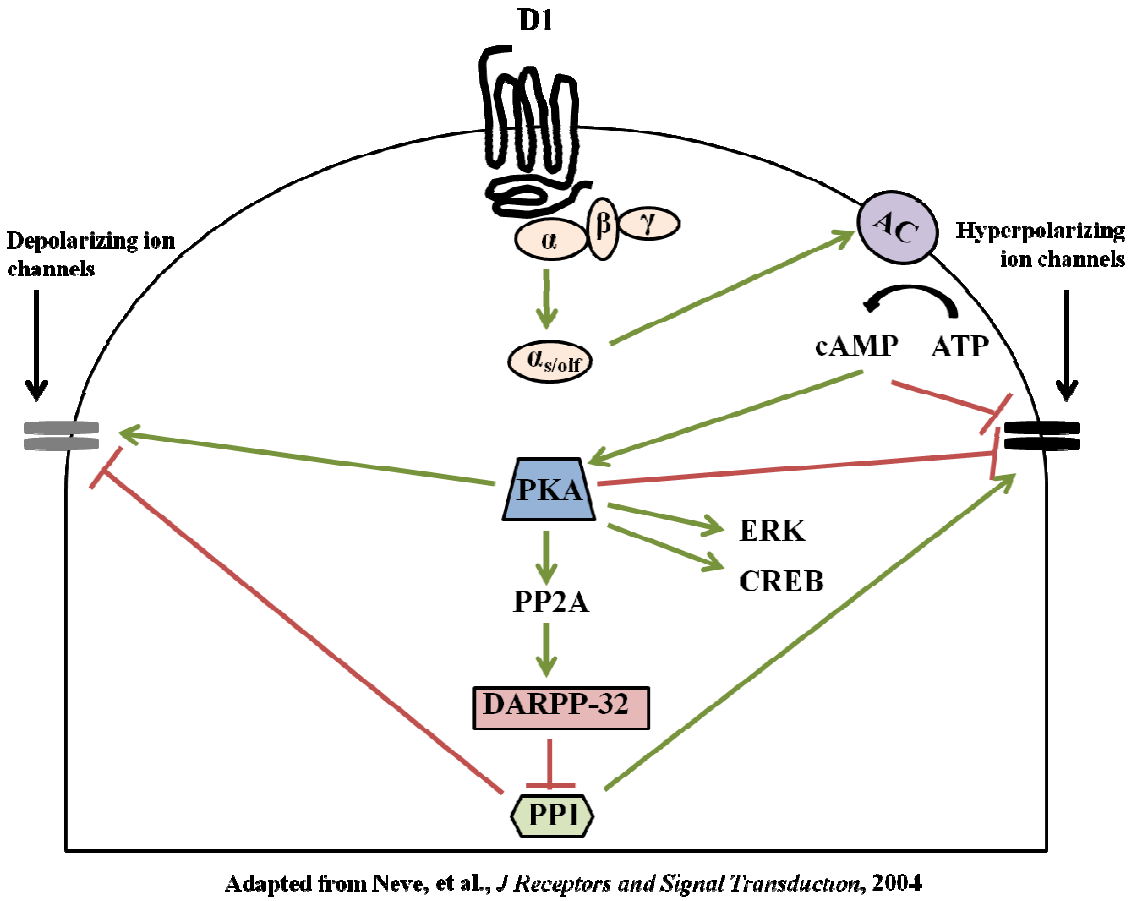

Figure 5.1 Dopamine D1 signaling pathway. D1 receptor activation leads to $G\alpha_s$ signaling, resulting in activation of adenyl cyclase (AC) and formation of cAMP. The net result is the activation of depolarizing ion channels and inhibition of hyperpolarizing channels, resulting in increase cellular activation and increased firing rate.

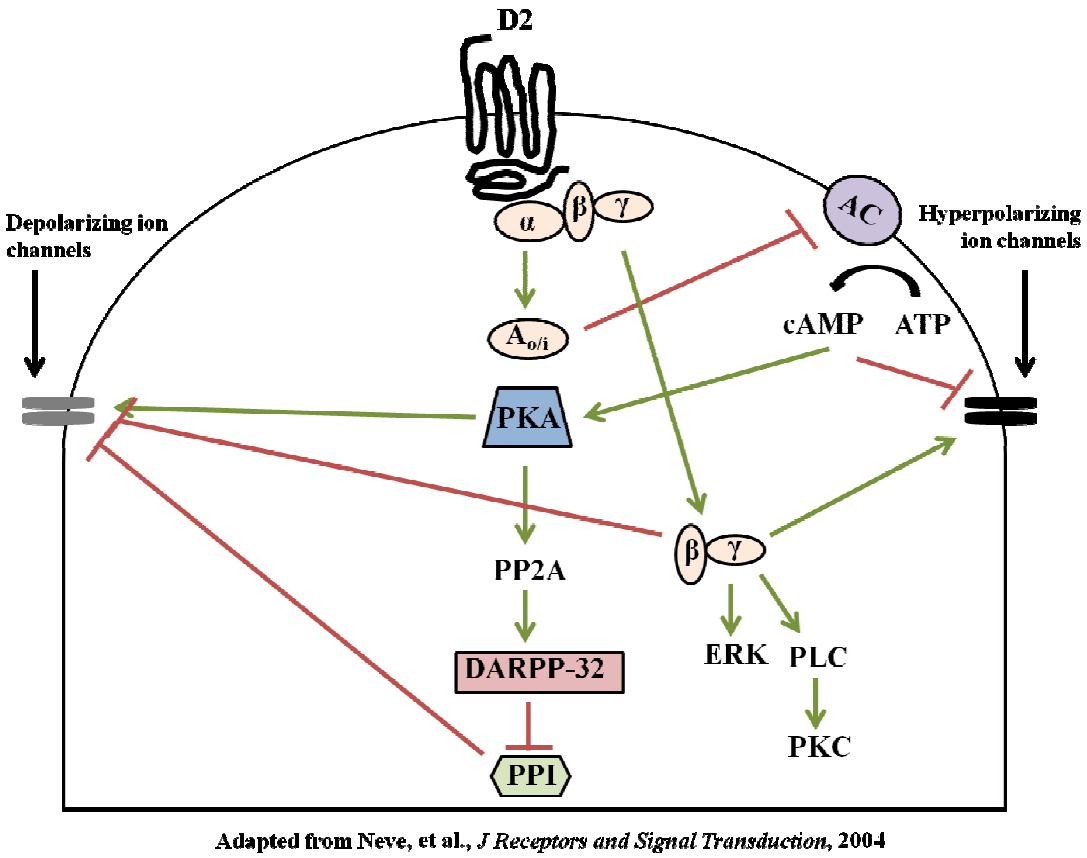

Figure 5.2 Dopamine D2 signaling pathway. D2 receptor activation leads to $G\alpha_i$ signaling, resulting in inhibition of adenyl cyclase (AC) and preventing the formation of cAMP. The net result is the inhibition of depolarizing ion channels and activation of hyperpolarizing channels, resulting in increase cellular inhibition and lower firing rate.

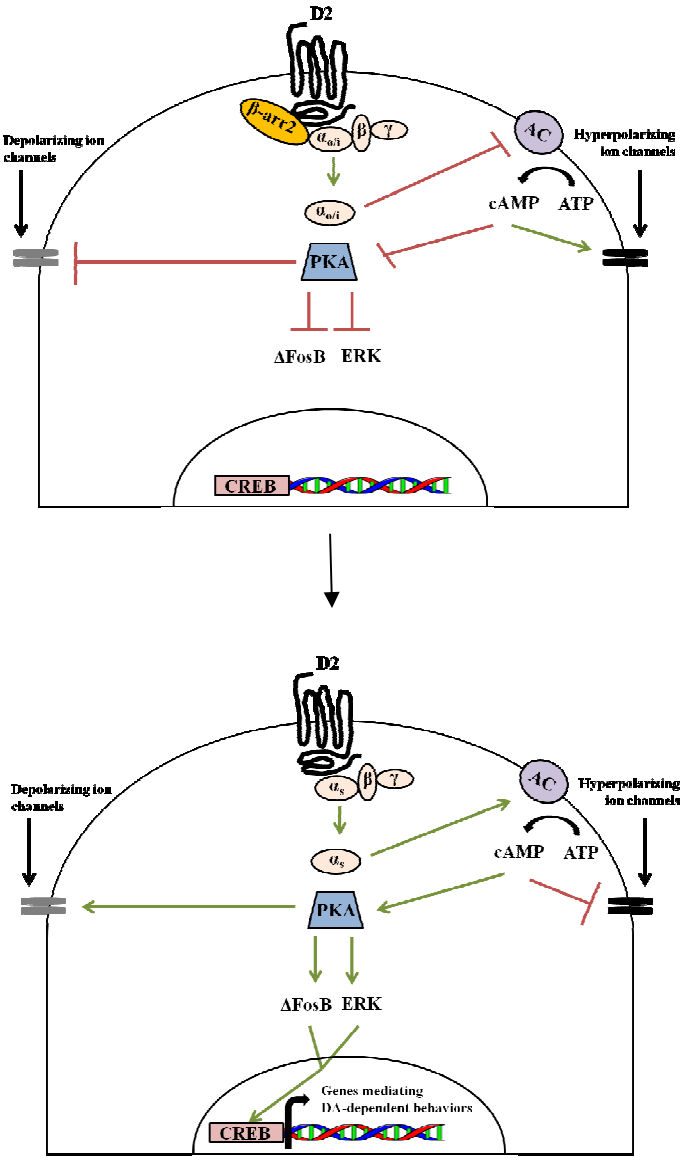

Figure 5.3 Model of dopamine D2 signaling following DBH inhibition. Following selective DBH inhibition, β-arrestin2 levels are decreased in the nucleus accumbens. Reduced β-arrestin2 availability leads to a switch in D2 receptor G-protein coupling, with $G\alpha_s$ now coupling to the receptor. This results in activation of adenyl cyclase (AC) and the formation of cAMP. The net result is now ion channel depolarization and inhibition of hyperpolarizing channels, resulting in increase cellular activation and firing rate.

CHAPTER VI:

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APPENDIX A1

EFFECTS OF DISULFIRAM AND DOPAMINE BETA-HYDROXYLASE GENOTYPE ON COCAINE-INDUCED SEIZURES

Adapted from:

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A1.1 Abstract

The antialcoholism drug disulfiram has shown recent promise as a pharmacotherapy for treating cocaine dependence, probably via inhibition of dopamine β-hydroxylase (DBH), the enzyme that catalyzes the conversion of dopamine (DA) to norepinephrine (NE). We previously showed that DBH knockout (*Dbh* -/-) mice, which lack NE, are susceptible to seizures and are hypersensitive to the psychomotor, rewarding, and aversive effects of cocaine, suggesting that disulfiram might exacerbate cocaine-induced seizures (CIS) by inhibiting DBH. To test this, we examined CIS in wild-type and *Dbh* -/- mice following administration of disulfiram or the selective DBH inhibitor nepicastat. We found that *Dbh* genotype had no effect on CIS probability or frequency, whereas disulfiram, but not nepicastat, increased the probability of having CIS in both wild-type and *Dbh* -/- mice. Both disulfiram and nepicastat increased CIS frequency in wild-type but not *Dbh* -/- mice. There were no genotype or treatment effects on serum cocaine levels, except for an increase in disulfiram-treated *Dbh* -/- mice at the highest dose of cocaine. These results suggest that disulfiram enhances CIS via two distinct mechanisms: it both increases CIS frequency by inhibiting DBH and increases CIS frequency in a DBH-independent manner.

A1.2 Introduction

Cocaine is considered the most potent stimulant of natural origin, yet despite a host of negative social, psychological, and medical consequences of the drug's abuse, there are currently no widely used pharmacotherapies for cocaine addiction. Recently, the compound disulfiram, also known as Antabuse has shown promise as a candidate pharmacotherapy. Disulfiram has been FDA approved for the treatment of alcohol dependence for over 50 years. Its efficacy is due to the inhibition of the enzyme aldehyde dehydrogenase, which induces an aversive reaction following alcohol consumption by elevating toxic aldehydes in the liver. This "Antabuse reaction" manifests as flushing, headache, nausea, weakness, dizziness, anxiety, vertigo, and ataxia. Interestingly, disulfiram has been shown to decrease cocaine intake regardless of concurrent alcohol consumption (Carroll et al., 1998, 2000; George et al., 2000; Petrakis et al., 2000), although the mechanisms by which it does so have not been fully elucidated. Nevertheless, because the combination of disulfiram and cocaine does not result in aldehyde accumulation, aldehyde dehydrogenase inhibition probably cannot account for the efficacy of disulfiram here. Because its major metabolite (diethyldithiocarbamate) is a copper chelator (Johansson, 1989), disulfiram affects enzymatic reactions that require copper as a cofactor. One hypothesis to account for disulfiram's efficacy in reducing cocaine intake is its inhibition of dopamine β-hydroxylase (DBH). DBH is a copper-containing monooxygenase enzyme that converts dopamine (DA) to norepinephrine (NE), thus controlling NE production and consequently the NE/DA ratio in noradrenergic neurons. Alteration of this ratio has been found to alter behavioral responsivity to cocaine in rodents and humans. For example, DBH knockout (*Dbh* -/-) mice are hypersensitive to

the locomotor, rewarding, and aversive effects of cocaine (Schank et al., 2006). Pharmacological inhibition of DBH with disulfiram, which decreases the NE/DA ratio in the rodent brain (Karamanakos et al., 2001; Bourdélat-Parks et al., 2005), facilitates the development of behavioral sensitization to cocaine (Haile et al., 2003). Furthermore, a common polymorphism in the *Dbh* gene influences both DBH enzymatic activity and cocaine-induced paranoia (Zabetian et al., 2001; Kalayasiri et al., 2007).

Noradrenergic transmission has been implicated in the modulation of seizure activity (reviewed by Weinshenker and Szot, 2002). Enhancement of noradrenergic transmission suppresses seizure activity (Lindvall, et al., 1988; Weinshenker et al., 2001; Kaminski et al., 2005), whereas norepinephrine depletion with 6-hydroxydopamine or disulfiram exacerbates seizures and facilitates seizure kindling (Corcoran, et al., 1974; Callaghan and Schwark, 1979; McIntyre, 1980; Abed, 1994; Amabeoku and Syce, 1997), and *Dbh* -/- mice have increased susceptibility to seizure induced by flurothyl, pentylenetetrazole, kainic acid, and sound (Szot et al., 1999).

Approximately 27% of all drug-related emergency room episodes are related to cocaine abuse (SAMHSA, 1996). Cocaine-induced seizures are a manifestation of the toxicity associated with the drug, and estimates are that 8-12% of patients admitted to emergency departments with cocaine intoxication have seizures (Derlet and Albertson, 1989; Dhuna et al., 1991; Koppel et al., 1996). These seizures can be resistant to common anticonvulsant drugs, such as benzodiazepines and barbiturates, and constitute a major fraction of cocaine-related deaths (Dhuna et al., 1991; Benowitz et al., 1993). In addition, there have been several reports of individuals without a history of epilepsy developing seizures following treatment with therapeutic doses of disulfiram (Liddon and Satran,

1967; Price and Silberfarb, 1976a, 1976b; McConchie et al., 1983; Daniel et al., 1987). Concurrent use of cocaine and disulfiram is now on the rise, as disulfiram is under evaluation as a pharmacotherapy for cocaine dependence. Because pharmacological or genetic inhibition of DBH increases the sensitivity to seizures and the behavioral effects of cocaine, we sought to examine the effects of DBH and disulfiram on susceptibility to cocaine-induced seizures (CIS). We measured the probability of having a seizure and the frequency of CIS following a high dose of cocaine (60 mg/kg) in both wild-type (*Dbh* +/+) and *Dbh* -/- mice. We hypothesized that (1) *Dbh* -/- mice would be hypersensitive to cocaine-induced seizures (CIS) and (2) disulfiram would exacerbate CIS in a *Dbh* genotype-dependent manner. To further examine whether disulfiram affects cocaine responses via a DBH-dependent mechanism, we also tested the selective DBH inhibitor nepicastat (Stanley et al., 1997). To determine whether the effects of these drugs could be attributed to changes in cocaine metabolism, we also measured peak serum cocaine levels.

A1.3 Methods

Animals and housing

Adult *Dbh* +/+ and -/- mice maintained on a mixed 129/SvEv and C57BL6/J background were developed and generated as previously described (Thomas et al, 1995, 1998). Genotypes were confirmed by PCR. All mice were reared in a specific pathogen-free facility with a 12-h light/dark cycle (lights on at 0700 h, lights off at 1900 h); food and water were available ad libitum. Naïve mice between 3 and 6 months of age were

used for all experiments, as were both male and female mice. No sex differences were observed, and results were combined. Experimental protocols were approved by the Emory University IACUC and meet the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Cocaine-induced seizures

Mice were given 3 injections of saline, disulfiram (100 mg/kg, i.p.), or the selective DBH inhibitor nepicastat (100 mg/kg, i.p.), with 2 hours between each injection. Two hours following the last injection, all mice were injected with a high dose of cocaine (60 mg/kg, i.p.). This dose was found to induce seizures in ~50% of mice of the same strain during a pilot study. Mice were observed for 30 minutes following cocaine administration, and the latency to first seizure and seizure frequency were recorded. The first seizure and/or ataxia typically occurred within 2-4 minutes postinjection. Seizures were defined as repetitive, rapid periods of jumping, wild-running, tonic-clonic activity, or a loss of the righting reflex. N = 9-16 for each treatment group.

Cocaine metabolism

Mice were given 3 injections of either saline or disulfiram (100 mg/kg, i.p.) each 2 hours apart. Two hours following their last injection, they were injected with cocaine (20 or 60 mg/kg, i.p.). These two doses were chosen because the 20-mg/kg dose supports a conditioned place preference in control mice, but elicits a conditioned place aversion in *Dbh -/-* mice, while the 60-mg/kg dose was the one used to produce seizures in this study. Mice were decapitated 5 minutes later. We chose this time point because cocaine levels

peak in mice ~5 minutes after i.p. cocaine administration (Benuck et al., 1987). Trunk blood was collected in microcentrifuge tubes containing 5 µl each of NaF (132mg/ml) and $(COOK)_2$ (106.7 mg/ml). Blood was centrifuged for 5 minutes at 10K, and serum was isolated and frozen until analysis. Serum cocaine levels were measured by HPLC. N = 3-8 for each genotype and treatment group.

Measurement of serum cocaine levels by HPLC

Cocaine was quantified in mouse serum by extraction with preparatory columns and isocratic HPLC with UV detection. Varian Bond Elut Certify C18 (130 mg) preparatory columns were placed on a vacuum manifold and pretreated with 2 ml of methanol and then 2 ml of 100 mM phosphate buffer (pH 6) at constant pressure (5 psi). Two hundred µL of calibrators, controls, and samples were mixed by vortexing with 20 µl of a 10 µg/ml nalorphine solution (internal standard), 1.8 ml of saline, and 1 ml of 100 mM phosphate buffer (pH 6). Columns were washed with 6 ml of Milli-Q water, then 3 ml of 1 M acetic acid solution, and finally vacuum dried. Next, the columns were washed with 6 ml of methanol, and then cocaine was eluted with 2 ml of dichloromethane:isopropanol (80:20) containing 2% ammonium hydroxide. Final sample eluates were dried to residue with streaming nitrogen; residues were redissolved in 250 µl of mobile phase, then 100 µl of each sample was injected into the HPLC system [Waters model 510 pump, Waters 717 sample injector, Waters 2587 UV detector; a Phenomenex C18 column (5 micron, 4.5 mm ID x 150 mm L)]. The flow rate of the mobile phase was 1.5 ml/min. Cocaine, BE, and nalorphine were detected at a fixed wavelength of 214 nm. The mobile phase contained 8% acetonitrile, 12% methanol, and 80% of a solution of 12

mM KH_2PO_4 (pH 2.5). Calibrators were spiked mouse serum at concentrations of 100, 500, 1000, 5000, and 10000 ng/ml. Cocaine and benzoylecgonine concentrations were expressed in ng/ml.

Drugs

All drugs were freshly prepared before being used and injected i.p. in a volume of 10 ml/kg. Cocaine HCl (Sigma Aldrich, St Louis, MO) and nepicastat (Roche Biosciences, Palo Alto, CA) were dissolved in 0.9% NaCl. Disulfiram (Sigma Aldrich, ST Louis, MO) was injected as a suspension in 0.9% NaCl following sonication.

Statistical analyses

The effect of disulfiram and nepicastat on the probability of an animal exhibiting a cocaine-induced seizure was analyzed using the χ^2 distribution. The effect of the DBH inhibitors on the number of cocaine-induced seizures exhibited in a 30-minute period and serum cocaine levels were analyzed using a 2-way completely randomized design ANOVA with post-hoc Bonferroni comparisons. A p-value of <0.05 was considered significant. Outliers were detected using the Grubb's test. One subject (disulfiram-treated *Dbh* -/-) met this criteria and was removed from all statistical analysis.

A1.4 Results

Disulfiram and cocaine-induced seizures

Dbh genotype alone had no effect on CIS probability; 64.2% of *Dbh* +/+ and 66.6% of *Dbh* -/- mice had seizures following cocaine administration (Figure A1.1a). Disulfiram pretreatment increased the probability of having at least one cocaine-induced seizure in both *Dbh* +/+ and *Dbh* -/- mice with similar magnitude (*Dbh* +/+, 64.2% saline vs. 95% disulfiram; *Dbh* -/-, 66.6% saline vs. 92.9% disulfiram) (Figure A1.1a). This effect was statistically significant in *Dbh* +/+ mice, but did not quite reach significance in *Dbh* -/- mice (*Dbh* +/+, $\chi^2 = 5.346$, p=0.02; *Dbh* -/-, $\chi^2 = 3.027$, p=0.08).

Dbh genotype alone also had no effect on CIS frequency (Figure A1.1b); however, disulfiram increased CIS frequency in *Dbh* +/+ but not *Dbh* -/- mice (Figure A1.1b). ANOVA revealed a significant effect of pretreatment, with disulfiram significantly increasing the number of seizures during the 30-minute test period following cocaine administration in wild-type (WT) but not knockout (KO) mice (F=12.58, df=1; p <0.001 for WT). An additional ANOVA was performed only on data from subjects exhibiting at least one cocaine-induced seizure. Analysis of seizure frequency in these animals showed the same effect of disulfiram, as pretreatment with the drug significantly increased seizure frequency only in wild-type mice (F= 14.13, df=1; p <0.001) (Figure A1.1c).

Nepicastat and cocaine-induced seizures

To further determine whether the effects on CIS from disulfiram were mediated by DBH inhibition, we tested the selective DBH inhibitor nepicastat. In contrast to disulfiram, nepicastat pretreatment did not affect the probability of having a cocaine-

induced seizure in wild-type mice, and in fact significantly reduced this probability in *Dbh* -/- mice ($\chi^2 = 5.35$; $p<0.05$) (Figure A1.2a).

The pattern of nepicastat effects on CIS frequency was similar to that of disulfiram. Nepicastat pretreatment increased CIS frequency in wild-type but not *Dbh* -/- mice. The effect on *Dbh* +/+ mice did not quite reach significance when all mice were included in the analysis (F=0.3672, df= 1; $p>0.05$) (Figure A1.2b), but did reach significance when data from animals exhibiting at least one seizure were analyzed (F=1.518, df= 1; $p<0.05$) (Figure A1.2c).

Cocaine metabolism

To determine whether the disulfiram-related rise in CIS probability and frequency could be attributed to drug effects on cocaine metabolism, we measured peak serum cocaine levels in *Dbh* +/+ and -/- mice after administration of 20 or 60 mg/kg of cocaine. We found that cocaine serum levels were unaffected either by *Dbh* genotype or by disulfiram in most cases. The exception was an increase in serum cocaine levels by disulfiram in *Dbh* -/- mice at the high dose of cocaine, and ANOVA revealed a genotype x treatment interaction (F=5.312, df= 1; $p<0.05$) (Figure A1.3). These results indicate that changes in cocaine metabolism do not underlie the effects of disulfiram on CIS.

A1.5 Discussion

Cocaine-induced seizures account for approximately 10% of cocaine-related emergency room visits to hospitals and are a common manifestation of cocaine toxicity.

While there are no widely accepted pharmacotherapies for cocaine addiction, disulfiram has shown recent promise as a treatment for cocaine dependence. Unfortunately, this treatment may be hazardous to some patients, due to its mechanism of action. Disulfiram inhibits the enzyme DBH, which could lead to increases in seizures and cocaine sensitivity. Therefore, we chose to examine how pharmacological DBH inhibition and DBH genotype affects CIS probability and frequency in an animal model. In order to assess this, we pretreated *Dbh* +/+ and -/- mice with disulfiram or nepicastat, a direct, selective DBH inhibitor, prior to administering a high dose of cocaine. We hypothesized that *Dbh* -/- mice would be hypersensitive to CIS and that disulfiram would exacerbate CIS in a *Dbh* genotype-dependent manner. We also assessed changes in serum cocaine levels to determine whether the effects of these drugs could be attributed to changes in cocaine metabolism.

Disulfiram had two distinct effects on CIS; it increased the probability of having a seizure in *Dbh* +/+ and *Dbh* -/- mice and increased CIS frequency in *Dbh* +/+ mice only. Nepicastat did not increase seizure probability, but increased the frequency of CIS in *Dbh* +/+ mice only. These results indicate that pharmacological DBH inhibition is responsible for increasing the frequency of CIS, while disulfiram's ability to raise the probability of CIS is mediated by a DBH-independent mechanism. Given disulfiram's mechanism of action as a copper chelator, the inhibition of cocaine metabolic enzymes, such as cholinesterase and carboxylesterase, could underlie its effects on CIS probability. However, we did not find this to be the case, as disulfiram did not alter serum cocaine concentrations in wild-type mice. Interestingly, nepicastat actually tended to inhibit CIS in *Dbh* -/- mice. Nepicastat does not chelate copper and is a direct, potent inhibitor of

DBH, and initial screening did not reveal any other high-affinity targets aside from DBH. It appears that the lack of DBH in *Dbh* -/- mice revealed a secondary anticonvulsant target for nepicastat.

Because pharmacological DBH inhibition increased CIS frequency, *Dbh* -/- mice would be expected to demonstrate more frequent CIS. We did not find this to be the case. One possible explanation is that compensatory changes in monoamine neurotransmitters arise following chronic and complete knockout of DBH and NE function. One likely compensatory candidate is the serotonin (5-HT) system. Serotonergic activity modulates seizure activity in response to cocaine and seems to have proconvulsant effects. Selective 5-HT reuptake inhibitors, such as fluoxetine, citalopram, paroxetine and the tricyclic antidepressant imipramine, all facilitate CIS (O'Dell et al., 1999; Ritz and George, 1997), whereas 5-HT_2 receptor antagonists decrease CIS (O'Dell, et al., 1999, 2000; Ritz and George, 1997; Schechter and Meehan, 1995). Since cocaine is known to inhibit 5-HT transporters (SERT), increases in 5-HT following cocaine administration can lead to accumulation of serotonin in synapses, which in turn can increase seizure activity via 5-HT_2 receptor activation. The serotonergic raphe nuclei receive dense amygdala projections from brainstem noradrenergic nuclei (Baraban and Aghajanian, 1981; Marcinkiewicz et al., 1989; Fritschy and Grzanna, 1990; Peyron et al., 1996), and activation of α_1-adrenergic receptors increases tonic excitatory activity in the dorsal raphe nucleus (Baraban and Aghajanian, 1980; Vandermaelen and Aghajanian, 1983; Hertel et al., 1998; Pudovkina et al., 2003; Judge and Gartside, 2006). Therefore, *Dbh* -/- mice should have lower levels of extracellular 5-HT, because they lack the noradrenergic excitatory drive on the serotonergic system. Indeed, when compared with control mice,

Dbh -/- mice have decreased 5-HT release in the nucleus accumbens following amphetamine treatment (D. Weinshenker and S. Puglisi-Allegra, unpublished observations), as well as in the hippocampus following fluoxetine administration (Cryan et al., 2004). This decreased concentration of the proconvulsant 5-HT in *Dbh -/-* mice may explain their "normal" CIS susceptibility at baseline.

Because disulfiram acts as a copper chelator, this drug is relatively nonspecific and inhibits two cocaine-metabolizing enzymes, cholinesterase and carboxylesterase (Zemaitis and Greene, 1976; Nousiainen and Törrönen, 1984; Savolainen et al., 1984). Disulfiram treatment increased cocaine plasma levels and decreased cocaine clearance in humans following intranasal cocaine administration (McCance-Katz et al., 1998a,b; Hameedi et al., 1995; Baker et al., 2007). Thus, it is possible that the disulfiram-induced increase in CIS was a direct result of decreased cocaine metabolism. However, disulfiram did not alter peak serum cocaine levels in most cases in our study. The single exception was an interaction between disulfiram treatment and genotype; the higher dose of cocaine (60 mg/kg) significantly increased cocaine plasma levels in *Dbh -/-* mice only. The 209mygdale209 underlying this synergy is unclear. One possibility is that NE limits the spread of cocaine through the bloodstream via its vasoconstrictive properties, while cholinesterase and carboxylesterase are responsible for its metabolism. Perhaps at low doses of cocaine, either mechanism alone is sufficient to maintain "normal" peak cocaine serum levels, but at high doses, the impairment of both noradrenergic function and cocaine metabolic enzymes results in increased serum levels. It is not clear why disulfiram did not increase peak serum cocaine levels in wild-type mice. The differences between the effects of disulfiram on serum cocaine levels in humans and rodents may be

due to different routes of cocaine administration (intranasal in humans vs. intraperitoneal in mice) or to species differences in cocaine metabolism.

Our results show that acute disulfiram administration increases the probability and frequency of CIS, which may present a clinical problem during cocaine addiction treatment with disulfiram pharmacotherapy. It should be noted that, while CIS have not been reported during cocaine dependence clinical trials examining disulfiram efficacy, there have been several reports of individuals without a history of epilepsy developing seizures following treatment with therapeutic doses of disulfiram (Liddon and Satran, 1967; Price and Silberfarb, 1976a, 1976b; McConchie et al., 1983; Daniel et al., 1987). Thus, clinicians should be cautious when considering disulfiram as a cocaine pharmacotherapy, particularly in patients with a history of epilepsy or cocaine overdose. The more selective DBH inhibitor nepicastat may be a safer alternative to disulfiram for treating cocaine dependence, as it does not increase CIS probability and is in fact anticonvulsant in *Dbh -/-* mice. DBH activity is genetically controlled and highly variable in humans (Weinshilboum, 1978; Zabetian et al., 2001). The haplotype associated with low DBH activity in humans is also associated with more cocaine-induced paranoia (Cubells et al., 2000; Kalayasiri et al., 2007). This increase in one of the aversive properties of cocaine may underlie the effectiveness of DBH inhibition via disulfiram in curbing cocaine intake. Given that the proconvulsant effect of disulfiram on CIS frequency is absent in *Dbh -/-* mice, disulfiram pharmacotherapy might perhaps be safer for cocaine addicts with low DBH activity. Preliminary data suggest that disulfiram is most effective for these individuals (R. Schottenfeld and J. Cubells, personal communication), possibly due to their enhanced aversion to cocaine. Our results indicate

that they may also be resilient to disulfiram-induced exacerbation of CIS and possibly other toxic effects of cocaine.

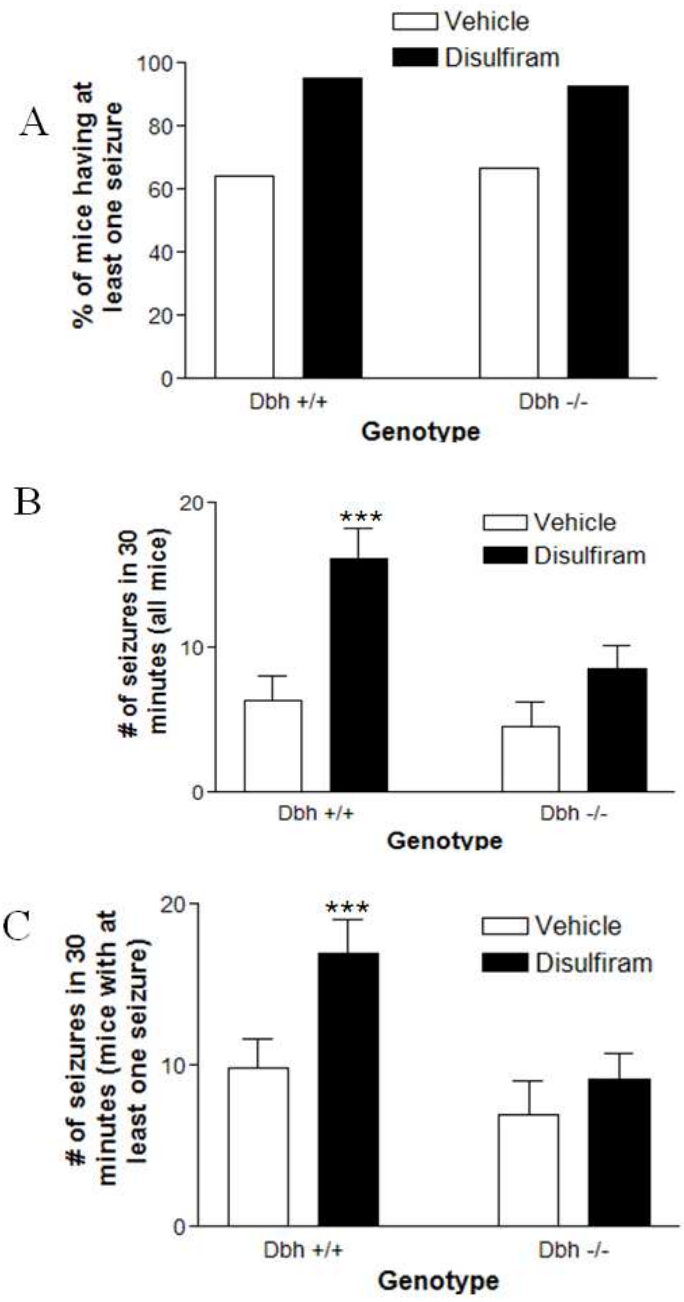

Figure A1.1 The effects of disulfiram *Dbh* genotype on cocaine-induced seizures. *Dbh* +/+ and *Dbh* -/- mice were pretreated with vehicle or disulfiram (3 doses of 100 mg/kg, i.p., each dose spaced 2 hours apart). Two hours after the last pretreatment, mice

were injected with cocaine (60 mg/kg, i.p.), and behavior was observed for 30 min. Shown is **(A)** the percent of all mice tested having at least one seizure, (B) the mean ± SEM seizures observed in 30 min in all mice tested, and (C) the mean ± SEM seizures only in mice that had at least one seizure. N=14–20 per genotype and treatment group. * $P < 0.05$, *** $P < 0.001$ compared to vehicle control for that genotype.

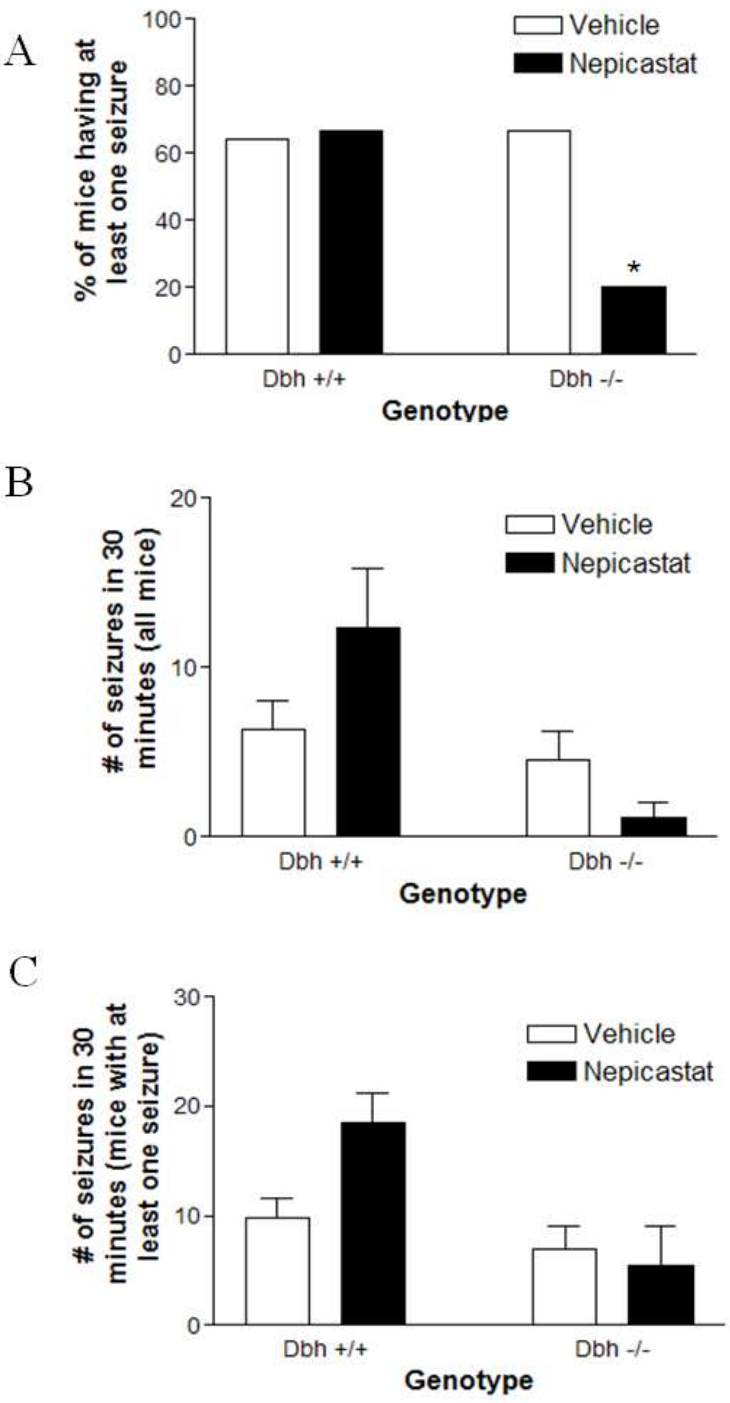

Figure A1.2 The effects of nepicastat and *Dbh* genotype on cocaine-induced seizures.

Dbh +/+ and *Dbh* -/- mice were pretreated with vehicle or nepicastat (3 doses of 100

mg/kg, i.p., each dose spaced 2 hours apart). Two hours after the last pretreatment, mice were injected with cocaine (60 mg/kg, i.p.), and behavior was observed for 30 min. Shown is **(A)** the percent of all mice tested having at least one seizure, (B) the mean ± SEM seizures observed in 30 min in all mice tested, and (C) the mean ± SEM seizures only in mice that had at least one seizure. N=9– 15 per genotype and treatment group. * $P < 0.05$ compared to vehicle control for that genotype.

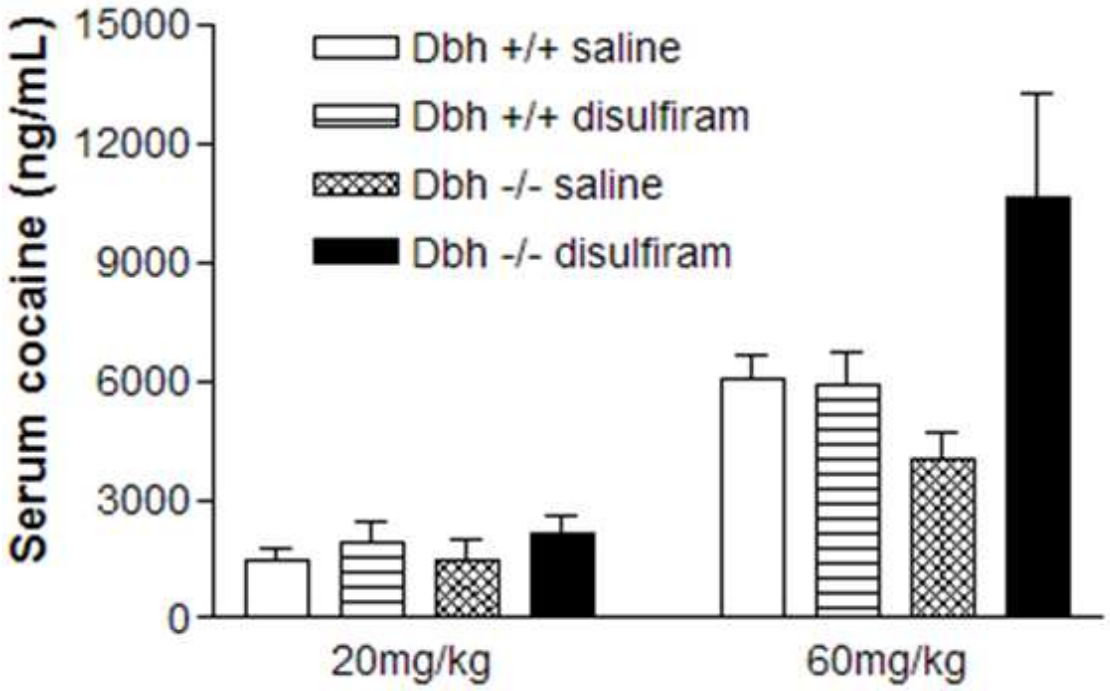

Figure A1.3 The effects of disulfiram and *Dbh* genotype on cocaine metabolism. *Dbh* +/+ and *Dbh* -/- mice were pretreated with vehicle or disulfiram (3 doses of 100 mg/kg, i.p., each dose spaced 2 hours apart). Two hours after the last pretreatment, mice were injected with cocaine (20 or 60 mg/kg, i.p.), and blood was collected 5 min later. Shown is the mean ± SEM peak serum cocaine levels as measured by HPLC. N = 6-8 per genotype and treatment group. * $P < 0.05$ compared to vehicle control for that genotype.

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APPENDIX A2

DISULFIRAM ATTENUATES DRUG-PRIMED REINSTATEMENT OF COCAINE-SEEKING VIA INHIBITION OF DOPAMINE β-HYDROXYLASE

Adapted from:

Schroeder JP*, Cooper DA*, Schank JR, Lyle MA, Gaval-Cruz M, Ogbonmwan YE, Pozdeyev N, Freeman KG, Iuvone PM, Edwards GL, Holmes PV, Weinshenker D. Neuropsychopharmacology. 2010 Nov;35(12):2440-2449.

*These authors contributed equally to this work.

A2.1 Abstract

The anti-alcoholism medication disulfiram (Antabuse) inhibits aldehyde dehydrogenase (ALDH), which results in the accumulation of acetaldehyde upon ethanol ingestion and produces the aversive "Antabuse reaction" that deters alcohol consumption. Disulfiram has also been shown to deter cocaine use, even in the absence of an interaction with alcohol, indicating the existence of an ALDH-independent therapeutic mechanism. We hypothesized that disulfiram's inhibition of dopamine β-hydroxylase (DBH), the catecholamine biosynthetic enzyme that converts dopamine (DA) to norepinephrine (NE) in noradrenergic neurons, underlies the drug's ability to treat cocaine dependence. We tested the effects of disulfiram on cocaine and food self-administration behavior and drug-primed reinstatement of cocaine seeking in rats. We then compared the effects of disulfiram with those of the selective DBH inhibitor, nepicastat. Disulfiram, at a dose (100 mg/kg, i.p.) that reduced brain NE by ~40%, did not alter responding for food or cocaine on a fixed ratio 1 (FR1) schedule, whereas it completely blocked cocaine-primed (10 mg/kg, i.p.) reinstatement of drug seeking following extinction. A lower dose of disulfiram (10 mg/kg) that did not reduce NE had no effect on cocaine-primed reinstatement. Nepicastat recapitulated the behavioral effects of disulfiram (100 mg/kg) at a dose (50 mg/kg, i.p.) that produced a similar reduction in brain NE. Food-primed reinstatement of food seeking was not impaired by DBH inhibition. Our results suggest that disulfiram's efficacy in the treatment of cocaine addiction is associated with the inhibition of DBH and interference with the ability of environmental stimuli to trigger relapse.

A2.2 Introduction

Disulfiram (Antabuse) has been used for more than 50 years in the treatment of alcoholism (Fuller et al., 1986). Disulfiram inhibits ALDH, which results in the accumulation of acetaldehyde upon ethanol ingestion. This toxic metabolite produces aversive symptoms, such as flushing, nausea, and vomiting, and a desire to avoid this reaction encourages abstinence. Because 50-90% of patients who abuse cocaine also abuse alcohol (Weiss et al., 1988; Grant and Harford, 1990; Closser and Kosten, 1992; Khalsa et al., 1992), the belief was that discouraging alcohol consumption in cocaine- and alcohol-dependent individuals might lower cocaine use. Indeed, disulfiram was found to reduce alcohol and cocaine intake in this patient population (Carroll et al., 1993; 1998; 2000). Surprisingly, further studies went on to reveal that disulfiram is at least as effective at treating cocaine addicts who do not consume alcohol, and may even be more effective (George et al., 2000; Petrakis et al., 2000; Carroll et al., 2004). Therefore, an aldehyde dehydrogenase-independent mechanism must be responsible for the ability of disulfiram to promote cocaine abstinence (Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009).

Cocaine increases extracellular levels of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) in the brain by blocking plasma membrane monoamine transporters. Thus, pathways critical for the production or transmission of these neurotransmitters are a reasonable place to look for targets underlying the efficacy of disulfiram in the treatment of cocaine dependence. Because the primary metabolite of disulfiram, diethyldithiocarbamate (DDC), is a copper chelator (Hald and Jacobsen, 1948; Johnston, 1953), disulfiram impairs the function of many copper-containing

enzymes, including ALDH, carboxylesterase, cholinesterase, and dopamine β-hydroxylase (DBH). Of particular interest, the inhibition of DBH by disulfiram reduces production of NE, with a concomitant increase in tissue levels of DA in rodents (Goldstein, 1966; Musacchio et al., 1966; Bourdelat-Parks et al., 2005). Disulfiram also decreases NE and its metabolites in the urine, blood, and CSF of humans (Takahashi and Gjessing, 1972; Major et al., 1979; Rogers et al., 1979; Hoeldtke and Stetson, 1980; Rosen and Lobo, 1987; Paradisi et al., 1991). We have shown that disulfiram has no effect on catecholamine levels in DBH knockout (*Dbh* -/-) mice, which lack NE, indicating that disulfiram's effects on NE and DA are mediated solely by DBH inhibition (Bourdelat-Parks et al., 2005). Disulfiram also inhibits cocaine metabolizing enzymes and increases peak plasma cocaine levels under some conditions in humans (McCance-Katz et al., 1998a, 1998b; Baker et al., 2007) but not rodents (Gaval-Cruz et al., 2008).

The efficacy of disulfiram in treating cocaine dependence has been attributed to several different mechanisms, including a decrease in cocaine reward, an increase in cocaine aversion, and as a "DA replacement therapy" that elevates DA levels and restores normal reward function in hypodopaminergic addicts (Weinshenker and Schroeder, 2007; Sofuoglu et al., 2008; Gaval-Cruz and Weinshenker, 2009); however, the data have been ambiguous. Different human laboratory studies report that genetic or pharmacological DBH inhibition increases cocaine-induced paranoia and decreases, increases, or has no effect on psychostimulant-induced euphoria (Hameedi et al., 1995; McCance-Katz et al., 1998a, 1998b; Cubells et al., 2000; Petrakis et al., 2000; Baker et al., 2007; Kalayasiri et al., 2007; Sofuoglu et al., 2008). In rodents, disulfiram decreases the locomotor-

activating effects of acute cocaine administration, but facilitates cocaine sensitization (Maj et al., 1968; Haile et al., 2003).

The available human and animal data give us a hazy picture of how disulfiram discourages cocaine use. The influence of disulfiram on the reinforcing properties of cocaine have yet to be investigated in an animal model, and while DBH inhibition has been suggested to underlie disulfiram's efficacy, this hypothesis has not been tested directly. In an effort to resolve these issues, we assessed the effects of disulfiram in operant rat paradigms of drug taking (cocaine self-administration) and relapse (cocaine-primed reinstatement) at doses that inhibit DBH in the brain. To determine whether the effects of disulfiram were mediated by inhibition of DBH, we employed the selective DBH inhibitor, nepicastat. Nepicastat is a direct, competitive inhibitor of DBH with greater potency than disulfiram (IC_{50} = 9 nM for nepicastat versus $IC_{50} \cong 1$ μM for disulfiram; Green, 1964; Goldstein, 1966; Stanley et al., 1997), as well as better selectivity (does not chelate copper, no significant interaction with a panel of other enzymes and receptors tested, including aldehyde dehydrogenase and tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis) (Stanley et al., 1997; K. Walker, Roche Biosciences, personal communication).

A2.3 Materials and methods

Subjects

Male Sprague-Dawley rats (175-200 g) were purchased from Charles River (Wilmington, MA, USA). All subjects were maintained in a temperature-controlled environment on a 12-h reverse light/dark cycle with the lights on from 7 pm to 7 am with

ad libitum access to food and water. Rats were acclimated to the vivarium for 1 week prior to catheter implantation surgery. All self-administration sessions occurred during the dark cycle and were performed using standard methods with minor modifications (McFarland and Kalivas, 2001; Fuchs et al., 2004). All animals were treated in accordance with NIH policy, and experiments were approved by the Emory IACUC committee.

Drug doses

In initial pilot experiments, we tested the effects of disulfiram (10, 25, 50, 75, 100, or 200 mg/kg, i.p.) and nepicastat (50 or 100 mg/kg, i.p.) on brain catecholamine levels and operant responding for food. Disulfiram was obtained from Sigma-Aldrich (St. Louis, MO), sonicated in sterile saline, and injected as a suspension. Nepicastat was obtained from Synosia Therapeutics (South San Francisco, CA), sonicated in sterile saline containing 1.5% DMSO and 1.5% Cremaphor EL (Sigma), and injected as a suspension. We chose the 100 mg/kg dose of disulfiram based on 3 criteria. First, 100 mg/kg was the maximum dose that significantly inhibited dopamine β-hydroxylase but did not impair the ability of rats to perform operant responses. Second, the 100 mg/kg dose has been shown by others to alter other behavioral effects of cocaine in rats, such as locomotor activity and sensitization (e.g. Haile et al., 2003). Third, the 100 mg/kg dose inhibits aldehyde dehydrogenase in rats and is in the range typically used for alcohol studies (e.g. Deitrich and Erwin, 1971; Yourick and Faiman, 1991; Karamanakos et al., 2001). Fourth, the 100 mg/kg dose is therapeutically relevant. The typical therapeutic dose for the cocaine studies performed in humans is 250-500 mg/day (e.g. Carroll et al.,

1998; McCance-Katz et al., 1998), which translates to ~ 3-7 mg/kg for a 70 kg human, or ~ 10-fold lower than we used in our study. Because of their higher metabolic rate, rodents require much larger doses of psychoactive drugs to produce behavioral and neurochemical effects compared to humans, and the 3-7 mg/kg dose has been shown to inhibit DBH in humans with a magnitude similar to the 100 mg/kg dose in rats (e.g. compare Vesell et al., 1971; Major et al., 1979; Rogers et al., 1979; Paradisi et al., 1991 human studies to our current rat study). Thus, use of the 100 mg/kg dose in rats is a close functional match to therapeutic doses in humans. We chose the 10 mg/kg dose of disulfiram for an additional experiment because it was the maximum dose in our pilot studies that did not significantly reduce brain NE levels. The 50 mg/kg dose of nepicastat was chosen to match the level of DBH inhibition observed with the 100 mg/kg dose of disulfiram.

Quantification of catecholamine levels

Rats were injected with disulfiram (10 or 100 mg/kg, i.p.), nepicastat (50 mg/kg, i.p.), or vehicle (saline for disulfiram, 1.5% DMSO + 1.5% Cremaphor EL in saline for nepicastat; 1 ml/kg, i.p.). Two hours later, rats were euthanized by CO_2, brains were removed, and the prefrontal cortex was dissected on ice and frozen. The prefrontal cortex was chosen because it contains comparable amounts of NE and DA, and thus can be used to accurately assess DBH inhibition. NE and DA levels were determined using HPLC followed by coulometric detection. DA and NE concentrations were normalized to wet tissue weight for each sample.

Analytical samples from saline- and disulfiram-treated rats were prepared by adding 10 volumes of ice-cold mobile phase [0.1 mM NaHSO4, monohydrate 0.1 mM EDTA, 0.2 mM octane sulfonic acid, 6.5% acetonitrile (pH 3.1)], and sonicated until completely homogenized. Samples were centrifuged at 13.2 rpm x 1000 for 30 min at 4°C, and the supernatant removed from the tubes. The supernatant was centrifuged again at 13.2 rpm x 1000 for 30 min at 4°C using a 22-micron filter column. The resulting eluant was injected using an ESA 542 Autosampler (ESA Biosciences Inc., Chelmsford, MA) onto a Synergi Max-RP 4u (150 x 4.6mm) with Security Guard precolumn filter with Max-RP cartridges (Phenomenex, Inc., Torrance, CA) at a constant rate of 1 ml/min maintained by ESA 584 pumps. An ESA CoulArray 5600A detector with a potential set at -150 mV, 200 mV was used to visualize the peaks. The retention time and height of NE and DA peaks were compared with reference standard solutions (Sigma). Peak heights were quantified by CoulArray software (ESA Biosciences Inc.).

Analytical samples of vehicle and nepicastat-treated rats were prepared by adding 70 µL of ice-cold 0.1 N perchloric acid and 0.04% sodium metabisulfite to the tissue, and then sonicating until completely homogenized. Samples were centrifuged at 15 rpm x 1000 for 10 min at 4°C. This supernatant was injected at a constant flow rate of 1 mL/min onto an Ultrasphere ODS 250 × 4.6 mm column, 5 µm (Beckman Coulter, Fullerton, CA, USA) with mobile phase (0.1 mM EDTA; 0.35mM sodium octyl sulfate; 0.6% phosphoric acid; 5% acetonitrile (pH 2.7)). A coulometric electrochemical array detector (Agilent Technologies; guard cell set at 600 mV and analytical cell at 300 mV) was used to visualize the peaks. The retention time, height, and area of NE and DA peaks

were compared with reference standard solutions (Sigma) and quantified by ChemStation chromatography software (Agilent Technologies).

Food training

Rats were trained to lever-press for food in standard rat operant chambers (Med Associates, St. Albans, VT) prior to drug exposure to facilitate acquisition of drug self-administration, as described (Fuchs et al., 2004). Each chamber was equipped with a house light, two levers (active and inactive), and stimulus lights above both levers. Fan motors provided ventilation and masked noise for each chamber. A microcomputer with Logic '1' interface and MED-PC software (MED Associates) controlled schedule contingencies and recorded data. Animals had access to a water bottle and received 45-mg food pellets following active lever presses on a fixed ratio 1 (FR1) schedule, meaning the rat received a reinforcer following each active lever press. The food training sessions lasted for 8 h, or until the animal met criteria, defined as at least 70% selection of the active lever and at least 100 food pellets obtained. Most rats met criteria on the first day of food training, but a few rats required 2-3 days.

Surgery

Following food training, rats were anesthetized with isoflurane and implanted with indwelling jugular catheters using standard methods. Briefly, catheters were inserted into the jugular vein and anchored with suture material and tissue adhesive. The catheter was then threaded subcutaneously through the skin between the shoulder blades, and the catheter was anchored. Catheters were flushed daily with 0.05 mL gentamicin (4 mg/mL)

and 0.1 mL heparin solution (30 U/mL in sterile saline). Catheter patency was verified periodically by infusing 0.08-0.12 ml of methohexital sodium (10 mg/ml, IV; Eli Lilly and Co., Indianapolis, Ind., USA), which produces a rapid loss of muscle tone only when administered intravenously.

Cocaine self-administration

Daily self-administration sessions were run for 2 h on a FR1 schedule. At the start of each session, both active and inactive levers were extended, and rats received a non-contingent infusion of cocaine (0.5 mg/kg). During training, each press of the active lever resulted in a cocaine infusion (0.5 mg/kg in a volume of 167 μl/kg) accompanied by a discrete flashing light above the lever. Following a 20-s timeout period (during which time active lever presses did not result in drug infusion), the stimulus light was extinguished, and responses were again reinforced. Responses on the inactive lever had no programmed consequences. To prevent overdose, the session was terminated early if the number of cocaine infusions exceeded 40.

Once rats reached a stable level of responding (number of drug infusions varied by <20% of the mean, and preference for the active lever was at least 75% for 3 consecutive days, with a minimum of 5 total days of cocaine self-administration), the effects of disulfiram were assessed. Rats received an injection of saline (2 ml/kg, i.p.) or disulfiram (100 mg/kg, i.p.) 2 h prior to the self-administration session. The rats were then allowed 1-2 days of self-administration sessions with no pretreatment. The following day, rats received the opposite pretreatment (saline or disulfiram) 2 h prior to the self-administration session in a counterbalanced fashion.

Extinction

Following the completion of the maintenance phase of cocaine self-administration, lever pressing was extinguished in daily 2-h sessions during which presses on the previously active lever no longer resulted in delivery of cocaine or presentation of cocaine-paired cues. Behavior was considered extinguished when active lever presses over 3 consecutive days was <25% of the average number of active lever presses during the last 3 days of maintenance.

Cocaine-primed reinstatement

The day after extinction criteria were met, rats were pretreated with saline (2 ml/kg, i.p.) or disulfiram (10 or 100 mg/kg, i.p.). Two hours later, they were given a noncontingent priming injection of cocaine (10 mg/kg, i.p.) and placed in the operant chambers under extinction conditions (i.e., presses on the "active" lever had no programmed consequences) for 2 h. Rats then underwent a second round of extinction, as described above. When extinction criteria were met, rats were again tested for cocaine-primed reinstatement, but received the opposite pretreatment (saline or disulfiram) in a counterbalanced fashion (order was randomized). Some of the rats used for the reinstatement tests were the same ones that received disulfiram at the end of the maintenance phase of cocaine self-administration, while others were from a separate group that did not receive any pretreatments during maintenance. We found no differences in reinstatement, and these groups were combined. To determine whether the effects of disulfiram on reinstatement were mediated by DBH inhibition, separate groups

of rats went through cocaine self-administration and extinction, then were pretreated with vehicle (1.5% DMSO, 1.5% Cremaphor EL in saline, 1 ml/kg, i.p.) or nepicastat (50 mg/kg, i.p.) prior to counterbalanced reinstatement sessions, as described for disulfiram.

Food self-administration

Separate groups of rats were used for the food self-administration and reinstatement experiments. Rats were maintained on a restricted diet of 16 g of normal rat chow per day, given in the evening at least 1 h after self-administration sessions had ended. Parameters of food self-administration were identical to the cocaine self-administration experiments, except that rats received a food pellet instead of a cocaine infusion for each active lever press, and sessions lasted 1 h and were terminated if the reinforcers obtained exceeded 60.

Food-primed reinstatement

Food-primed reinstatement of food seeking was performed using a modified version of published protocols (e.g. Sun and Rebec, 2005; Peters and Kalivas, 2006). Once maintenance criteria for operant food self-administration were met (maintenance criteria and extinction criteria were identical to those used for cocaine-primed reinstatement), rats were pretreated with vehicle (1.5% DMSO, 1.5% Cremaphor EL in saline, 1 ml/kg, i.p.) or nepicastat (50 mg/kg, i.p.). 2 h later, they were placed in the operant chambers and the reinstatement session was started. Three food pellets were delivered non-contingently in the first ten seconds of the session and the levers were presented to the subjects. As during extinction, responses on either of the levers had no

programmed consequence. Throughout the 60 min food reinstatement session, a food pellet was delivered every 3 min non-contingently, and responses upon the formerly active and inactive levers were recorded. Rats then underwent a second round of maintenance and extinction training for operant food self-administration, as described above, then were tested for food-primed reinstatement following the opposite pretreatment (vehicle or nepicastat) in a counterbalanced fashion (order was randomized).

Data analyses

Catecholamine level data were analyzed by Student's t-test, and self-administration data were analyzed by ANOVA followed by Bonferroni post hoc tests using Prism 4.0 for Macintosh.

A2.4 Results

Disulfiram inhibits DBH and decreases brain NE levels

DBH is the enzyme in the catecholamine biosynthetic pathway that converts DA to NE in noradrenergic neurons. Thus, inhibition of DBH has the unique effect of simultaneously decreasing NE production and increasing DA (Figure 2A.1). To confirm previous reports that systemic disulfiram administration inhibits DBH in the rat brain, we measured NE, DA, and the NE/DA ratio in the frontal cortex following administration of saline or disulfiram (100 mg/kg, i.p.). We chose the frontal cortex because it contains NE and DA in similar concentrations, thereby allowing the detection of both decreases and increases in these neurotransmitters. As expected, disulfiram was a bona fide DBH

inhibitor, as it decreased NE, increased DA, and decreased the NE/DA ratio (Figure A2.2). Inhibition of other catecholamine biosynthetic enzymes would have had different patterns, such as decreases in both NE and DA following tyrosine hydroxylase (TH) inhibition.

Disulfiram has no effect on self-administration of food or cocaine

To ensure that we were using a dose of disulfiram that did not impair the ability of rats to perform an operant task, we assessed responding for food pellets following saline or disulfiram (100 mg/kg, i.p.) administration. Disulfiram had no effect on food responding; all rats obtained the maximum number of reinforcers possible during the session (61), regardless of pretreatment (n = 4 per group). To determine whether disulfiram altered the reinforcing or aversive effects of cocaine, we assessed maintenance levels of responding for cocaine infusions (0.5 mg/kg/infusion) following saline or disulfiram (100 mg/kg, i.p.). Disulfiram had no effect on cocaine self-administration (Figure A2.3). Repeated measures ANOVA revealed no significant effects for active lever presses ($F_{23,2} = 0.77$, $p = 0.48$) or reinforcers obtained ($F_{23,2} = 0.97$, $p = 0.4$). Inactive lever presses were negligible (0-2 presses per animal) and did not differ between groups.

Disulfiram blocks cocaine-primed reinstatement of cocaine seeking

We next tested the effects of disulfiram on drug-primed reinstatement of cocaine seeking. Following the attainment of stable self-administration and extinction, rats were treated with saline or disulfiram (100 mg/kg, i.p.) prior to a noncontingent priming

injection of cocaine (10 mg/kg, i.p.). Rats that were pretreated with saline showed a robust reinstatement of responding on the previously active lever following cocaine prime. In contrast, disulfiram pretreatment completely blocked cocaine-primed reinstatement (Figure A2.4). ANOVA revealed a significant effect of treatment phase ($F_{4,51} = 8.17$, $p < 0.0001$), and Bonferroni post hoc analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following saline pretreatment ($t = 3.62$, $p < 0.05$), but not between extinction responding and disulfiram pretreatment ($t = 0.22$, $p > 0.05$). In addition, there was a significant difference between reinstatement responding with saline pretreatment and disulfiram pretreatment ($t = 2.81$, $p < 0.05$). There was no effect of pretreatment on inactive lever responding.

We next tested the ability of a lower dose of disulfiram (10 mg/kg, i.p.) to attenuate cocaine-primed reinstatement. This dose of disulfiram, which we found in pilot studies to be the highest one that does not significantly reduce NE levels in the PFC (vehicle = 0.32 ± 0.04 ng/mg tissue, disulfiram = 0.29 ± 0.08, $p > 0.05$, $n = 4$ per group), did not impair cocaine-primed reinstatement (Figure A2.4). Bonferroni post hoc analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following low dose disulfiram pretreatment ($t = 2.69$, $p < 0.05$, but not between saline and low dose disulfiram pretreatment ($t = 0.18$, $p > 0.05$).

Nepicastat blocks cocaine-primed reinstatement of cocaine seeking

The previous experiments indicated that a dose high enough to inhibit DBH is required for the efficacy of disulfiram in blocking cocaine-primed reinstatement. However, because DBH has many other targets, it was unclear whether DBH inhibition

alone was sufficient to block reinstatement. Thus, we repeated the self-administration experiments with the selective DBH inhibitor, nepicastat, at a dose (50 mg/kg, i.p.) that inhibited DBH to a similar extent as the effective dose of disulfiram (100 mg/kg, i.p.) (Figure A2.5), and found that nepicastat pretreatment mimicked the effects of disulfiram in several ways. First, nepicastat had no affect on the maintenance phase of cocaine self-administration (Figure A.6). Repeated measures ANOVA revealed a non-significant trend for active lever presses ($F_{26,2} = 3.36$, $p = 0.06$) and no effect on reinforcers obtained ($F_{26,2} = 0.38$, $p = 0.69$). Inactive lever presses were negligible and did not differ between groups. Second, nepicastat blocked cocaine-primed reinstatement (Figure A2.7). Repeated measures ANOVA revealed a significant effect of treatment phase ($F_{3,23} = 18.14$, $p < 0.0001$), and Bonferroni post hoc analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following saline pretreatment ($t = 5.17$, $p < 0.001$) and between vehicle pretreatment and nepicastat pretreatment ($t = 4.67$, $p < 0.01$), but not between extinction responding and cocaine-primed reinstatement following nepicastat pretreatment ($t = 0.5$, $p > 0.05$). Pretreatment had no effect on inactive lever responding. Third, nepicastat (50 mg/kg, i.p.) had no effect on food responding; all rats obtained the maximum number of reinforcers possible during the session (61), regardless of pretreatment ($n = 8$ per group).

Because the neural and molecular pathways underlying reinstatement of cocaine and food seeking are partially overlapping (Nair et al., 2009), we tested whether the attenuation of reinstatement by DBH inhibition was specific to cocaine, and found that nepicastat did not significantly reduce food-primed reinstatement of food seeking (Figure A2.8). Repeated measures ANOVA revealed a significant effect of treatment phase ($F_{3,27}$

= 29.49, $p < 0.0001$), and Bonferroni post hoc analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following vehicle or nepicastat pretreatment (vehicle $t = 4.27$, $p < 0.05$; nepicastat $t = 2.57$, $p < 0.05$), but not between cocaine-primed reinstatement following vehicle and nepicastat pretreatment ($t = 1.70$, $p > 0.05$). These results indicate that the blockade of cocaine-primed reinstatement by nepicastat cannot be attributed to an inability to perform the operant task and that DBH inhibition does not impair reinstatement of responding for a natural reward.

A2.5 Discussion

Disulfiram has shown promise as a treatment for cocaine dependence in several clinical trials (Carroll et al., 1993; 1998; 2000; 2004; Petrakis et al., 2000; George et al., 2000; Grassi et al., 2007; Pettinati et al., 2008). Because concurrent alcohol use is not necessary for disulfiram to have beneficial effects on cocaine addiction, an ALDH-independent mechanism is likely. Furthermore, whatever the underlying molecular mechanism, why disulfiram treatment reduces cocaine use remains unclear; several human laboratory studies have produced conflicting results over how DBH inhibition influences the rewarding and aversive effects of cocaine. The purpose of our study was therefore two-fold. First, to gain insight into which aspects of addiction were being altered in the clinic, we determined which "phase" of cocaine self-administration (i.e., maintenance vs. reinstatement) was affected by disulfiram in rats. Second, to test the hypothesis that disulfiram was acting via DBH inhibition, we used a lower dose of disulfiram that does not inhibit DBH and the selective DBH inhibitor, nepicastat.

Treatments that alter the reinforcing effects of cocaine, such as dopaminergic manipulations, typically change cocaine self-administration behavior (Koob et al., 1994). Given the history of NE manipulations and cocaine self-administration, it is not surprising that disulfiram had no effect on maintenance responding for cocaine. NE transporter (NET) inhibitors themselves do not support self-administration, and neither NET inhibitors nor adrenergic receptor antagonists alter cocaine self-administration (Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009 Yokel and Wise, 1976; Roberts et al., 1977; Woolverton, 1987; Wee et al., 2006; Howell and Byrd, 1991; Skjoldager et al., 1993; Tella, 1995).

Drug addiction is a chronic relapsing disorder (Hunt et al., 1971; Leshner, 1997), as patients in treatment often slip back into drug taking after periods of sobriety. Several types of stimuli can trigger drug craving and lead to relapse, including re-exposure to the drug, stress, and drug-associated cues; these stimuli also trigger reinstatement in the rat model. The reliability, species generality, as well as face and construct validity of the reinstatement model are high, because it recapitulates many of the features of human addiction (Panlilio and Goldberg, 2007). In contrast to the lack of data to support an influence on the maintenance phase of psychostimulant self-administration, the role of NE in the reinstatement of drug seeking is clear (Erb et al., 2000; Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009). Central infusion of NE itself, or the facilitation of NE transmission with reuptake inhibitors or inhibitory autoreceptor antagonists, induces reinstatement in rats and non-human primates (Lee et al., 2004; Platt et al., 2007; Brown et al., 2009). Conversely, blockade of β-adrenergic receptors prevents stress-induced reinstatement, whereas blockade of α1-adrenergic receptors prevents drug-

primed reinstatement (Leri et al., 2002; Zhang and Kosten, 2005). Because we examined cocaine-primed reinstatement, it is likely that reinstatement was blunted following disulfiram or nepicastat pretreatment due to reduced NE production and a failure to engage α1-adrenergic receptors. The ability of DBH inhibition to block cocaine-primed reinstatement provides further support for the critical role of NE in this paradigm, and we propose that the clinical efficacy of disulfiram, via DBH inhibition and reduction of NE, reduces the risk for relapse. Most disulfiram clinical trials to date have not been designed to examine cocaine relapse specifically. It will be important to build measures into future trials that can distinguish between abstinence due to altered subjective drug effects vs. healthier responses to environmental triggers.

The evidence available suggests that blockade of cocaine-primed reinstatement by disulfiram involves the impairment of neurotransmission in the nucleus accumbens (NAc). DA and glutamate release in the NAc are both essential for cocaine-primed reinstatement (Schmidt et al., 2005; Kalivas et al., 2009). Noradrenergic neurons project to the mesocorticolimbic DA system, and NE promotes DA transmission, primarily via activation of α1-adrenergic receptors. For example, depletion of NE, or attenuation of α1-adrenergic receptor signaling via genetic, pharmacological, or neurotoxic means impairs psychostimulant-induced DA release in the NAc (Darracq et al., 1998; Drouin et al, 2002; Ventura et al., 2003). It is important to note that while DBH inhibition increases tissue levels of DA, it decreases DA release because NE-mediated excitation of DA neurons is reduced (Schank et al., 2006; Weinshenker and Schroeder, 2007; Weinshenker et al., 2008). Thus the failure of a cocaine prime to provoke DA release in the NAc may underlie the efficacy of disulfiram in this paradigm. While proof of a direct role for NE in

regulating cocaine-induced glutamate release in the NAc is lacking, we have recently found that α1-adrenergic receptors are enriched in presumptive glutamatergic terminals throughout the mesocorticolimbic system (Rommelfanger et al., 2009), and we predict that a loss of noradrenergic tone may also attenuate the glutamate release essential for cocaine-primed reinstatement.

Although the blockade of cocaine-primed reinstatement by disulfiram could involve several targets, our results strongly suggest that it is mediated primarily by DBH inhibition, NE reduction, and a decrease in α1AR signaling, as the effects of disulfiram require a dose that significantly inhibits DBH and are mimicked by the selective DBH inhibitor, nepicastat (present study), and the α1AR antagonist, prazosin (Zhang and Kosten, 2005). What remains unclear is why a reduction of NE/α1AR signaling hampers drug-primed reinstatement, but not the maintenance phase of cocaine self-administration. Earlier findings revealed that blockade of α1ARs does not affect "conventional" operant responding for cocaine, but does attenuate the escalation of cocaine self-administration elicited by long-access "binge" paradigms or prior drug sensitization (Zhang and Kosten, 2007; Wee et al., 2008). Combined, these results suggest that while NE does not play a critical role in the primary reinforcing effects of cocaine, as measured by standard operant self-administration, it does have significant effects under conditions that escalate or reinstate drug-seeking behavior. Furthermore, medications that impair NE production, such as disulfiram or nepicastat, may short circuit the ability of environmental triggers to promote relapse, and therefore make promising pharmacotherapies for the treatment of dependence on cocaine and other stimulants.

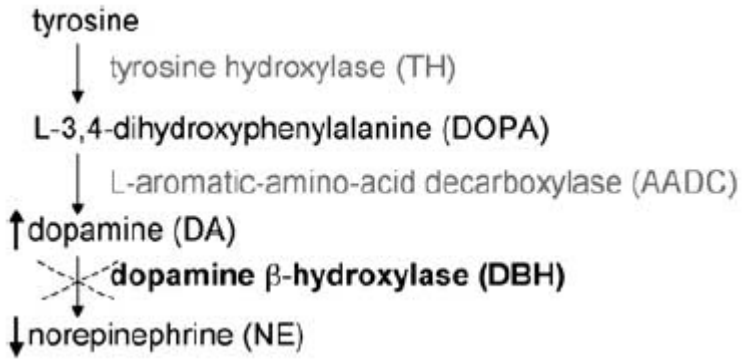

Figure A2.1 Catecholamine biosynthetic pathway. Because DBH converts DA to NE in noradrenergic neurons, inhibition of DBH is unique in its ability to decrease NE while increasing DA.

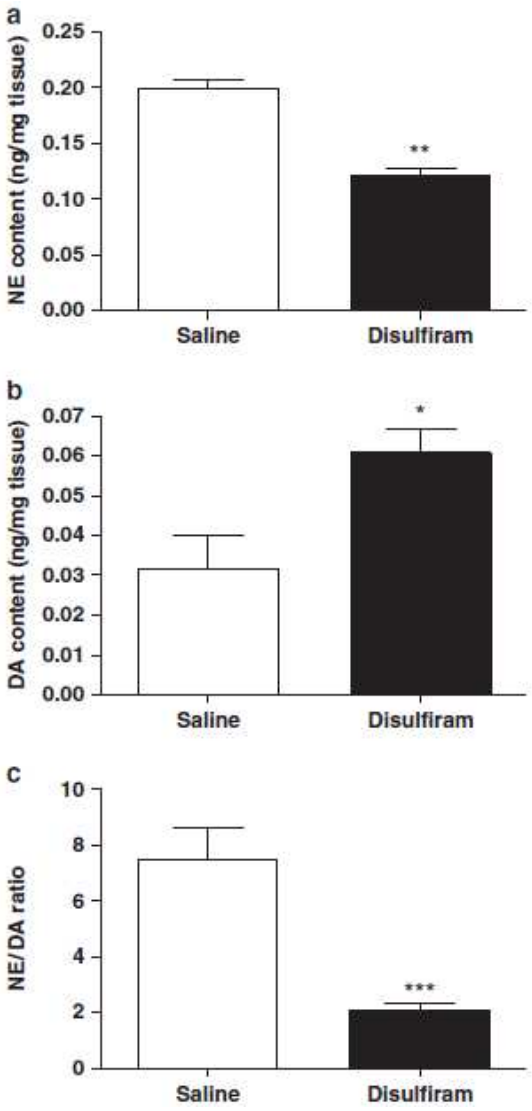

Figure A2.2 Effect of disulfiram on catecholamine levels in the rat prefrontal cortex. Shown is the mean ± SEM for (A) NE levels, (B) DA levels, and (C) the NE/DA ratio in the prefrontal cortex of rats after treatment with saline or disulfiram (single injection of 100 mg/kg, i.p., catecholamines measured 2 hours after disulfiram administration by HPLC followed by electrochemical detection; N = 6 per group). *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle.

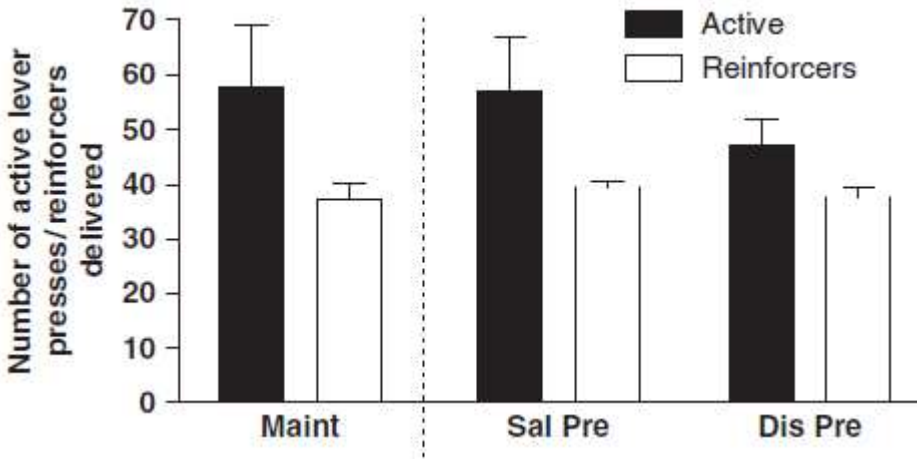

Figure A2.3 Disulfiram does not affect maintenance of cocaine self-administration. After reaching maintenance levels for operant cocaine self-administration ("Maint"), rats were pretreated with saline ("Sal Pre") or disulfiram (100 mg/kg, i.p.; "Dis Pre") 2 hours prior to cocaine self-administration sessions. Shown are mean ± SEM active lever responses and number of reinforcers obtained over a 2-hour session. Maintenance values reflect an average number of responses and reinforcers obtained over the last 3 days of maintenance. Occasional active lever pressing during the 20-second timeout periods result in more active lever presses than reinforcers received. N = 8 per group.

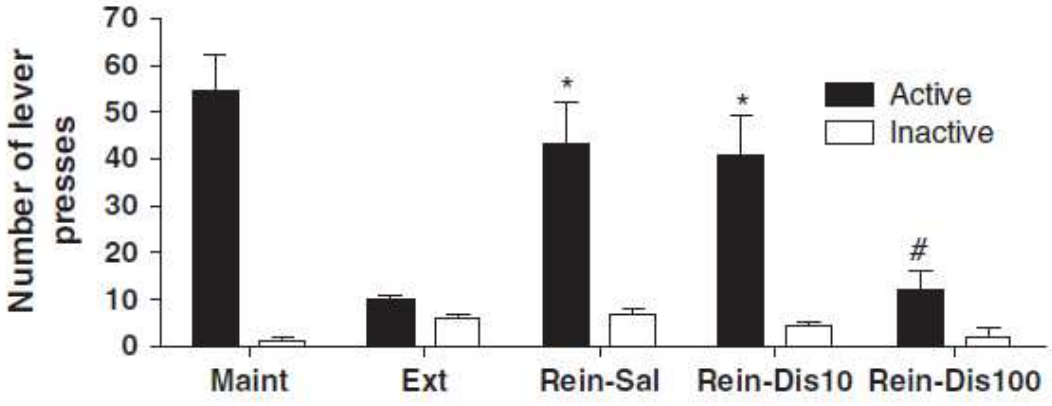

Figure A2.4 Disulfiram blocks cocaine-primed reinstatement. Once maintenance ("Maint") and extinction ("Ext") criteria for operant cocaine self-administration were met, rats were pretreated with saline ("Rein-Sal", N = 13) or disulfiram (10 or 100 mg/kg, i.p.) ("Rein-Dis10", N = 6 and "Rein-Dis100", N = 7) 2 hours prior to cocaine prime (10 mg/kg, i.p.) and placement into the self-administration chambers. Shown are active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. *$P < 0.05$ compared with active lever responses during extinction, $^{\#}P < 0.05$ compared with active lever responses during cocaine-induced reinstatement tests with saline pretreatment (N = 7 per group).

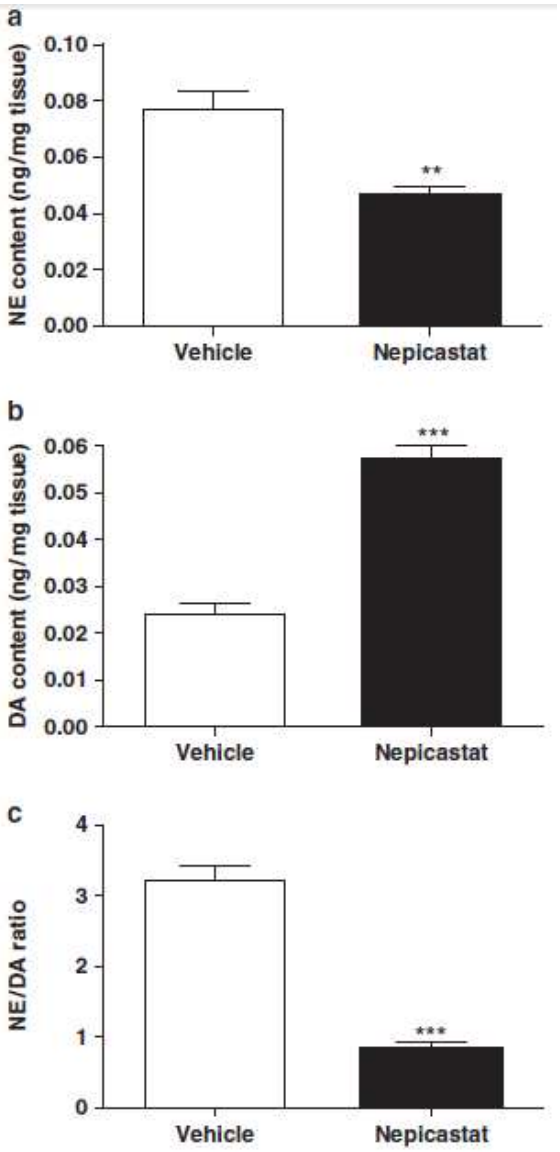

Figure A2.5 Effect of nepicastat on catecholamine levels in the rat prefrontal cortex. Shown is the mean ± SEM for (A) NE levels, (B) DA levels, and (C) the NE/DA ratio in the prefrontal cortex of rats after treatment with vehicle or nepicastat (single injection of 50 mg/kg, i.p., catecholamines measured 2 hours after nepicastat administration by HPLC followed by electrochemical detection; N = 8 per group). **P < 0.01, ***P < 0.001 compared with vehicle.

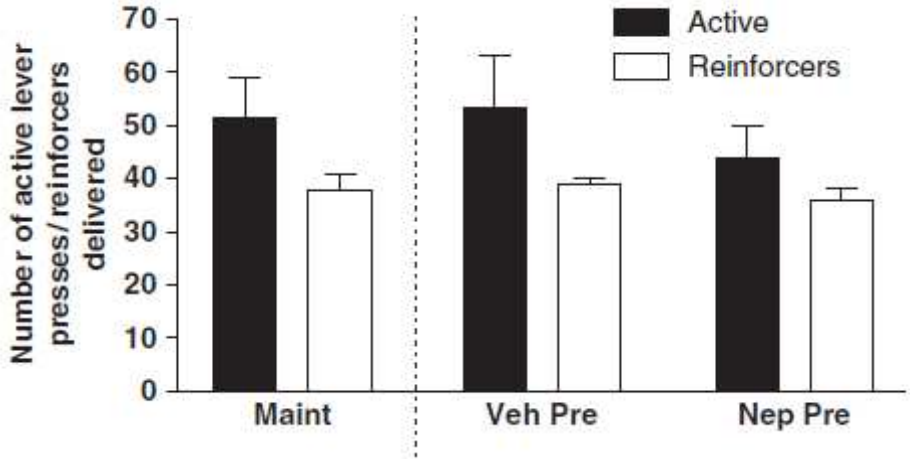

Figure A2.6 Nepicastat does not affect maintenance of cocaine self-administration. After reaching maintenance levels of operant cocaine self-administration ("Maint"), rats were pretreated with vehicle ("Veh Pre") or nepicastat (50 mg/kg, i.p.; "Nep Pre") 2 hours prior to cocaine self-administration sessions. Shown are mean ± SEM active lever responses and number of reinforcers obtained over a 2-hour session. Maintenance values reflect an average number of responses and reinforcers obtained over the last 3 days of maintenance. Occasional active lever pressing during the 20-second timeout periods result in more active lever presses than reinforcers received. N = 6 per group.

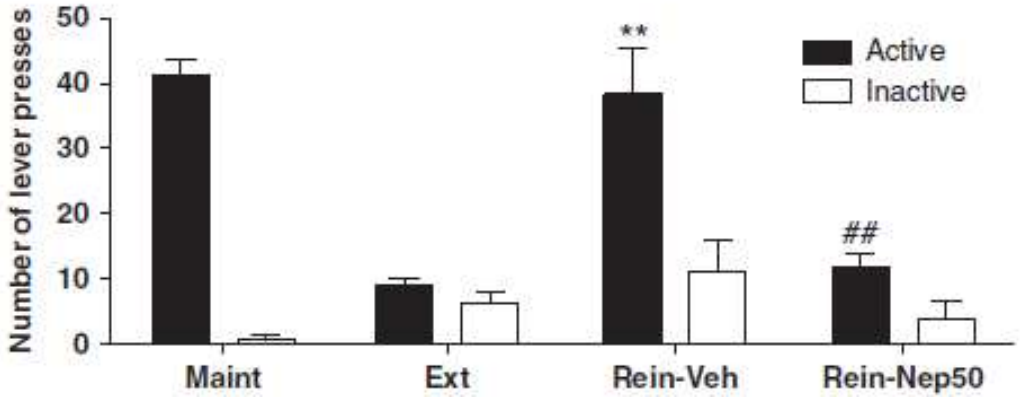

Figure A2.7 Nepicastat blocks cocaine-primed reinstatement. Once maintenance ("Maint") and extinction ("Ext") criteria for operant cocaine self-administration were met, rats were pretreated with vehicle ("Rein-Veh") or nepicastat (50 mg/kg, i.p.; "Rein-Nep50") 2 hours prior to cocaine prime (10 mg/kg, i.p.) and placement into the self-administration chambers. Shown are mean ± SEM active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. **$P < 0.01$ compared with active lever responses during extinction, $^{\#\#}P < 0.01$ compared with active lever responses during cocaine-induced reinstatement tests with saline pretreatment (N = 6 per group).

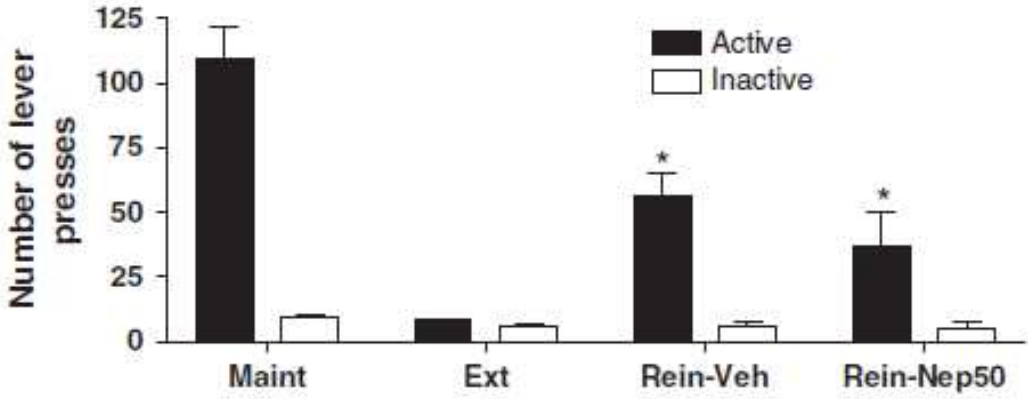

Figure A2.8 Nepicastat does not affect food-primed reinstatement of food seeking. Once maintenance ("Maint") and extinction ("Ext") criteria for operant food self-administration were met, rats were pretreated with vehicle ("Rein-Veh") or nepicastat (50 mg/kg, i.p.; "Rein-Nep50") 2 hours prior to food prime (3 pellets at beginning of session, then 1 pellet every 3 min over the 60 min session) and placement into the self-administration chambers. Shown are mean ± SEM active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. *$P < 0.05$ compared with active lever responses during extinction ($N = 7$ per group).

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