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The Role of Norepinephrine in Cocaine-Induced Reward, Anxiety, and Relapse

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An Abstract of
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in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
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Abstract

The Role of Norepinephrine in Cocaine-Induced Reward, Anxiety, and Relapse
By Jesse Schank

Cocaine is a widely abused psychostimulant that acts by blocking monoamine transporters, thus increasing extracellular availability of dopamine (DA), norepinephrine (NE), and serotonin. DA and NE are synthesized in the same enzymatic pathway, with DA being converted to NE by the enzyme dopamine β-hydroxylase (DBH). Although DA is generally thought of as the primary neurotransmitter underlying the behavioral effects of psychostimulants, a role for NE signaling is now increasingly recognized. Furthermore, disulfiram, a non-specific DBH inhibitor, is efficacious at decreasing cocaine use in human addicts. Using mice with a genetic knockout of the Dbh gene that completely lack NE (Dbh−/− mice), and by administering disulfiram, we explored how DBH and NE modulate responses to cocaine. First, we found that Dbh−/− mice were more sensitive than control mice to the locomotor stimulating effects of cocaine, and this increased psychomotor response was associated with an increased density of high affinity-state striatal DA receptors. We then explored cocaine-induced reward behavior and found that Dbh−/− mice were more sensitive to both the rewarding and aversive properties of cocaine. However, in spite of this increased cocaine aversion, Dbh−/− mice exhibit a paradoxical insensitivity to acute cocaine-induced anxiety. This behavioral phenotype was mimicked in control mice following pretreatment with disulfiram or the β-adrenergic receptor antagonist propranolol. Interestingly, we found that while propranolol blocked cocaine-induced anxiety-like behavior in control mice, the α1-adrenergic receptor antagonist prazosin blocked the expression of cocaine-induced place preference. These results suggest a dissociation between the anxiogenic and rewarding properties of cocaine, and distinguish the specific adrenergic signaling pathways involved in these behaviors. Finally, we used the rat self-administration paradigm to assess whether pretreatment with disulfiram could alter cocaine-seeking behavior. Administration of disulfiram had no effect on the maintenance phase of cocaine self-administration behavior, but completely blocked the expression of cocaine-primed reinstatement. Understanding the mechanism of action by which disulfiram attenuates cocaine use will increase our knowledge about the role of NE in drug addiction, and will help identify novel targets for future exploration in medication development for drug dependence.
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CHAPTER 1:
INTRODUCTION
1.1 Cocaine Abuse

Cocaine has an extensive history of abuse in humans, and it is currently one of the most commonly used illicit drugs in the United States. While many experiment with psychostimulant drugs and do not enter a cycle of abuse, these drugs are highly addictive and can lead to dependence. Cocaine dependence represents a financially and emotionally costly problem in today’s society, as well as a serious health risk to those who abuse the drug. Drug addiction refers to a cycle of behaviors where a user shifts between abstinence and relapse to drug use, often with periods of binge drug taking behavior. This behavior is continued in the face of adverse health, economic, and societal consequences, and significant time is spent obtaining and using the drug at the expense of other normal activities (Meyer and Quenzer, 2005).

In humans, cocaine use results in a broad spectrum of acute effects, both subjectively positive (e.g. euphoria, increased energy, enhanced alertness) and negative (e.g. anxiety, nausea). These behavioral effects are commonly reported in human users and can, in many cases, be assessed in animals using preclinical behavioral testing paradigms. Also, acute cocaine intoxication causes significant hypertension and potential cardiac arrhythmia, thus increasing the risk of sudden heart attack or stroke, and can cause seizures. These physiological effects account for the majority of emergency room admissions for people who have abused the drug excessively (Rowbotham and Lowenstein, 1990; Spivey and Euerle, 1990; O’Connor et al., 2005; Pozner et al., 2005).

In 2005, it was estimated that approximately 2.4 million Americans were regular cocaine abusers (NIDA). Chronic, high dose use of the drug can trigger a host of negative effects including paranoia, psychosis, and addiction. Paranoia refers to an
extremely agitated state expressed in the absence of threatening stimuli, and in cocaine users often takes the form of users fearing apprehension by law enforcement, or in intense suspicion of others attempting to steal their drug supply. Chronic psychostimulant-induced psychosis refers to a break from reality caused by repeated, high level use of the drug, and is often indistinguishable from a schizophrenic type of psychosis. Addiction (defined above) represents a severe negative consequence of repeated drug use, and prevention of addictive behavior has been the topic of decades of research in behavioral pharmacology and clinical psychiatry.

Cocaine belongs to the class of drugs known as psychostimulants and acts by blocking the plasma membrane transporters for norepinephrine (NE), dopamine (DA), and serotonin (5HT). Transporter proteins are responsible for the reuptake of signaling molecules following release, and blockade of transporter function leads to increased extracellular levels of neurotransmitter in the synapse (Di Chiara and Imperato, 1988). This, in turn, enhances post-synaptic signaling and induces a wide range of behavioral and neurochemical effects. Cocaine also acts as a local anesthetic by inhibiting the activity of sodium channels, but this target is not believed to mediate cocaine’s behavioral effects, and will not be discussed here. NE, DA, and 5HT belong to the class of neurotransmitter molecules known as monoamines and, with the exception of the ionotropic 5HT3 receptor, are thought to signal exclusively through G-protein coupled receptors. Because cocaine does not bind directly to postsynaptic receptors, but rather alters signaling by influencing the amount of transmitter available in the synapse, it is often referred to as an indirect agonist. Monoamine neurotransmitters are involved in a
wide range of behaviors including movement, reward, stress, cognition, emotional affect, arousal, and seizure sensitivity.

Like most drugs of abuse, cocaine increases DA signaling in the brain reward circuitry originating in the midbrain. The mesolimbic DA pathway consists of dopaminergic projections from the ventral tegmental area (VTA) in the midbrain to the nucleus accumbens (NAC), a forebrain structure involved in motivated behaviors. The VTA also sends dopaminergic projection neurons to the prefrontal cortex (PFC), and this pathway is commonly referred to as the mesocortical DA circuit. These two dopaminergic projection pathways are strongly influenced by acute and chronic use of drugs of abuse, and cocaine is especially potent at altering dopaminergic signaling in these regions. This same circuitry is also activated by natural rewards in the environment (Mirenowicz and Schultz, 1997, for review see Schultz 2002). A common metaphor used to describe the influence of abused drugs is that they “hijack” the reward pathway that typically controls motivated behavior to obtain natural rewards and “co-opts” this system to drive compulsive drug seeking and drug taking behavior.

1.2 Basic Characteristics of Norepinephrine and Dopamine Systems in the Brain

NE is a major monoamine neurotransmitter that is involved in a wide range of behaviors including seizure sensitivity, anxiety, stress, depression, attention, arousal, pain, learning, and memory (Foote et al., 1983; Levine et al., 1990; Ressler and Nemeroff, 1999; Weinshenker et al., 2001; Gibbs and Summers, 2002; Jasmin et al., 2002; Berridge and Waterhouse, 2003; Murchison et al., 2004). The major noradrenergic nuclei of the central nervous system are the locus coeruleus (LC), A1, and A2, which are
located in the pontine region of the brainstem. These nuclei send diffuse projections to a wide range of brain structures and consist of two major projection pathways: the dorsal bundle, which originates in the LC, and the ventral bundle, which projects from the A1 and A2 regions. NE is produced in noradrenergic neurons in a synthetic pathway that begins with the conversion of tyrosine to DOPA by the enzyme tyrosine hydroxylase (TH, see Figure 1.1). DOPA is then converted to DA by aromatic acid decarboxylase (AADC), and finally from DA to NE by dopamine β-hydroxylase (DBH). In neurons that release epinephrine, NE is converted by phenylethanolamine N-methyltransferase to epinephrine. Although TH is generally considered the rate-limiting enzyme in the synthesis of NE, there is evidence that DBH can become rate limiting under some conditions (Cubells and Zabetian, 2004; Bourdelat-Parks et al., 2005).

NE signals through 3 major classes of G-protein coupled receptors: the α1-adrenergic receptor (α1-AR), α2-adrenergic receptor (α2-AR), and β-adrenergic receptor (β-AR). Each of these classes is further subdivided into three subtypes. The β-AR couples primarily to the Gs subunit and positively modulates adenylyl cyclase, cyclic AMP production, and protein kinase A activation. The α2-AR couples to Gi subunits and negatively modulates this same pathway. In addition to serving as a postsynaptic heteroceptor, the α2-AR is the major presynaptic autoreceptor for NE, and negatively regulates NE release. The α1-AR couples to the Gq pathway, which stimulates the metabolism of PIP2 by phospholipase C, ultimately activating calcium release from the endoplasmic reticulum via IP3 receptors, and stimulating protein kinase C activity via diacylglycerol (see Cooper et al., 2003).
Based on their structure, which includes a catechol ring, both DA and NE belong to the subclass of monoamines known as catecholamines. Dopaminergic neurons are involved in local signaling processes in the retina, olfactory bulb, and hypothalamus. These local effects of DA will not be discussed here; this report will rather focus on the DA projection pathways that originate in midbrain nuclei such as the VTA and substantia nigra (SN). DA neurons from the SN project to the caudate/putamen (CP), also known as the dorsal striatum, and are vital for the initiation of movement. Massive DA cell death in the SN underlies the motor symptoms of Parkinson’s disease. VTA neurons project in two major pathways: the mesolimbic pathway that innervates the NAC, also known as the ventral striatum, and the mesocortical pathway that innervates various cortical regions, including the FC. These pathways are involved in complex behaviors such as cognition and reward, and will be discussed in depth in this report. DA signals through two major classes of dopaminergic receptors: the D1 class that includes both D1 and D5 receptors and stimulates Gs function, and the D2 class that includes D2, D3, and D4 receptors and acts primarily via the Gi pathway (see Cooper et al., 2003)

1.3 Norepinephrine and Dopamine Interactions

Although DA signaling has been primarily implicated in behavioral responses to psychostimulants, cocaine is known to increase extracellular NE levels as well, and NE transmission has been shown to modulate psychostimulant-induced behaviors and neurochemistry (for extensive review see Weinshenker and Schroeder et al., 2006).

The NE system is both anatomically and functionally connected to the dopaminergic reward pathways (reviewed by Moore and Bloom, 1979). For example, noradrenergic
neurons directly innervate the VTA, NAC, and PFC (Swanson and Hartman, 1975; Jones et al., 1977; Simon et al., 1979; Morrison et al., 1981; Berridge et al., 1997; Delfs et al., 1998; Liprando et al., 2004), and connect to the VTA and NAC indirectly via glutamatergic afferents projecting to these regions from the PFC (Darracq et al., 2001; see Figure 1.2). In addition to these extensive anatomical connections, several studies have outlined the functional interaction between the NE and DA systems. For example, stimulation of the LC increases burst firing of DA neurons in the VTA, an effect that is blocked by \( \alpha_1 \)-AR antagonists (Grenhoff et al., 1993; Grenhoff and Svensson, 1993; Grenhoff et al., 1995), and lesions of the LC decrease basal DA release in the NAC (Lategan et al., 1990). Further, amphetamine-induced DA release in the NAC is attenuated following NE depletion of the PFC (Ventura et al., 2003), lesion of the LC (Lategan et al., 1990; Haidkind et al., 2002), \( \alpha_1b \)-AR knockout (Auclair et al., 2002), or delivery of an \( \alpha_1 \)-AR antagonist either systemically or to the PFC (Darracq et al., 1998; Drouin et al., 2002a). Not surprisingly, these effects extend to the locomotor activating effects of psychostimulants as well. For example, amphetamine-induced locomotion is attenuated following pharmacological or genetic inhibition of the \( \alpha_1 \)-AR (Darracq et al., 1998; Drouin et al., 2002a; Drouin et al., 2002b; Auclair et al., 2004). Conversely, NE transporter (NET) knockout mice, which have elevated extracellular NE, exhibit an enhanced sensitivity to the effects of psychostimulants (Xu et al., 2000).

Interestingly, \( Dbh \) knockout (\( Dbh^{-/-} \)) mice, which completely lack NE, also display an increased sensitivity to the locomotor activating effects of both amphetamine and the D2/D3 agonist quinpirole (Weinshenker et al, 2002a). While this may seem counterintuitive in light of the findings outlined above, it agrees with the behavioral
hypersensitivity to amphetamine observed following LC lesions. Specifically, Harro and colleagues have reported hypersensitivity of DA receptors in terminal regions and an increased sensitivity to the locomotor activating effects of amphetamine after lesions of the LC (Harro et al., 2000). These effects are likely the result of a compensatory response to decreased DA availability in the synapse. These findings suggest that there may be a complex relationship between NE signaling, DA function, and responses to dopaminergic drugs such as psychostimulants, with acute versus chronic inhibition of NE having varying effects. When taken together, the results outlined above indicate that acute inhibition of NE signaling may depress DA release and attenuate locomotor response to psychostimulants, while chronic NE depletion may lead to dopaminergic hypersensitivity and enhanced psychostimulant-induced behaviors.

The evidence outlined above strongly supports an interaction between NE and DA signaling under basal conditions and in response to psychostimulants. It is clear that NE exerts an activating effect on DA release in brain regions that are pertinent for drug reward in general, and behavioral responses to psychostimulants specifically. However, the ways in which this interaction relates to drug-induced reward, anxiety, and reinforcement is less established.

### 1.4 Disulfiram, Cocaine Abuse, and Dopamine β-Hydroxylase

When considering drug abuse in our society, one major concern is how to help cocaine-dependent people abstain from drug use. While decades of research have gone into the search for a pharmacological treatment for cocaine dependence, no medication has gained widespread acceptance as a clinically efficacious treatment. Treatments that
have been tested in animals and humans cover a broad range of targets including monoamine transporters (desipramine, modafinil, GBR compounds), opioid receptors (naltrexone), cocaine metabolizing enzymes (antibodies, vaccines), ionotropic glutamate receptors (acamprosate), glutamate exchange (N-acetyl cysteine), and GABA signaling (baclofen, topiramate). For comprehensive discussion of the efficacy of these medications see (Gorelick et al., 2004; Kreek et al., 2005; Vocci and Elkashef, 2005; Sofuglu and Kosten, 2006).

Promising research in the last 10 to 15 years has indicated an efficacy of disulfiram in the treatment of cocaine addicts. Disulfiram has been used clinically for decades in the treatment of alcoholism, and acts by blocking the activity of aldehyde dehydrogenase (see Haley, 1979; Eneanya et al., 1981; Petersen, 1992; Mays et al., 1995), leading to an accumulation of acetaldehyde after alcohol consumption. This generates a severe, aversive reaction that includes intense nausea, flushing, and hypotension. Recently, it has been shown that disulfiram is also effective for the treatment of cocaine dependence, and at this time, six independent studies have confirmed its efficacy (e.g. Carroll et al., 1998; George et al., 2000; Petrakis et al., 2000). Studies concerning the use of disulfiram in the treatment of cocaine addiction were first undertaken because there is a high incidence of cocaine and alcohol codependence. Clinical observations and patient reports initially suggested that this drug might be effective in attenuating cocaine use through enhancement of aversive effects of cocaine, such as paranoia, anxiety, and nausea (Hameedi et al., 1995; McCance-Katz et al., 1998). However, these studies produced no reliable or controlled effects of disulfiram on any cocaine-induced behavioral response.
Interestingly, disulfiram reduces cocaine intake regardless of alcohol co-dependence or consumption (George et al., 2000; Petrakis et al., 2000). In fact, recent research from Carroll and colleagues (2004) has shown an even greater effectiveness of disulfiram in attenuating cocaine use if the patient is not alcohol dependent. These findings indicate that disulfiram’s effect on alcohol metabolism plays no role in its inhibition of cocaine intake. While disulfiram is beginning to show significant clinical efficacy in the treatment of cocaine abuse, its mechanism of action is unknown, and few groups have explored the effects of this drug on cocaine-related behaviors in rodents, such as place preference and self-administration.

Disulfiram inhibits a wide range of endogenous enzymes in addition to aldehyde dehydrogenase, and there is good evidence that disulfiram may be mediating its effects on cocaine use via inhibition of DBH, which is responsible for converting DA to NE in noradrenergic neurons (described above; see Figure 1.1). The primary metabolite of disulfiram (diethyldithiocarbamate) is a copper-chelating agent, and because DBH requires copper as a co-factor, disulfiram inhibits DBH enzymatic activity (Johansson 1989). Our group and others have consistently shown that systemic administration of disulfiram dose-dependently decreases NE to DA ratio in rodent brain tissue (Musacchio et al., 1966, Maj et al., 1968; Karamanakos et al., 2001; Bourdelat-Parks et al., 2005). Disulfiram also decreases NE and related metabolites in urine, blood, and cerebrospinal fluid in 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).

In the human population, DBH function is under strong genetic control (reviewed in Cubells and Zabetian, 2004), and low genetic DBH activity correlates with increased
susceptibility to certain aversive effects of cocaine, specifically cocaine-induced paranoia (Cubells et al. 2000, Kalayasiri et al., 2007). Further, DBH activity interacts with the effectiveness of disulfiram, with people that express lower levels of DBH activity being more sensitive to disulfiram’s effect on cocaine intake, disulfiram-induced psychosis, and cocaine-induced paranoia (Ewing et al., 1977; Major et al., 1979; R. Schottenfeld and J. Cubells, personal communication). Taken together, these findings indicate an interaction between level of DBH activity, sensitivity to certain behavioral effects of cocaine, and responsiveness to disulfiram pharmacotherapy for cocaine addiction, suggesting that DBH inhibition underlies disulfiram’s effect on cocaine use.

1.5 Potential Mechanisms for Disulfiram Action on Cocaine Abuse

In humans, disulfiram could be acting in a number of ways to decrease cocaine intake. First, it could be blunting the rewarding properties of cocaine, thus decreasing motivation to take the drug. Second, disulfiram could be acting to enhance some aversive property of cocaine, in a similar fashion to the way in which it enhances the nausea-inducing effects of alcohol. Finally, disulfiram could be preventing relapse to compulsive drug seeking behavior, possibly in part through alleviating the negative affective states that are induced by drug withdrawal.

There is considerable evidence to suggest that inhibiting NE function through blocking DBH activity would impact the rewarding effects of cocaine. For example, specific NE lesions of the PFC block the expression of amphetamine-induced place preference (Ventura et al., 2003). Also, while no groups have convincingly demonstrated that NE is directly involved in the maintenance of cocaine self-administration behavior
(for review see Weinshenker and Schroeder, 2007), NE signaling through the $\alpha_1$-AR mediates escalated drug intake following certain drug exposure regimens. Specifically, $\alpha_1$-AR antagonists prevent increased rates of cocaine self-administration following repeated non-contingent administration of cocaine prior to self-administration training (Zhang and Kosten, 2007) and extended access to cocaine during long access sessions (Wee et al., 2008). This suggests that even though NE may not play an integral role in the immediate reinforcing effects of cocaine in the rat self-administration paradigm per se, alterations in NE activity may be involved in the transition from initial exposure to addictive drug seeking behavior.

Alternatively, an enhancement of cocaine’s aversive properties could underlie the ability of disulfiram to inhibit cocaine use in the clinic. It is known that psychostimulant enhancement of dopaminergic signaling can induce anxiety in laboratory animals, which can interfere with the rewarding aspects of certain drugs. For example, repeated amphetamine administration can increase anxiety-like behavior in the elevated plus maze in a dopamine receptor dependent manner (Cancela et al., 2001). Further, cocaine reward is attenuated in BALB/cByJ mice because of the high anxiety levels in this strain (David et al., 2001). These results indicate that genetic background can alter cocaine responses and suggests that enhanced aversive properties of a drug, which in some cases can be induced by hyperactivity of dopaminergic signaling, can directly counteract drug reward. In addition, cocaine-induced paranoia is more prevalent in human cocaine abusers with genetically low DBH activity (Cubells et al., 2000; Kalayasiri et al., 2007; described above), and $Dbh^{-/-}$ mice show an increased sensitivity to amphetamine-induced stereotypy (Weinshenker et al., 2002a). Stereotypical behaviors are highly repetitive,
non-goal directed movements such as circling and head bobbing that often result from excessive DA activation.

A third possibility is that DBH inhibition could be preventing stress- and drug-induced relapse to compulsive drug use. Stressors can induce both central and peripheral NE release, and the induction of stress can lead to relapse in both humans and in rodent models of self-administration (reviewed in Shaham et al., 2000; Shaham et al., 2003). Both systemic injection of α2-AR agonists, which act at autoreceptors to decrease NE release, and site specific blockade of β-ARs in the amygdala or bed nucleus of the stria terminalis, attenuate the reinstatement of cocaine seeking behavior following an acute stressor (Erb et al., 2000; Leri et al., 2002). NE interacts with the extrahypothalamic corticotropin releasing factor system in these structures to influence the expression of stress-induced reinstatement (Erb et al., 1998; Erb and Stewart, 1999). Further, augmentation of NE release alone precipitates reinstatement in rats and non-human primates (Lee et al., 2004; Shepard et al., 2004). These results indicate that NE is necessary and sufficient for stress-induced reinstatement. Also, blockade of α1-ARs inhibits cocaine-induced reinstatement of drug seeking (Zhang and Kosten, 2005), indicating that NE is involved in non-stress circuits of drug reinstatement as well. Stress or cocaine-induced relapse pose particularly challenging obstacles in the clinical treatment of cocaine dependence, and these phenomena represent potential targets for treatments that alter NE levels or transmission.

In addition to its well-documented rewarding and locomotor activating effects in rodents, acute cocaine also induces anxiety-like behavior (Costall et al., 1988; Rogiero and Takahashi, 1992; Blanchard and Blanchard 1999). This anxiogenic property of
cocaine can be reversed by administration of anxiolytic agents, such as diazepam and alcohol (Ettenberg and Geist, 1990; Paine et al., 2002). NE is highly involved in stress and anxiety responses in general (for review see Stanford 1995, Morilak et al., 2005), and signaling through the β-AR specifically mediates anxiety responses in rodents (Gorman and Dunn, 1993). Interestingly, cocaine withdrawal also induces anxiety, and this phenomenon is associated with increased NE activity (Harris and Aston-Jones, 1993; McDougle et al., 1994; Kampman et al., 2001). In fact, recent findings indicate that activation of the β1-AR in the amygdala is specifically involved in the expression of cocaine withdrawal-induced anxiety (Rudoy et al., 2007).

Negative affective states induced by withdrawal, such as anxiety and depression, are commonly cited by addicts as precipitating factors for relapse into compulsive drug seeking. This is an example of negative reinforcement, where the probability that a behavior (drug taking) will be performed is increased by motivation to remove a certain stimulus (withdrawal symptoms; Koob and LeMoal, 2008). Alleviation of withdrawal-induced anxiety could potentially decrease risk of relapse, and may represent a potential behavioral target for treatment of drug addiction. Whereas cocaine-induced reward has been studied extensively, less is known about the processes underlying the subjectively negative behavioral states associated with acute administration of the drug or withdrawal from chronic psychostimulant use.

These three hypotheses (increased aversion, blunted reward, and relapse prevention) to explain the efficacy of disulfiram in the treatment of cocaine dependence are not mutually exclusive; all three could act in concert to attenuate drug-taking behavior. The experiments outlined in this proposal will assess the alterations in cocaine-
induced behaviors that result from genetic or pharmacological DBH inhibition, and will attempt to determine the involvement of the behavioral targets listed above in these responses.

1.6 Dopamine Beta-Hydroxylase Knockout Mouse: A Novel Tool for Studying the Role of Norepinephrine and DBH in Drug Addiction

In our lab, we have a unique tool for exploring the impact of DBH function on responses to psychostimulants: a colony of mice that contain a targeted mutation of the Dbh gene (Dbh -/- mice). These mutants were developed in the laboratory of Dr. Richard Palmiter at the University of Washington in the mid-1990s, and contain a neomycin resistance cassette inserted in place of 0.6 Kb of the proximal gene promoter and the first exon, completely inhibiting DBH protein synthesis and enzymatic activity (Thomas et al., 1995). Since these animals cannot convert DA to NE, they completely lack NE and produce DA in their “noradrenergic” cells. As mentioned above, epinephrine is produced following synthesis of NE; therefore, Dbh -/- mice also lack epinephrine. Because epinephrine levels in the central nervous system are very low and nuclei that release this transmitter do not project to brain pathways involved in behavioral responses to psychostimulants, the epinephrine system will not be discussed here.

Complete ablation of NE is lethal in utero, necessitating the addition of adrenergic agonists and precursors in the drinking water of pregnant dams to ensure survival of knockout pups. Drinking water provided to pregnant dams is supplemented with the α1-AR agonist phenylephrine and the β-AR agonist isoproterenol from E9.5 to E14.5, and dihydroxyphenylserine (DOPS) from E15 to birth (Thomas et al., 1995; Thomas et al.,
DOPS is a catecholamine precursor that can be converted to NE by the enzyme AADC, thus bypassing the requirement for DBH in NE synthesis. Noradrenergic nuclei in the CNS of these animals develop relatively normally, with cell counts, projection pathways, and co-transmitters remaining largely intact (Weinshenker et al., 2001a; Jin et al., 2004; D. Weinshenker, personal communication). Expression of the NET and adrenergic receptors is mostly normal in Dbh -/- mice, with the exception of an upregulation of β-ARs (Weinshenker et al., 2002b; Sanders et al., 2006). These observations will be discussed in the context of Dbh -/- phenotypes where appropriate.

Because brain catecholamine levels and behavioral phenotypes of heterozygous (Dbh +/-) mice are indistinguishable from those of true wild-type mice (Dbh +/+), Dbh +/- mice have historically been used as controls for Dbh -/- mice, including many of the experiments shown in this report (e.g. Thomas et al., 1998; Szot et al., 1999; Mitchell et al., 2006). Dbh -/- mice exhibit a wide range of phenotypes including impaired maternal behavior (Thomas and Palmiter, 1997a), increased seizure sensitivity (Weinshenker et al., 2001a; Weinshenker et al., 2001b; Schank et al., 2005), ptosis (Thomas et al., 1995), and impaired thermoregulatory response (Thomas and Palmiter, 1997b). Many of these behavioral phenotypes can be reversed by acute administration of DOPS or adrenergic receptor agonists (Thomas et al., 1998; Szot et al., 1999; Weinshenker et al., 2001b). These results further support the interpretation that the NE system of Dbh -/- mice remains largely intact in spite of chronic lack of NE, and in these animals adrenergic receptors are available and can be activated by appropriate agonists. Furthermore, since acute DOPS administration restores NE but does not normalize DA levels in
noradrenergic neurons (Thomas et al., 1998), these results indicate that most phenotypes associated with the $Dbh$ $^{-/-}$ mutation are due to a lack of NE, and not ectopic DA.

In addition to the phenotypes listed above, $Dbh$ $^{-/-}$ mice also display a hypersensitivity to drugs that activate the dopaminergic system. For example, these animals show increased locomotor activity in response to the D2/D3 receptor agonist quinpirole (Weinshenker et al., 2002a). In addition, $Dbh$ $^{-/-}$ mice are more sensitive to the locomotor activating and stereotypy inducing effects of amphetamine (Weinshenker et al., 2002a; mentioned above). Many of the experiments in this report further explore this DA hypersensitivity and attempt to determine if this heightened responsiveness to dopaminergic-acting drugs extends to the behavioral effects of cocaine.

1.7 Rodent Behavioral Testing Paradigms

Cocaine-induced behaviors that were examined in this report using rodent models include cocaine-induced reward, anxiety, reinforcement and relapse. To study these behaviors we used well-established rodent behavioral testing paradigms such as conditioned place preference, elevated plus maze, and rat intravenous self-administration. These paradigms will now be discussed in detail to provide readers with a basis for the rationale behind their selection and the interpretation of results.

The conditioned place preference (CPP) paradigm is commonly used to test the rewarding and aversive properties of environmental stimuli, such as drugs (for review see Tzschentke 1998, Bardo and Bevins 2000). The apparatus used for testing is a chamber consisting of two conditioning compartments that are very distinct from one another, and are separated by a center neutral section that is smaller than the two conditioning areas.
The conditioning compartments are made distinct from one another using a variety of sensory cues including varying floor texture, pattern of wall covering, lighting condition, and scent. The animal is introduced to the chamber on pretest day, allowed to freely explore the entire apparatus, and the amount of time spent in each compartment is recorded using a photobeam tracking system. Then, over three conditioning days the animal is injected with saline in the morning and immediately confined to one side of the chamber for 30 minutes. Several hours later, the animal is injected with cocaine and confined to the opposite side of the chamber. Over the three conditioning days the animal forms an association between the properties of the test drug and the side of the chamber on which it was confined following drug injection. After this conditioning phase the animal is returned to the chamber again on the following day, in a drug-free state, and allowed to freely explore. This test is based on the interpretation that if an animal finds the properties of the drug rewarding, on test day it will go to the side that was previously paired with drug injection. If the animal finds the properties of the drug aversive, it will avoid the side that was paired with drug injection.

To test cocaine-induced anxiety behavior we utilized the elevated plus maze (EPM) paradigm (for review see Johnston and File, 1988). The apparatus for this behavioral test consists of a platform that sits approximately three feet off the ground and includes four arms arranged in a plus orientation, two of which are open and two of which are enclosed. This test is based on the premise that a rodent naturally prefers a dark, enclosed compartment relative to an open well-lit one. Therefore, increased exploration of the open arms is believed to indicate a decrease in anxiety, because the animal is spending more time exploring compartments of the apparatus in which it would normally
be less comfortable. This interpretation has been validated by the efficacy of known anxiolytic and anxiogenic agents in the EPM. To express our data, we use the dependent variable of percent open arm time, which we calculate by dividing the amount of time spent in the open arms by the combined amount of time spent in the open and closed arms. Using percent open arm time instead of absolute amount of time spent in the open arms helps to control for any differences in total locomotor activity on the plus maze apparatus between treatment groups (Pellow et al., 1985).

We used the rat intravenous self-administration paradigm to test the reinforcing properties of cocaine (for review see Roberts et al., 2007). These tests were performed in rats because intravenous self-administration in mice is an extremely challenging technique that few labs have had success at consistently implementing. In rat self-administration, the chambers used for testing are equipped with two levers, one that results in reward delivery (the “active lever”) and one that does not (the “inactive lever”). In this procedure, the animal is first trained to press a lever to receive food pellets, which is necessary to initiate high levels of responding on days where the animal is first exposed to drug availability. Pretraining for food reward also helps to establish a preference for the active lever in the test animals. After this training phase the rat is implanted with a jugular catheter and allowed several days for recovery. During drug administration sessions, the intravenous catheter is attached to an infusion line via a back mount cannula that connects with the catheter tubing. When the animal depresses the active lever, a pump is activated that initiates an intravenous infusion of the drug. In this way the animal can control when drug is delivered, with some experimenter imposed restrictions and requirements. This is known as operant reinforced responding for
cocaine because the animal is performing an active behavior (lever press) to receive a reward (drug infusion).

A number of experimenter-controlled conditions can be applied to these sessions to measure various behavioral properties of the administered drug. First, the schedule of drug delivery can be modified to alter the response requirement for drug infusions. In fixed ratio (FR) schedules the animal must press the lever a specific number of times to receive drug rewards. For example, in an FR3 schedule, the animal receives an infusion following every third press of the active lever. Progressive Ratio (PR) schedules can be used to measure motivation for drug delivery (for review see Richardson and Roberts, 1996). In this schedule the number of responses required for a single infusion of the test drug is gradually increased. The maximum number of lever responses that the animal will perform to receive a drug infusion is referred to as the breaking point (BP). The general interpretation is that the higher the BP, the harder the animal is willing to work to obtain drug infusions. Although we did not use a PR schedule in our experiments, this paradigm could be an informative addition to future studies with disulfiram and other noradrenergic manipulations. To prevent excessive intake of drug over a short period of time, a timeout can be initiated following drug infusions where a cue light is illuminated for several seconds. During this timeout, further responses on the active lever do not result in drug infusions, although lever responses are recorded. Following the timeout, the animal is returned to the normal schedule of reinforcement. Also, the maximum number of rewards that can be obtained over the session can be capped so that the session ends prematurely to prevent overdose. Session duration is another variable that can be manipulated where differing patterns of drug taking behavior are commonly observed.
following short access (1 to 2 hours), or long access (greater than 6 hours; see Wee et al., 2007)

There are several phases of self-administration behavior that can be analyzed. Acquisition refers to when the rat first learns to press the active lever to receive drug infusions at a reliable rate, and will consistently select the active lever at a greater frequency than the inactive lever. Maintenance responding refers to the animal maintaining a consistently high frequency of active lever selection that stays stable over several days. A common criteria used to indicate maintenance behavior is less than 10-20% variability from the mean number of active lever presses over three consecutive days, and a greater than 75% selection of the active lever on each day. Extinction describes a phase where drug is no longer delivered following active lever responses and instead vehicle infusion, or no infusion at all, results from active lever presses. After an initial increase in responding, active lever responses will gradually decrease and reach a stable, low level after several sessions. Responding on the active lever can be reinstated by certain stimuli, including priming injection of the drug, stressors such as intermittent footshock, or the presentation of cues or environments that were previously paired with drug availability. In this reinstatement phase, active lever responding is reactivated by these particular stimuli, and occurs even though the active lever continues to deliver no drug reward.

1.8 Summary

It is clear, based on the evidence outlined above, that NE signaling has a significant effect on DA transmission in brain regions pertinent to reward, and
manipulations of the NE system can alter behavioral responses to psychostimulants. Further, there is also good evidence to suggest that DBH function is involved in the clinical efficacy of disulfiram as a pharmacotherapy for cocaine addiction. What is unclear is the behavioral target of disulfiram, and how specifically this treatment alters drug-seeking behavior. The purpose of this study was to explore the role of DBH function and NE signaling in the behavioral effects of cocaine, including drug-induced locomotion, reward, anxiety, and reinforcement. To ask these questions we used a variety of tools including mice with a genetic deletion of the Dbh gene, pharmacological inhibitors of the DBH enzyme, and antagonists of specific subtypes of adrenergic receptors. The outcome of the experiments performed will be related back to the clinical effect of disulfiram, and will be used to suggest a potential mechanism for the efficacy of this potential treatment for cocaine addiction.
Figure 1.1. NE Synthesis pathway

Tyrosine

Tyrosine Hydroxylase

DOPA

Aromatic Acid Decarboxylase

Dopamine

Dopamine β-hydroxylase

Norepinephrine
Figure 1.2. NE and DA anatomical connections
CHAPTER 2:

DOPAMINE β-HYDROXYLASE KNOCKOUT MICE HAVE ALTERATIONS IN DOPAMINE SIGNALING AND ARE HYPERSENSITIVE TO COCAINE

Adapted from:

2.1 Abstract

Multiple lines of evidence demonstrate that the noradrenergic system provides both direct and indirect excitatory drive onto midbrain dopamine (DA) neurons. We used dopamine β-hydroxylase knockout (Dbh -/-) mice that lack norepinephrine (NE) to determine the consequences of chronic NE deficiency on midbrain DA neuron function in vivo. Basal extracellular DA levels were significantly attenuated in the nucleus accumbens (NAC) and caudate putamen (CP), but not prefrontal cortex (PFC), of Dbh -/- mice, while amphetamine-induced DA release was absent in the NAC and attenuated in the CP and PFC. The decrease in dopaminergic tone was associated with a profound increase in the density of high-affinity state D1 and D2 DA receptors in the NAC and CP, while DA receptors in the PFC were relatively unaffected. As a behavioral consequence of these neurochemical changes, Dbh -/- mice were hypersensitive to the psychomotor, rewarding, and aversive effects of cocaine as measured by locomotor activity and conditioned place preference. However, the α1-AR antagonist prazosin blocked the expression of cocaine-induced place preference in control mice, suggesting a difference between chronic lack of NE and acute inhibition of adrenergic receptors. Antagonists of DA, but not serotonin (5-HT) receptors attenuated the locomotor hypersensitivity to cocaine in Dbh -/- mice.

Because DBH activity in humans is genetically controlled, and the DBH inhibitor disulfiram has shown promise as a pharmacotherapy for cocaine dependence, these results have implications for the influence of genetic and pharmacological DBH inhibition on DA system function and drug addiction.
2.2 Introduction

Brainstem 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). Dopamine β-hydroxylase (DBH) controls norepinephrine (NE) to DA ratio in noradrenergic neurons, and modulates the activity of the DA system and certain psychostimulant-induced behaviors. This pathway has recently become of particular interest for three reasons: (1) a common, single base polymorphism in the human Dbh gene has been identified that controls DBH enzymatic activity (Zabetian et al., 2001), (2) DBH activity affects responses to cocaine in humans, and recent studies indicate that the DBH inhibitor disulfiram has efficacy as a treatment for cocaine dependence (Carroll et al., 1998; Cubells et al., 2000; George et al., 2000; Petrakis et al., 2000; Carroll et al., 2004), and (3) Dbh knockout (Dbh -/-) mice have changes in DA receptor signaling and are hypersensitive to the psychomotor effects of amphetamine (Weinshenker et al., 2002a). Therefore, it is of interest to understand the influence of DBH and the noradrenergic system on DA neuron function.

The psychostimulants cocaine and amphetamine facilitate release and/or block reuptake of DA, NE, and serotonin (5-HT), resulting in increased synaptic availability of these neurotransmitters. The mesolimbic and mesocortical DA systems, comprised of projections from the VTA to the nucleus accumbens (NAC) and prefrontal cortex (PFC), respectively, have been primarily implicated in both the reinforcing and aversive effects of these drugs of abuse (Koob et al., 1998; Spanagel and Weiss, 1999). While DA
pathways and signaling have been the focus of most psychostimulant addiction research, it is also clear that NE plays an important role in modulating responses to psychostimulants. For example, amphetamine-induced accumbal DA release and place preference is lost in mice with NE depletion in the PFC, suggesting that psychostimulant reward depends on a functional noradrenergic system (Ventura et al., 2003). Also, lesions of noradrenergic neurons, administration of the α1-adrenergic receptor (α1-AR) antagonist prazosin, or targeted disruption of the α1b-AR attenuate the psychomotor effects of psychostimulants in rodents (Snoddy and Tessel, 1985; Mohammed et al., 1986; Darracq et al., 1998; Drouin et al., 2002a; Drouin et al., 2002b). Paradoxically, we found that Dbh knockout (Dbh −/−) mice are hypersensitive to the psychomotor effects of amphetamine (Weinshenker et al., 2002a), indicating that differences exist between chronic NE deficiency and disruption of a single adrenergic receptor or relatively acute losses of NE signaling.

Because the noradrenergic system has a facilitory effect on DA neurons, we hypothesized that DA release would be compromised in Dbh −/− mice and that a compensatory increase in DA receptor signaling might underlie the hypersensitivity of Dbh −/− mice to amphetamine. To identify neurochemical changes associated with the amphetamine hypersensitivity in Dbh −/− mice, we assessed DA release by microdialysis and high-affinity state DA receptors by radioligand binding in vitro. To further explore the effects of chronic DBH inhibition and NE deficiency on behavioral responses to psychostimulants, we tested cocaine-induced locomotion, reward, and aversion. Finally, since α1-AR antagonists typically attenuate DA release in the NAC and block the
expression of psychostimulant-induced hyperlocomotion, we tested the effect of the \( \alpha_1 \)-AR antagonist prazosin on cocaine-induced place preference.

2.3 Materials and Methods

Mouse Breeding and Genotyping

\( Dbh -/- \) mice, maintained on a mixed 129/SvEv and C57BL/6J background, were developed and generated as described (Thomas et al., 1995; Thomas et al., 1998). \( Dbh -/- \) males were bred to \( Dbh +/- \) females. Pregnant \( Dbh +/- \) females were given the AR agonists isoproterenol and phenylephrine (20 \( \mu \)g/ml each) + vitamin C (2 mg/ml) from E9.5 to E14.5, and L-3,4-dihydroxyphenylserine (DOPS; 2 mg/ml + vitamin C 2 mg/ml) from E14.5 to birth in their drinking water to rescue the embryonic lethality associated with the homozygous \( Dbh -/- \) mutation. Because of this treatment, NE and epinephrine were present in \( Dbh -/- \) animals before, but not after, birth. \( Dbh -/- \) mice were identified by the delayed growth and ptosis phenotypes, which are 100% correlated with the \( Dbh -/- \) genotype. Genotypes were confirmed by PCR. \( Dbh +/- \) mice were used as controls because they have normal catecholamine levels and are indistinguishable from \( Dbh +/- \) mice for all previously tested phenotypes (Thomas et al, 1995; Thomas et al., 1998). All mice were reared in a specific pathogen-free facility with a 12 hr light/dark cycle (lights on at 7:00, lights off at 19:00); food and water were available ad libitum. Naïve male and female mice between 3 and 6 months of age were used for all experiments. No sex differences were observed, and results for males and females were combined.

Experimental protocols were approved by the animal care committees at Emory University, University of Toronto, and Universita La Sapienza, and meet the guidelines of
the American Association for Accreditation of Laboratory Animal Care and Italian national law (DL n. 116, 1992) governing the use of animals for research.

**Locomotor Activity**

Experiments were conducted in an isolated behavior room between 10:00 and 16:00. Ambulations (consecutive beam breaks) were measured in transparent plexiglass cages (40 x 20 x 20 cm) placed into a rack with 7 infrared photobeams spaced 5 cm apart, with each end beam 5 cm from the cage wall (San Diego Instruments Inc., LaJolla, CA). Mice were placed in the activity chambers for 4 hours, injected with cocaine (5, 10, or 20 mg/kg i.p.), and ambulations were recorded for an additional 2 hr. Data were analyzed by ANOVA followed by Bonferroni post-hoc tests. For the antagonist studies, saline, the 5-HT1A antagonist WAY100635 (0.03 mg/kg), the 5-HT2 antagonist ketanserin (0.3 mg/kg), the D1 antagonist SCH23390 (0.03 mg/kg), or the D2 antagonist eticlopride were injected i.p. 30 minutes prior to cocaine (20 mg/kg). Antagonist doses were chosen based on the literature and our pilot experiments; higher doses were tried, but typically resulted in sedation and ataxia, indicating non-specific effects. All drugs were purchased from Sigma-Aldrich (St. Louis, MO).

**Radioligand Binding**

Receptor binding experiments were performed by the lab of Dr. Philip Seeman at the University of Toronto in Toronto, Ontario.

Mice were euthanized by CO2 asphyxiation, and brains were quickly removed and dissected on ice. Dissections were guided by the mouse brain atlas (Paxinos and Franklin, 1997). The frontal cortex was isolated by removing the olfactory bulb and making a cut 1 mm caudal to the beginning of the brain proper. Because this region is comprised mostly
of prefrontal cortex (PFC) and related structures, it will be subsequently referred to as the PFC. The striatum was isolated by making a second cut 1.5 mm caudal to the first cut, then cutting away the cortex, the most medial structures including the septum and diagonal band, and the most ventral structures including the olfactory tubercle and ventral pallidum. Each remaining hemisphere was then cut horizontally just above the anterior commissure to isolate the dorsal striatum (caudate putamen, CP) and nucleus accumbens (NAC). Brain tissue was then placed in microfuge tubes, frozen on dry ice, and stored at -70°C until used. Because approximately 30 mg of tissue was required for each assay, tissue from 8 mice per genotype was batched.

D2 receptor radioligand binding experiments were performed as described (Seeman et al., 2002). Briefly, tissue was homogenized in buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl$_2$) with or without 200 µM guanilylimidodiphosphate (guanine nucleotide; GN), and placed in glass test tubes, followed by the addition of [$^3$H]raclopride (76.8 Ci/mmol; 12 final concentrations, from 0.2-20 nM). Samples were incubated at room temperature and passed through a filter, which was then rinsed, placed in vials of scintillant, and monitored 6 hours later for tritium using a spectrometer. Nonspecific binding for D2 receptors was defined as that which occurred in the presence of 10 µM S-sulpiride (Ravizza, Milan, Italy). The density ($B_{max}$) and dissociation constant ($K_d$) of [$^3$H]raclopride binding sites were obtained by Scatchard analysis. Scatchard analysis was performed in triplicate on each tissue batch, and the receptor density values were consistently within 5-8% of each other. D1 receptor density was measured by the same procedure, except that the ligand was [$^3$H]SCH23390 (75.5 Curies/mmol) and non-specific binding was defined by 1 µM (+)butaclamol.
[^3]H]raclopride K\textsubscript{d} values ranged from 1 nM to 2.5 nM, and[^3]H]SCH23390 K\textsubscript{d} values ranged from 0.21 nM to 0.49 nM, similar to published values. The addition of G.N. consistently elevated the K\textsubscript{d} of[^3]H]raclopride, reflecting the release of endogenous DA, which then competes with[^3]H]raclopride. The addition of GN did not affect the K\textsubscript{d} of[^3]H]SCH23390. Each binding assay was performed three times on separate days, and the absolute values for the total receptor densities varied 5-8% from day to day. Therefore, it was more reliable to measure the difference caused by GN on the receptor density in the Dbh +/- and Dbh -/- samples on the same day. This procedure permitted the reliable detection of differences in DA receptor densities, which ranged from 0.7 to 11.7 pmol/g for D1 and from 0.4 to 10.3 pmol/g for D2. The net difference caused by GN varied by less than 10% for the triplicate experiments.

**Microdialysis**

The following experiments were performed by the lab of Dr. Stefano Puglisi-Allegra at Universita La Sapienza in Rome, Italy.

Upon their arrival, animals were housed in groups of 4 in standard breeding cages (27x21x13.5 cm) with food and water ad libitum on a 12/12 hour dark/light cycle (light on between 7:00 and 19:00). All mice used were handled and accustomed to the environment where the experiment was to be performed and then randomly assigned to different treatments. All experiments were carried out between 14:00 and 18:00.

Animals were anesthetized with chloral hydrate (450 mg/kg), mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) equipped with a mouse adapter, and implanted unilaterally with a guide cannula (stainless steel, shaft OD 0.38 mm, Metalant AB, Stockholm, Sweden,) in the CP, NAC, or PFC. The length of the
guide cannula was 2.5 mm for the CP, 4 mm for the NAC, and 1 mm for the PFC. The guide cannula was fixed with epoxy glue, and dental cement was added for further stabilization. The coordinates from bregma, measured in mm according to the mouse brain atlas (Paxinos and Franklin, 1997), were: +1.0 anteroposterior and +1.4 lateral for the CP, +1.4 anteroposterior and +0.6 lateral for the NAC (mostly including the shell subdivision; Paxinos and Franklin, 1997; Ventura et al., 2003), and +2.52 anteroposterior and 0.6 lateral for the PFC. The probe (dialysis membrane length 2 mm for CP and PFC and 1 mm for NAC; o.d. 0.24 mm, MAB 4 cuprophane microdialysis probe, Metalant AB) was introduced 24-48 hours after implantation of the guide cannula. The animals were lightly anesthetized with chloral hydrate (225 mg/kg) to facilitate manual insertion of the microdialysis probe into the guide cannula. The membranes were tested for in vitro recovery of DA on the day before use in order to verify recovery.

The microdialysis probe was connected to a CMA/100 pump (Carnegie Medicine, Stockholm, Sweden) through PE-20 tubing and an ultra-low torque dual channel liquid swivel (Model 375/D/22QM, Instech Laboratories, Inc., Plymouth Meeting, PA) to allow free movement. Artificial CSF (147 mM NaCl, 2.2 mM CaCl₂ and 4 mM KCl) was pumped through the dialysis probe at a constant flow rate of 2 µl/min. Experiments were carried out 22-24 hours after probe placement. Each animal was placed in a circular cage (21.5 cm in height, 17.5 cm in diameter) provided with microdialysis equipment (Instech Laboratories, Inc.) and with home cage bedding on the floor. Dialysis perfusion was started 1 hour later. Following the start of dialysis perfusion mice were left undisturbed for approximately 2 hours before the collection of baseline samples. Following baseline sample collection (1 sample every 20 minutes for 60 minutes), mice were injected with
saline or amphetamine (2.5 mg/kg, i.p.), and dialysate was collected every 20 minutes for 120 minutes. Only data from mice with correctly placed cannula (judged by methylene blue staining) were reported. Twenty µl of the dialysate samples were analyzed by high performance liquid chromatography (HPLC). The remaining 20 µl were kept for possible subsequent analysis. Concentrations (pg/20 µl) were not corrected for probe recovery. The mean concentration of the 3 samples collected immediately before treatment (less than 10% variation) was taken as basal concentration.

The HPLC system consisted of an Alliance (Waters Corporation, Milford, MA) system and a coulometric detector (ESA Model 5200A Coulochem II) provided with a conditioning cell (M 5021) and an analytical cell (M 5011). The conditioning cell was set at 400 mV, electrode 1 at 200 mV, and electrode 2 at -250 mV. A Nova-Pack C18 column (3.9 x 150 mm, Waters) maintained at 33°C was used. The flow rate was 1.1 ml/min. The mobile phase was as previously described (Ventura et al., 2003). The assay detection limit was 0.1 pg.

Statistical analyses were performed on raw data (concentrations, pg/20 µl). The effects of amphetamine on extracellular monoamine levels in the CP, NAC, or PFC was analyzed by repeated measures ANOVA with two between factors (strain, two levels: Dbh +/− and Dbh −/−; treatment, two levels: amphetamine and saline) and one within factor (minutes, seven levels: 0, 20, 40, 60, 80, 100 and 120). Simple effects were assessed by one way ANOVA for each time point. Individual between group comparisons, when appropriate, were performed by post hoc test (Duncan’s multiple range test).
**Conditioned Place Preference**

Experiments were conducted in an isolated behavior room between 10:00 and 16:00. Mice were placed in the “neutral” middle compartment of a three compartment conditioned place preference chamber (San Diego Instruments, La Jolla, CA) and allowed to freely explore the other two compartments that were distinguishable by floor texture and wall pattern for 20 minutes, and time spent in each compartment was recorded (“pretest”). One to six days later, mice were subjected to “conditioning” sessions for three consecutive days. Mice (N=7-10 per group) were given an injection of saline (10 ml/kg, i.p.) and restricted to one compartment for 30 minutes in the morning, then given an i.p. injection of saline or cocaine (5, 10, 20, 40, or 60 mg/kg, 10 ml/kg) and restricted to the other compartment for 30 minutes in the afternoon (approximately 4 hr after the morning conditioning session). Mice were designated to receive cocaine on either the “A” side or the “B” side using an unbiased design (i.e. for each genotype, equal numbers of mice received cocaine on each side, and equal numbers of mice received cocaine on the “preferred” side and “non-preferred” side based on pretest results). The day following the last conditioning session, mice were placed in the neutral middle compartment in a drug-free state, allowed to freely explore all compartments for 20 min, and time spent in each compartment was recorded. Testing sessions were begun roughly halfway between the morning and afternoon conditioning sessions of the third day of conditioning. The preference score was calculated by subtracting the amount of time spent on the saline-paired side from the amount of time spent on the cocaine-paired side. Data were analyzed by paired t-tests (pretest preference vs. post-test preference for each group).
In experiments exploring the effect of specific adrenergic receptor antagonists on the expression of cocaine-induced place preference, Dbh +/- mice (N=4-11 per group) were injected with either saline (0.9 % NaCl, i.p. at 10 ml/kg), vehicle (0.9% saline with 1.5% DMSO, 1.5% Cremaphor EL, 10 ml/kg, i.p), the α1-AR antagonist prazosin (1 mg/kg, i.p. at 10 ml/kg, dissolved in vehicle), or the β-AR antagonist propranolol (5 mg/kg, i.p. at 10 ml/kg, dissolved in saline) 30 minutes prior to cocaine injection (20 mg/kg) on each conditioning day. Data for animals receiving no pretreatment injection, vehicle injection, or saline injection prior to cocaine conditioning were statistically compared and no differences were found, therefore the results were combined and are presented as the “control” group.

Because NE lesions impair the expression of amphetamine-induced place preference (Ventura et al., 2003) and NE is involved in various types of learning and memory (for review see Ramos and Arnsten, 2007), we assessed the effect of genetic deletion of Dbh on the expression of place preference to a natural reward. In this experiment, animals were conditioned with a high calorie sweetened drink, Ensure. Dbh -/- mice (N=8) were first trained to consume Ensure via water bottle by removing all food on the night following normal pretest sessions. For 1 hour the following day the animals were presented with a water bottle containing Ensure in the home cage and were allowed to drink freely. At least 1 hour after Ensure water bottles were removed, mice were given free access to normal rodent chow for 2 hours. Following this 2 hour period, the food was again removed and animals were fasted overnight. Water was available at all times during this training phase, except for when the Ensure bottle was placed in the cage. This training was repeated for 3 days before conditioning sessions began.
Conditioning sessions were carried out in the same fashion as described above, except that instead of receiving saline injections during morning conditioning and cocaine injections during afternoon conditioning sessions, mice were presented with water bottles in the conditioning chambers. These bottles were filled with normal drinking water in the side on which they were confined in the morning, and Ensure on the side on which they were confined in the afternoon. Most mice readily drank the Ensure during conditioning sessions. If mice did not consume Ensure during conditioning sessions, they were removed from the experiment. Beginning at least 1 hour after afternoon conditioning sessions, mice were allowed 2 hours of free access to normal rodent chow. After this feeding session, food was again removed and mice were fasted overnight. This procedure was repeated for 3 consecutive days prior to a test session, as described above.

2.4 Results

**Basal and amphetamine-induced DA release is attenuated in Dbh -/- mice**

Because the activity of mesolimbic dopaminergic neurons is enhanced by NE, we predicted that striatal DA release would be compromised in Dbh -/- mice. We assessed DA release in the NAC, CP, and PFC of awake, behaving Dbh +/- and Dbh -/- mice by microdialysis. Basal extracellular DA levels were significantly reduced in the NAC and CP, but not the PFC, of Dbh -/- mice (NAC: Dbh +/- 1.25 ± 0.25 pg/20 µl, Dbh -/- 0.56 ± 0.5, p<0.05; CP: Dbh +/- 1.99 ± 0.27, Dbh -/- 1.32 ± 0.11, p<0.05; PFC: Dbh +/- 0.56 ± 0.03, Dbh -/- 0.47 ± 0.03). Basal extracellular NE levels were 1.02 ± 0.13 in the PFC and 0.62 ± 0.09 in the NAC of Dbh +/- mice, while NE was undetectable in Dbh -/- mice.
The effects of amphetamine on DA release are shown in Figure 2.1. Extracellular DA levels in the NAC, CP, and PFC of $Dbh$ +/- mice were increased dramatically by amphetamine administration (2.5 mg/kg, i.p.), peaking at ~ 100% maximal increase in the NAC and CP and ~ 200% maximal increase in the PFC. In contrast, the amphetamine-induced increase in extracellular DA was absent in the NAC, and present but reduced in the CP (50% maximal increase) and PFC (75% maximal increase) of $Dbh$ -/- mice. Statistical analyses revealed a significant strain x treatment x minutes interaction for the NAC ($F(6,226)=3.44; p<0.005$), CP ($F(6,120)=2.23; p<0.05$), and PFC ($F(6,114)=2.30; p<0.05$). Posthoc analysis revealed significant differences between $Dbh$ +/- and $Dbh$ -/- mice challenged with amphetamine at multiple time points (Figure 2.1).

**Increased density of striatal high-affinity state DA receptors in $Dbh$ -/- mice**

Reductions in striatal DA availability typically result in the upregulation of striatal DA receptors. In addition, $Dbh$ -/- mice share a number of phenotypes with wild-type animals that have undergone psychostimulant sensitization, including greater psychostimulant- and D2 agonist-induced locomotion, and an increase in striatal high-affinity state D2 receptors is thought to contribute to these phenotypes in sensitized animals (Seeman et al., 2002; Weinshenker et al., 2002a; Seeman et al., 2005). We measured saturation binding of the D1 antagonist SCH23390 and the D2 antagonist raclopride to brain tissue homogenates in the presence and absence of guanine nucleotide (GN). Under normal conditions, antagonist binding to high-affinity state receptors is prevented by endogenous DA, but previous data are consistent with the principle that GN permits the release of receptor-bound DA and allows the binding of the antagonist to all
receptors (Seeman et al., 1989). Thus, the difference in antagonist binding in the presence and absence of GN represents the density of high-affinity state receptors.

While no large differences in total striatal DA receptors were found between $Dbh^{+/-}$ and $-/-$ mice, high-affinity state DA receptors were markedly elevated in $Dbh^{-/-}$ mice (D1 receptors: 1.9 pmol/g for $Dbh^{+/-}$ mice, 5.7 pmol/g for $Dbh^{-/-}$ mice in the CP; 6.6 pmol/g for $Dbh^{+/-}$ mice, 11.7 pmol/g for $Dbh^{-/-}$ mice in the NAC; D2 receptors: 3.7 pmol/g for $Dbh^{+/-}$ mice, 10.3 pmol/g for $Dbh^{-/-}$ mice in the CP; 0.8 pmol/g for $Dbh^{+/-}$ mice, 5.3 pmol/g for $Dbh^{-/-}$ mice in the NAC; Figure 2.2A-2.2D). Similar results were obtained when antagonist binding to total striatal homogenates was examined independently from separate groups of animals, and when two other methods of measuring high-affinity state receptors were used (DA/[^3]H]raclopride or DA/[^3]H]domperidone competition; Seeman et al., 2005). In contrast, the density of high-affinity state DA receptors in the PFC of $Dbh^{-/-}$ mice was similar to that of controls (D1 receptors: 0.7 pmol/g for $Dbh^{+/-}$ mice, 0.7 pmol/g for $Dbh^{-/-}$ mice; D2 receptors: 0.4 pmol/g for $Dbh^{+/-}$ mice, 0.6 pmol/g for $Dbh^{-/-}$ mice, and total D2 receptor density was modestly reduced (Figure 2.2E, 2.2F).

**$Dbh^{-/-}$ mice are hypersensitive to cocaine-induced locomotion**

We have previously shown that $Dbh^{-/-}$ mice are hypersensitive to amphetamine-induced locomotion and stereotypy (Weinshenker et al., 2002a). To determine whether this hypersensitivity extends to cocaine, we measured the locomotor response of $Dbh^{+/-}$ and $Dbh^{-/-}$ mice to cocaine. Cocaine produced a dose-dependent increase in locomotor activity in both $Dbh^{+/-}$ and $-/-$ mice (Figure 2.3). However, as with amphetamine,
cocaine-induced locomotion was greater in $Dbh$ -/- mice at all doses tested. There was a significant genotype x time interaction for 5 mg/kg ($F(11,168)=6.82$, $p<0.0001$), 10 mg/kg ($F(11,168)=8.80$, $p<0.0001$), and 20 mg/kg cocaine ($F(11,144)=5.52$, $p<0.0001$). Posthoc analysis revealed significant differences between $Dbh$ +/- and $Dbh$ -/- mice at multiple time points following cocaine administration (Figure 2.3). Locomotor activity in response to a novel environment prior to drug administration was reduced in $Dbh$ -/- mice, as described previously (Weinshenker et al., 2002a; Figure 2.3).

To determine whether the alterations in DA receptors contributed to the locomotor hypersensitivity to cocaine in $Dbh$ -/- mice, we administered saline, the D1 antagonist SCH23390, or the D2 antagonist eticlopride to mice 30 minutes prior to cocaine (20 mg/kg). Because serotonergic hyperinnervation of the striatum can occur after DA depletion, we also tested the ability of 5-HT antagonists to prevent the cocaine hypersensitivity of $Dbh$ -/- mice. A significant effect of treatment was found for both genotypes (one-way ANOVA: $Dbh$ +/- $F(5,49)=4.35$, $p<0.0001$; $Dbh$ -/- $F(5,48)=12.03$, $p<0.0001$). Posthoc analysis revealed that both SCH23390 and eticlopride attenuated cocaine-induced locomotion in $Dbh$ +/- and $Dbh$ -/- mice (Figure 2.4). In contrast, the 5-HT1A antagonist WAY100635 and the 5-HT2 antagonist ketanserin attenuated cocaine-induced locomotion in $Dbh$ +/- mice, but were completely without effect in $Dbh$ -/- mice (Figure 2.4).

**Altered cocaine reward and aversion in $Dbh$ -/- mice**

Because NE is involved in many types of learning and memory, we first tested the effects of a natural reward in the CPP paradigm, to be certain that $Dbh$ -/- mice were
capable of performing this type of task. We found that $Dbh$ -/- mice expressed a robust place preference following conditioning with palatable food (Ensure). Paired t-tests revealed a significant difference between pretest and posttest preference scores ($t(7)=4.128$, $p=0.0044$; Figure 2.5).

Next, to determine whether the alterations of dopaminergic signaling in $Dbh$ -/- mice affect psychostimulant reward, we assessed cocaine conditioned place preference in $Dbh$ +/- and $Dbh$ -/- mice. The side preference of both genotypes before pairing with cocaine and after pairing with saline was essentially random (Figure 2.6). While $Dbh$ +/- mice expressed a significant place preference to cocaine at the 10 mg/kg dose ($t(7)=6.020$, $p=0.0005$) and 20 mg/kg dose ($t(9)=2.862$, $p=0.0187$) but not the 5 mg/kg dose, $Dbh$ -/- mice expressed a significant preference only at the 5 mg/kg dose ($t(7)=2.776$, $p=0.0275$) and avoided the cocaine-paired chamber at the 20 mg/kg dose ($t(6)=2.659$, $p=0.0376$; Figure 2.6).

To determine whether the normal dose response to cocaine was shifted in $Dbh$ -/- mice, we examined whether higher doses of cocaine produced a place aversion in $Dbh$ +/- mice. Conditioned place aversion was not observed in $Dbh$ +/- mice following conditioning with doses of cocaine up to 60 mg/kg (Figure 2.7). $Dbh$ +/- mice in fact showed a trend towards a place preference at 40 and 60 mg/kg cocaine, but it was not quite significant in either case (posttest preference for cocaine-paired side at 40 mg/kg: $+327 \pm 178$ sec, $p=0.1$ compared to pretest, $N=10$; 60 mg/kg: $+248 \pm 245$ sec, $p=0.21$ compared to pretest, $N=9$; Figure 2.6).
Prazosin blocks the expression of cocaine-induced place preference

Because the α1-AR antagonist prazosin attenuates DA release in the NAC and blocks the expression of certain behavioral effects of cocaine, we explored the effect of specific adrenergic antagonists on the development of cocaine-induced place preference in control (Dbh +/-) mice by administering these drugs prior to cocaine injection on each conditioning day. We found that while pretreatment with vehicle (t(22)=2.360, p=0.0275) or the β-AR antagonist propranolol (t(8)=2.246, p=0.0549) had no effect on the normal expression of cocaine-induced place preference after conditioning with 20 mg/kg cocaine, pretreatment with prazosin attenuated the expression of cocaine-induced place preference at this dose (t(11)=0.7269, p=0.4825, Figure 2.8).

2.5 Discussion

Regulation of DA Release by NE

We found that basal extracellular DA levels were attenuated in the NAC and CP of Dbh -/- mice. Consistent with these results, LC lesions decrease striatal DA availability (Russell et al., 1989; Lategan et al., 1990; Lategan et al., 1992; Haidkind, et al., 2002), which results in DA receptor supersensitivity (Donaldson et al., 1976; Lategan et al., 1989; Harro et al., 2000). Basal DA release in the PFC was not significantly affected, suggesting that NE is more critical for the basal tone of mesolimbic and nigrostriatal DA neurons than for mesocortical DA neurons. However, it is important to note that the NE transporter (NET) can take up DA, and noradrenergic neurons may release DA as well as NE (Carboni and Silvagni, 2004; Devoto et al., 2005). Therefore, it is possible that basal DA levels in the PFC of Dbh -/- mice are maintained by the local
release of DA from “noradrenergic” neurons. This “ectopic” DA could be synthesized de novo by the “noradrenergic” neurons or be released from dopaminergic terminals and taken up into the noradrenergic neurons via the NET.

Amphetamine can increase DA release in an impulse-independent manner by acting directly on DA transporters. However, accumbal DA release following systemic amphetamine appears to be primarily impulse-dependent in some mouse strains, and NE is critical for this aspect of DA outflow (Ventura et al., 2004). Amphetamine-induced burst firing of DA neurons is dependent on α1-AR signaling, and amphetamine-induced DA release in the NAC is attenuated in α1b-AR knockout mice, mice with depletion of NE in the PFC, and by α1-AR antagonists (Darracq et al., 1998; Shi et al., 2000; Paladini et al., 2001; Auclair et al., 2002; Ventura et al., 2003). Our data confirm and extend these findings. Amphetamine-induced DA release was completely abolished in the NAC of Dbh−/− mice, while DA release persisted in the CP and PFC, although at reduced levels. These results indicate that NE is required for the maximal increase in extracellular DA observed after psychostimulant administration, and that mesolimbic DA neurons absolutely depend on the noradrenergic system for this response.

The LC, A1, and A2 brainstem cell groups project directly to the VTA and exert excitatory control over DA neuron firing (Jones and Moore, 1977; Simon et al., 1979; Grenhoff et al., 1993; Grenhoff and Svensson, 1993; Grenhoff et al., 1995; Liprando et al., 2004; Mejias-Aponte et al., 2004). In addition, PFC neurons express α1-ARs (Palacios et al., 1987; Pieribone et al., 1994), and α1-AR antagonists infused directly into the PFC or NE depletion in the PFC attenuates amphetamine-induced DA release in the NAC (Darracq et al., 1998; Ventura et al., 2003). Thus, the lack of both “direct” and
“indirect” (via the PFC and other structures; Taber et al., 1995; Tong et al., 1996; Marek and Aghajanian, 1999; Carr and Sesack, 2000; Darracq et al., 2001) excitatory noradrenergic inputs to the VTA likely contribute to the reduction of accumbal DA release in Dbh -/- mice.

**DA Receptor Supersensitivity in Dbh -/- Mice**

A reduction in DA availability typically results in the upregulation of DA receptor signaling in terminal regions. For example, DA receptor hypersensitivity is observed after lesions of DA neurons (Arnt, 1985) or genetic DA depletion (Kim et al., 2000). An increase in high-affinity state DA receptors was observed in the NAC and CP, but not the PFC, of Dbh -/- mice. Because DA availability in the PFC of Dbh -/- mice was normal under basal conditions, but decreased after amphetamine administration, the increase in striatal high-affinity state receptors correlated with the basal availability of extracellular DA as opposed to the availability of DA after evoked release. Although the density of both high-affinity state D1 and D2 receptors was increased in the striatum, Dbh -/- mice were hypersensitive to the behavioral effects of a D2 agonist, but not a D1 agonist. In fact, Dbh -/- mice are relatively insensitive to D1 agonist-induced locomotion (Weinshenker et al., 2002a). Because D1 signaling in the PFC opposes DA release in the NAC and locomotor activation (Vezina et al., 1991; Ventura et al., 2004), PFC D1 receptors may be preferentially activated by D1 agonists in Dbh -/- mice, although other explanations are possible.

Taken together, these neurochemical changes in DA signaling may explain the behavioral hypersensitivity of Dbh -/- mice to amphetamine. We have previously shown that amphetamine-induced locomotion is modestly enhanced in Dbh -/- mice, while
amphetamine-induced stereotypy is greatly magnified (Weinshenker et al., 2002a). DA signaling in the CP is thought to underlie stereotypies. For example, a number of groups have shown that 6-OHDA lesions of the CP abolish amphetamine-induced stereotypy (Kelly et al., 1975; Makanjuola and Ashcroft, 1982). Thus, the persistence of some amphetamine-induced DA release in the CP of Dbh -/- mice coupled with the profound striatal DA receptor hypersensitivity likely underlies their stereotypy-related phenotypes. In contrast, the locomotor response to amphetamine has been attributed to DA signaling in the NAC (e.g. Kelly et al., 1975; Pijnenburg et al., 1975; Makanjuola and Ashcroft, 1982), and it might seem surprising that amphetamine-induced locomotion is intact in Dbh -/- mice despite a lack of amphetamine-induced DA release in the NAC. Because the locomotor response to amphetamine is blocked by DA antagonists in Dbh -/- mice (Weinshenker et al., 2002a), we speculate that DA release in other brain regions, such as the CP and/or PFC, is responsible. This is consistent with the work of Ventura and colleagues (2003), who demonstrated that amphetamine-induced locomotion can occur in the absence of DA release in the NAC. Alternatively, DA release in the NAC of Dbh -/- mice may occur at levels below our limit of detection yet be sufficient for locomotor behavior due to receptor hypersensitivity.

**Chronic Dbh Deficiency Affects Cocaine Locomotion, Reward, and Aversion**

Our results indicate that, similar to amphetamine, Dbh -/- mice are hypersensitive to cocaine-induced locomotion. We assessed the effects of DA and 5-HT antagonists to determine whether this hypersensitivity was mediated by DA and/or 5-HT receptors. WAY100635 and ketanserin were used for three reasons. First, 5-HT1A and 5-HT2 receptors modulate DA release and cocaine-induced locomotion in rats (De
Deurwaerdère and Spampinato, 1999; Carey et al., 2001; Broderick et al., 2004). Second, serotonergic hyperinnervation of the striatum occurs after DA neuron lesions, and these receptors contribute to the hyperlocomotion observed in monoamine-depleted animals (Stachowiak et al., 1984; Mignon and Wolf, 2002; Zhang et al., 2002; Bishop et al., 2003). Third, the residual locomotor response to psychostimulants in α1b-AR knockout mice is mediated by compensatory 5-HT2A signaling (Auclair et al., 2004). We found that cocaine-induced locomotion in Dbh +/- mice was attenuated by DA and 5-HT antagonists, but only the DA antagonists effectively eliminated cocaine-induced locomotion in Dbh +/- mice. We conclude that the expression of cocaine hypersensitivity of Dbh +/- mice is mediated by changes in DA receptors, although changes in 5-HT signaling could contribute to the development of the hypersensitivity.

Interestingly, acute pharmacological inhibition of DBH or α1-ARs has the opposite effect of chronic DBH deficiency; a single injection of the DBH inhibitor disulfiram or the α1-AR antagonist prazosin to normal animals attenuates psychostimulant-induced locomotion (Maj et al., 1968; Darracq et al., 1998; Weinshenker et al., 2002a). In contrast, chronic disulfiram administration increases cocaine sensitization (Haile et al., 2003). These observations indicate that the dopaminergic adaptations in response to the chronic absence of NE, and not the acute lack of NE, underlie the psychostimulant hypersensitivity of Dbh +/- mice.

After first determining that Dbh +/- mice can develop a place preference to natural reward (also shown by Olson et al., 2006), we assessed the behavior of Dbh +/- and Dbh +/- in the CPP paradigm following conditioning with cocaine. We observed a significant CPP in Dbh +/- mice at a low dose of cocaine that does not support a preference in Dbh
+- mice, suggesting a hypersensitivity to cocaine reward. Perhaps the most striking phenotype we observed was the aversion of Dbh -/- mice to a dose of cocaine (20 mg/kg) that produced a place preference in control mice. To further explore this phenomenon we attempted to produce a place aversion in control mice by increasing the dose of cocaine used during the conditioning sessions. Even at the highest dose tested (60 mg/kg), Dbh +/− mice still tended to prefer the cocaine-paired side. We did not test higher doses because 60 mg/kg is near the threshold for cocaine-induced seizures in mice, which would confound results (e.g. Golden et al., 2001). Thus, it is not technically correct to say that chronic Dbh deficiency produces hypersensitivity to the cocaine place aversion, because the phenotype was never observed in control mice. Rather, there appears to be a novel shift in the balance between cocaine reward and aversion in Dbh -/- mice, where the aversive properties of cocaine can become overwhelming and preclude the reward (Ettenberg and Geist, 1991). Importantly, no seizure activity was observed in Dbh -/- mice following administration of the cocaine dose (20 mg/kg) that produced place aversion. To our knowledge, this is one of the only examples of a conditioned place aversion to cocaine in rodents. Both a lack of NE and hyperdopaminergic signaling could contribute to this phenotype. For example, mice specifically lacking NE in the PFC show a similar place aversion to amphetamine (Ventura et al., 2003), and Dbh -/- mice are hypersensitive to the aversive effects of ethanol (Weinshenker et al., 2000). In addition, because DA release occurs in response to aversive as well as rewarding events (Thierry et al., 1976; Abercrombie et al., 1989; Jensen et al., 2003) and Dbh -/- mice have hypersensitive DA receptors, excessive DA signaling may also underlie the aversion.
In this study we also assessed the effect of specific adrenergic receptor antagonists on the development of cocaine CPP at a high conditioning dose (20 mg/kg). We found that the α1-AR antagonist prazosin blocked the expression of cocaine-induced place preference in control mice. This agrees with previous findings from multiple groups indicating that prazosin attenuates midbrain DA release and blocks the expression of psychostimulant-induced locomotion, as described above. This also highlights another interesting divergence between chronic NE deficiency and acute blockade of specific adrenergic receptor subtypes. While acute inhibition of the α1-AR may blunt psychostimulant responses by decreasing DA release in the NAC, chronic NE ablation, as is seen in Dbh -/- mice, leads to an increased responsiveness to many properties of psychostimulants via hypersensitive DA receptors.

**DBH and Cocaine Dependence**

DBH activity varies between individuals, and this variation has a strong genetic component that is primarily controlled by a single, common polymorphism (Zabetian et al., 2001). Our results suggest that dopaminergic function and psychostimulant responses could be altered in individuals with low DBH activity, a hypothesis that is supported by the human literature. For instance, cocaine-induced paranoia is more prevalent in individuals with genetically low DBH activity (Cubells et al., 2000; Kalayasiri et al., 2007). Furthermore, the DBH inhibitor disulfiram has shown promise as a treatment for cocaine dependence (Carroll et al., 2004). These findings suggest that genetic or pharmacological inhibition of DBH may increase the aversive effects of cocaine, and identifies a potential target for future medications development.
Acknowledgements

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Figure 2.1

A) NAc

B) CP

C) PFC

% of basal DA output vs. Time after amphetamine (min) for different genotypes and treatments.
Figure 2.1 Amphetamine-induced DA release is attenuated in \textit{Dbh} -/- mice. Shown are extracellular DA levels in the (A) NAC, (B) CP, and (C) PFC of \textit{Dbh} +/- and \textit{Dbh} -/- mice after administration of saline (sal) or amphetamine (amph; 2.5 mg/kg, i.p.; N=5–11 per group). Values are expressed as mean ± SEM. *p<0.05 compared to amphetamine-treated \textit{Dbh} -/- mice for that time point. #p<0.05 compared to saline-treated mice of the same genotype for that time point.
Figure 2.2

**A**

Apparent density of D1 sites, pmol/g

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D1High = 1.9 pmol/g
D1High = 5.7 pmol/g

**B**

Apparent density of D2 sites, pmol/g

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D2High = 3.7 pmol/g
D2High = 10.3 pmol/g

**C**

Apparent density of D1 sites, pmol/g

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D1High = 6.6 pmol/g
D1High = 11.7 pmol/g

**D**

Apparent density of D2 sites, pmol/g

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D2High = 6.3 pmol/g
D2High = 10.8 pmol/g

**E**

Apparent density of D1 sites, pmol/g

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D1High = 0.7 pmol/g
D1High = 0.7 pmol/g

**F**

Apparent density of D2 sites, pmol/g

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D2High = 0.4 pmol/g
D2High = 0.6 pmol/g
Figure 2.2  Density of high-affinity state DA receptors in the NAC and CP are elevated in Dbh −/− mice. Shown are the results from Scatchard analysis of saturation curves of $[^3]$H]SCH23390 (D1 receptors; A, C, and E) and $[^3]$H]raclopride (D2 receptors; B, D, and F) binding to homogenized NAC (A and B), CP (C and D), and PFC (E and F) in the presence (total receptors, black bars) and absence (low-affinity state receptors, white bars) of guanilylimidodiphosphate (GN). The difference between the –GN and +GN conditions represents the density of high-affinity state receptors ($D_1^{\text{High}}$, $D_2^{\text{High}}$). The two bars on the left side of each graph represent data from Dbh +/− mice, and the two bars on the right side of each graph represent data from Dbh −/− mice. Tissue from eight animals was pooled for each genotype, and Scatchard analysis was performed in triplicate on each tissue batch. Binding values from the three runs agreed to within 5–8% of each other.
Figure 2.3

A  
Cocaine 5 mg/kg

B  
Cocaine 10 mg/kg

C  
Cocaine 20 mg/kg
Figure 2.3 *Dbh* +/- mice are hypersensitive to cocaine-induced locomotion. Mice were placed in activity chambers and injected with cocaine 4 hours later (black arrows), and ambulations were recorded via infrared beam breaks for 2 additional hours. Cocaine doses were (A) 5 mg/kg, (B) 10 mg/kg, and (C) 20 mg/kg. N=7–8 per group. Values are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to *Dbh* +/- mice at that time point.
Figure 2.4
Figure 2.4 Effects of DA and 5-HT antagonists on cocaine-induced locomotion in Dbh mice. Shown are the total ambulations of Dbh +/- mice (white bars, left of dashed line) and Dbh -/- mice (black bars, right of dashed line) for the 2 hours following administration of saline (SAL), cocaine (COC; 20 mg/kg), the 5-HT1A antagonist WAY100635 (0.03 mg/kg) + cocaine (WAY + COC), the 5-HT2 antagonist ketanserin (0.3 mg/kg) + cocaine (KET + COC), the D1 antagonist SCH23390 (0.03 mg/kg) + cocaine (SCH + COC), or the D2 antagonist eticlopride (0.3 mg/kg) + cocaine (ETC + COC). Antagonists were administered 30 minutes prior to cocaine. N=6–12 per group. Data were analyzed by one way ANOVA followed by Bonferroni post-hoc tests. *p<0.05 compared to saline control for that genotype. **p<0.05 compared to cocaine alone for that genotype.
Figure 2.5

![Bar chart showing preference between pretest and posttest.

- **Pretest:** Low preference.
- **Posttest:** High preference with significant increase.

Graph indicates a significant increase in preference from pretest to posttest.
Figure 2.5 *Dbh* -/- mice develop place preference to a natural reward. Food restricted *Dbh* -/- mice were conditioned with normal drinking water or the high calorie drink Ensure. Shown is the preference for the Ensure paired side in seconds at pretest (left) and post-test (right), N=8. Values are expressed as mean ± SEM. **p<0.01 compared to pretest.
Figure 2.6

![Graph showing preference for cocaine-paired side (sec) for Dbh +/− and Dbh −/− genotypes.](image)

- Pretest
- Posttest

Key:
- ○ Dbh +/−
- ■ Dbh −/−
Figure 2.6 Dbh -/- mice are hypersensitive to cocaine reward and aversion. Shown is the preference of Dbh +/- mice (white bars) and Dbh -/- mice (black bars) in seconds for the “cocaine paired” side before (Pretest, left of dashed line) and after (Post-test, right of dashed line) 3 days of conditioning, N=7-10 per group. Values are expressed as mean ± SEM. *p<0.05 compared to pretest for that group.
Figure 2.7

[Graph showing preference (sec) for 40 mg/kg and 60 mg/kg treatments in pretest and test conditions.]
Figure 2.7 *Dbh +/-* mice do not express a place aversion to high doses of cocaine.

*Dbh +/-* mice were conditioned with 40 mg/kg (white bars) or 60 mg/kg (black bars) cocaine. Shown is the preference for each group at pretest (left of line) and post-test (right of line), N=9-10 per group. Values are expressed as mean ± SEM.
Figure 2.8 Expression of cocaine-induced place preference is blocked by pretreatment with the α1-AR antagonist prazosin. Shown is the preference score for the cocaine-paired side in seconds following conditioning with 20 mg/kg cocaine in Dbh +/- mice. Each pretreatment was given prior to cocaine sessions on each conditioning day. Pretest values are shown to the left of the line and post-test values to the right of the line, N=9-23 per group. Values are expressed as mean ± SEM. *p<0.05 compared to pretest, +p=0.0549 compared to pretest.
CHAPTER 3:
NOREPINEPHRINE SIGNALING THROUGH β-ADRENERGIC RECEPTORS IS CRITICAL FOR THE EXPRESSION OF COCAINE-INDUCED ANXIETY

Adapted from:

3.1 Abstract

Cocaine is a widely abused psychostimulant that has both rewarding and aversive properties. While the mechanisms underlying cocaine’s rewarding effects have been studied extensively, less attention has been paid to the unpleasant behavioral states induced by cocaine, such as anxiety. In this study we evaluated the performance of dopamine β-hydroxylase knockout (Dbh -/-) mice, which lack norepinephrine (NE), in the elevated plus maze (EPM) to examine the contribution of noradrenergic signaling to cocaine-induced anxiety. We found that cocaine dose-dependently increased anxiety-like behavior in control (Dbh +/-) mice, as measured by a decrease in open arm exploration. Dbh -/- mice, on the other hand, though they showed normal baseline performance in the EPM, were completely resistant to the anxiogenic effects of cocaine. Also, cocaine-induced anxiety was attenuated in Dbh +/- mice following administration of disulfiram, a DBH inhibitor. In experiments where specific adrenergic antagonists were administered to Dbh +/- mice, we found that pretreatment with the β-adrenergic receptor antagonist propranolol blocked cocaine-induced anxiety-like behavior in Dbh +/- mice, while the α1 antagonist prazosin and the α2 antagonist yohimbine had no effect. In experiments using wild-type C57BL/6J mice, we were able to replicate our results seen following β-adrenergic antagonism in Dbh +/- mice, demonstrating that this phenomenon is not specific to our Dbh strain background. These results indicate that noradrenergic signaling via β-adrenergic receptors is required for cocaine-induced anxiety in mice.
3.2 Introduction

Cocaine is a widely abused psychostimulant drug that acts by blocking the plasma membrane transporters for dopamine (DA), norepinephrine (NE), and serotonin (5HT). In humans, cocaine use results in a broad spectrum of effects, both subjectively positive (e.g. euphoria, increased energy, enhanced alertness) and negative (e.g. anxiety, paranoia, nausea, hypertension). In addition to its well-documented rewarding and locomotor activating effects in rodents, cocaine also induces anxiety-like behavior that can be reversed by administration of typical anxiolytic drugs, such as diazepam (Costall et al., 1988; Ettenberg and Geist, 1991; Rogiero and Takahashi, 1992; Yang et al., 1992; Blanchard and Blanchard, 1999; David et al., 2001; Paine et al., 2002). Whereas cocaine-induced reward has been studied extensively, less is known about the processes underlying the subjectively negative behavioral states associated with acute administration of the drug.

Although DA signaling has been primarily implicated in psychostimulant responses, cocaine is known to increase extracellular NE levels as well, and NE transmission has been shown to modulate psychostimulant-induced behaviors and neurochemistry (Drouin et al., 2002a; Drouin et al., 2002b; Weinshenker et al., 2002a; Ventura et al., 2003; Schank et al., 2006). Given that NE modulates general stress and anxiety responses (Gorman and Dunn, 1993; Stanford, 1995), we surmised that NE might also play a critical role in cocaine-induced anxiogenesis.

Dopamine β-hydroxylase (DBH) is the enzyme that converts DA to NE in the catecholamine biosynthetic pathway, therefore Dbh knockout (Dbh -/-) mice lack NE completely (Thomas et al., 1995; Thomas et al., 1998). Previously, we have shown that
Dbh -/- mice exhibit an increase in high affinity-state DA receptors and a corresponding hypersensitivity to the locomotor activating, rewarding, and aversive effects of cocaine (Schank et al., 2006). In particular, we observed a novel cocaine-induced place aversion in Dbh -/- mice at a dose of 20 mg/kg: a dose that produces a robust place preference in control animals. An initial goal of the present experiments was to determine whether this place aversion in Dbh -/- mice could be attributed to an increase in cocaine-induced anxiety. Few studies to date have explored the specific pathways involved in this particular drug effect, and none have thoroughly examined the role of NE. Using various pharmacological treatments in control and Dbh +/- mice, we assessed the influence of global NE deficit and DA system hypersensitivity on cocaine-induced anxiety.

3.3 Materials and Methods

Animals

Male and female Dbh +/- and -/- mice (aged 2 to 5 months) were individually housed in a reverse light cycle (lights on at 19:00, lights off at 7:00), and were allowed a minimum of two weeks to habituate to the new lighting conditions, after moving from normal light cycle (lights on at 7:00, lights off at 19:00). Food and water were available ad libitum throughout the course of the study. Data from male and female mice were combined, since there were no detectable gender differences. Dbh mice were generated as described (Thomas et al., 1995) and maintained on a mixed C57BL/6J and 129SvEv background. Dbh +/- mice were used as controls, 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 et al., 2005). Wild type
C57BL/6J mice were also used to generalize the findings from these experiments to a different strain of mouse, and in these experiments male and female mice (Jackson Labs, Bar Harbor, ME) aged 3 months were used. Housing, handling, and testing conditions for these animals were identical to those used in experiments with Dbh +/- mice. All animals were treated in accordance with the 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 IACUC committee.

**Behavioral testing**

The EPM apparatus consisted of two open arms and two enclosed arms arranged in a plus orientation. The arms were elevated 30 inches above the floor, with each arm projecting 12 inches from the center. Because rodents naturally prefer dark, enclosed compartments, a greater willingness to explore the open, well-lit arms is believed to represent a decrease in the animal’s anxiety. This interpretation has been validated by the efficacy of known anxiolytic and anxiogenic treatments in this paradigm (Pellow et al., 1985; Johnston and File, 1988; Gorman and Dunn, 1993; Paine et al., 2002).

In all experiments, cocaine was injected 20 minutes prior to behavioral testing as described by Yang and colleagues (Yang et al., 1992). To begin each test, mice were placed in the EPM facing one of the open arms and allowed to freely explore the apparatus for 5 minutes, during which time their behavior was videotaped. Videotapes were later scored by an observer who was blind to genotype and treatment group. The measure used for analysis is the percentage of time spent exploring the open arms, which was calculated by dividing the amount of time spent in the open arms by the combined
time spent in open and closed arms. Because some drug treatments alter locomotor activity in the EPM, it is important to use this percentage measurement as the dependent variable for analysis (Pellow et al., 1985). Entry into an arm of the plus maze was defined as the animal placing all four paws into that particular compartment of the apparatus. All tests were run during the dark cycle, between 14:00 and 18:00. Mice were excluded from data analysis for any of the following reasons: if they jumped or fell off the maze after test had begun, if they had a seizure while on the testing apparatus, or if their open arm time was detected as an outlier using Grubb’s test. Of 263 total mice tested, only 6 were excluded from data analysis as outliers. Data were analyzed using independent samples t-tests, one-way ANOVA followed by Dunnett’s post-hoc tests, or two-way ANOVA followed by Bonferroni post-hoc tests using Prism 4.0 for Macintosh.

**Cocaine dose-response**

*Dbh* +/- and -/- mice (N=8 per group) were injected with 0.9% saline (i.p., 10 ml/kg) or cocaine (5, 10, or 20 mg/kg, i.p. at 10 ml/kg, dissolved in 0.9% saline) 20 minutes prior to behavioral testing. Behavioral testing then proceeded for 5 minutes, as described above.

**NE restoration in Dbh -/- mice**

Central NE was restored acutely to *Dbh* -/- mice (N=6-8 per group) prior to testing on the EPM. L-3,4-dihydroxyphenylserine (DOPS) can be converted to NE by the enzyme aromatic amino acid decarboxylase (AADC), thus bypassing the requirement for DBH in the NE synthesis pathway. However, DOPS administration does not alter DA levels in *Dbh* -/- mice (Thomas et al., 1998). We administered DOPS in combination with benserazide, an AADC inhibitor that does not cross the blood-brain barrier, thereby
confining NE restoration to the central nervous system (Murchison et al., 2004). *Dbh* -/- mice were injected with DOPS (1 g/kg, s.c. + 0.25 g/kg benzerazide, s.c.) or vehicle (distilled water with 2% HCl, 2% NaOH, and 2 mg/kg vitamin C, s.c.) 4 to 6 hours prior to injection of cocaine (10 mg/kg, i.p at 10 ml/kg, dissolved in 0.9% saline) or saline. To habituate mice to large volume s.c. injection, vehicle injections were given for 3 days prior to test day. Cocaine injection occurred 20 minutes before introducing mice to the EPM, and behavioral testing proceeded for 5 minutes, as described above.

**DBH inhibition in *Dbh* +/- mice**

DBH enzyme activity was inhibited pharmacologically via acute administration of disulfiram. Disulfiram is a copper-chelating agent that inhibits DBH activity and alters catecholamine tissue content (Musacchio et al., 1966; Maj et al., 1968; Bourdelat-Parks et al., 2005). Mice (N=8 per group) were given 3 injections, each spaced 2 hours apart, with either vehicle (0.9% NaCl) or disulfiram (200 mg/kg, i.p. at 10 ml/kg, sonicated and suspended in 0.9% NaCl). This dosing regimen is known to decrease NE by approximately 70% in the mouse brain (Bourdelat-Parks et al., 2005). Mice received cocaine (10 mg/kg, i.p. at 10 ml/kg, dissolved in 0.9% saline) or saline 2 hours after the final pretreatment injection, and behavioral testing took place 20 minutes later, as described above. To habituate mice to the multiple daily injection regimen and large total injection volumes, they were injected 3 times per day (spaced 2 hours apart) with 0.9% saline (10 ml/kg) for 3 days prior to test day. In preliminary experiments we utilized a dose of 100 mg/kg disulfiram, but this was not sufficient to alter cocaine-induced anxiety in the EPM (data not shown), so we therefore used the highest dose that we have administered to mice previously (200 mg/kg) in this experiment.
Administration of adrenergic antagonists in *Dbh* +/- mice

*Dbh* +/- mice (N=10-17 per group) were pretreated with 0.9% saline (4 ml/kg, i.p.), vehicle (0.9% saline with 1.5% DMSO, 1.5% Cremaphor EL, 10 ml/kg, i.p), the β-AR antagonist propranolol (5 mg/kg, i.p. at 4 ml/kg, dissolved in 0.9% saline), the α1-AR antagonist prazosin (0.5 mg/kg or 1 mg/kg, i.p. at 10 ml/kg, dissolved in vehicle), or the α2-AR antagonist yohimbine (2.5 mg/kg, i.p. at 10 ml/kg, dissolved in distilled water) 10 minutes prior to cocaine injection. Behavioral testing was then performed 20 minutes after cocaine injection (10 mg/kg, i.p. at 10 ml/kg, dissolved in saline). Open arm times for the saline and vehicle groups were compared, and no differences were found; therefore these two groups were combined to form a single “control” group.

β-adrenergic inhibition in C57BL/6J wild type mice

C57BL/6J wild-type mice (N=7 per group, purchased from Jackson Labs, Bar Harbor, ME) were pretreated with 0.9% saline or propranolol (5 mg/kg) 10 minutes prior to injection of cocaine (20 mg/kg) or saline, and behavioral testing was performed 20 minutes later, as described above. In preliminary experiments with this mouse strain, we found that a dose of 10 mg/kg cocaine was not sufficient to induce significant cocaine-induced anxiety in our laboratory (data not shown). Therefore, in this experiment, we utilized a higher dose of cocaine.

3.4 Results

Cocaine-induced anxiety is abolished in *Dbh* +/- mice

Consistent with our previous results (Marino et al., 2005), baseline performance on the EPM was the same for *Dbh* +/- and *Dbh* -/- mice (Figure 3.1). Cocaine treatment
dose-dependently decreased percent open arm time in \(Dbh\) +/− mice. In contrast, the anxiety behavior of \(Dbh\) −/− mice was unaffected by cocaine treatment at any dose (Figure 3.1). Two way ANOVA revealed main effects of dose (\(F(3,56)=4.391, p=0.0076\)) and genotype (\(F(1,56)=19.78, p<0.0001\)), as well as a dose-genotype interaction (\(F(3,56)=4.046, p=0.0113\)). Bonferroni post-hoc analysis revealed a significant decrease from the saline treated group in percent open arm time for \(Dbh\) +/− mice treated with 10 mg/kg cocaine (\(p<0.01\)) or 20 mg/kg cocaine (\(p<0.01\)). Also, \(Dbh\) +/− animals showed a lower level of open arm exploration when compared to \(Dbh\) −/− mice for doses of 10 mg/kg (\(p<0.01\)) and 20 mg/kg cocaine (\(p<0.001\)).

**Pharmacological restoration of NE alters anxiety behavior in \(Dbh\) −/− mice**

\(Dbh\) −/− mice lack NE from birth and have high tissue DA levels (Thomas et al., 1995), raising the possibility that the failure of cocaine to produce anxiety-like behavior in the knockouts is a result of compensatory mechanisms related either to NE deficiency during postnatal development or to an increase in DA. To test this possibility, we acutely restored central NE to adult \(Dbh\) −/− mice without normalizing their DA levels by administering DOPS + benserazide prior to cocaine injection and EPM testing. Two way ANOVA revealed a main effect of pretreatment (\(F(1,24)=7.035, p=0.0139\), see Figure 3.2), but no statistically significant main effect of drug or interaction. Post-hoc Bonferroni tests indicated that percent open arm time was significantly lower in the DOPS-Cocaine group relative to the Vehicle-Cocaine group (\(p<0.05\)). Because no interaction was found, and since the Vehicle-Cocaine group showed an equally low level of open arm exploration as the DOPS-cocaine group, the possibility remains that DOPS
treatment alone can increase anxiety-like behavior in the elevated plus maze. This particular experiment does not definitively determine if increased anxiety in the DOPS-Cocaine animals was a result of cocaine’s effect following NE restoration, if NE restoration alone was sufficient to alter anxiety behavior, or if both possibilities exist.

**The DBH inhibitor disulfiram abolishes cocaine-induced anxiety in control mice**

To determine whether NE depletion confers resistance to cocaine-induced anxiety in normal animals, Dbh +/- mice were pretreated with the DBH inhibitor disulfiram or saline prior to cocaine administration and EPM testing. Disulfiram abolished the ability of cocaine to reduce open arm exploration in Dbh +/- mice, but had no effect in animals treated with saline prior to testing (Figure 3.3). Two way ANOVA revealed a pretreatment by drug interaction (F(1,28)=5.227, p=0.0300), with the Disulfiram-Cocaine group showing a significantly increased level of open arm exploration relative to the Saline-Cocaine group (p<0.05). This suggests that temporary inhibition of NE production decreases cocaine-induced anxiety, phenocopying the behavior we observed in Dbh -/- mice.

**Blockade of β-adrenergic receptors attenuates cocaine-induced anxiety in Dbh +/- mice**

The results of the preceding experiments indicated that NE is likely required for the anxiogenic effect of cocaine in the EPM. To determine which subtype of adrenergic receptor is critical for cocaine-induced anxiety, we pretreated Dbh +/- mice with the α1-AR antagonist prazosin, the α2-AR antagonist yohimbine, or the β-AR antagonist
propranolol prior to administration of cocaine and EPM testing. We found that cocaine-induced anxiety was preserved following prazosin or yohimbine treatment, but abolished by propranolol (Figure 3.4). One way ANOVA revealed a significant effect of pretreatment on percent open arm time ($F(3,63)=3.485$, $p=0.0211$), and Dunnett’s post-hoc tests indicated that the propranolol group differed significantly from the control group ($p<0.01$), whereas the prazosin and yohimbine groups did not. This suggests that NE signaling through β-adrenergic receptors is required for the cocaine-induced anxiety behavior of mice as measured by the EPM.

For the purposes of comparing these results to the experiment with adrenergic antagonists from Chapter 2 (see Figure 2.8), we examined the effect of prazosin pretreatment at a dose of 1.0 mg/kg in a separate group of $Dbh +/-$ mice. In this experiment we found that prazosin at 1.0 mg/kg replicated the effect of 0.5 mg/kg, in that it had no effect on the expression of cocaine-induced anxiety in the EPM.

To examine the possibility that the pretreatments alone altered plus maze behavior we treated $Dbh +/-$ mice with either propranolol, prazosin, or yohimbine, at the same doses considered above, and tested plus maze behavior 20 minutes later. This data was then compared to behavior observed in $Dbh +/-$ mice treated with saline in the dose-response experiment and one way ANOVA revealed no significant effect of drug treatment on percent open time ($F(3,31)=1.892$, $p=0.1539$, $N=7-10$).
Blockade of β-adrenergic receptors attenuates cocaine-induced anxiety in wildtype C57BL/6J mice

Next, we tested the effect of propranolol pretreatment on cocaine-induced anxiety behavior in C57BL/6J mice. The purpose of this experiment was two fold. First, we wanted to provide more generalizable data by replicating the behavioral effects observed in Dbh +/- mice with a different strain of mouse. Second, we wanted to confirm that propranolol pretreatment alone had no effect on plus maze behavior. In this experiment we observed a decrease in open arm exploration only in animals pretreated with vehicle and then given cocaine prior to testing (Figure 3.5). Two way ANOVA revealed a main effect of drug treatment (F(1,24)=5.694, p=0.0253). While the percent open arm time of the Vehicle-Cocaine treated group was significantly lower than the Vehicle-Saline group (p<0.05), Propranolol-Saline and Propranolol-Cocaine treated groups did not significantly differ.

3.5 Discussion

Although cocaine-induced anxiety is widely recognized in both animals and humans, very little is known about the mechanism underlying these effects. The main finding of our current experiments is that a complete lack of NE, such as occurs in Dbh -/- mice, abolished cocaine-induced anxiety. This phenotype was mimicked by either inhibition of DBH activity or β-AR signaling in control animals. In these experiments, we used percent open time as our dependent variable to account for any effects of genotype or drug treatment on exploratory activity. Our findings therefore imply a specific role for NE in cocaine-induced anxiety.
We then used selective adrenergic antagonists in control mice to determine which subtype of adrenergic receptor is critical for cocaine-induced anxiety. We found that propranolol, but not prazosin or yohimbine, recapitulated the $Dbh^{-/-}$ phenotype and prevented the cocaine-induced decrease in open arm time. These results indicate that activation of the $\beta$-AR is required for the anxiogenic effects of cocaine in this test. The behavioral effect of $\beta$-AR antagonists was replicated in wild type animals with a different strain background, suggesting that the results observed are not specific to our $Dbh$ animals. The specific location of the $\beta$-ARs necessary for mediating cocaine-induced anxiety is not known. While the dorsal and ventral striatum and prefrontal cortex appear to be important for mediating the locomotor-activating and rewarding effects of cocaine, it seems likely that cocaine-induced anxiety is mediated elsewhere. The amygdala and bed nucleus of the stria terminalis are appealing candidates because of their dense noradrenergic innervation and their involvement in general and drug-specific stress responses (Erb et al., 2000; Shaham et al., 2000; Leri et al., 2002; Aston-Jones and Harris, 2004; Morilak et al., 2005).

One of the initial motives driving these experiments was the question of whether the hypersensitivity to cocaine-induced place aversion observed in $Dbh^{-/-}$ mice might be accounted for by increases in cocaine-induced anxiety. Our results, however, reveal a dissociation between the anxiogenic effects of cocaine in the EPM and the aversive effects of cocaine in the place conditioning test. While $Dbh^{-/-}$ mice find a 20 mg/kg dose of cocaine strongly aversive in the place conditioning paradigm (Schank et al., 2006), they were completely insensitive to the anxiogenic properties of the same dose of cocaine in the EPM. We conclude that some unpleasant effect of cocaine, aside from increased
anxiety, must account for the hypersensitivity to the aversive effects of cocaine seen in
Dbh −/− mice. Human cocaine addicts with genetically low DBH activity experience
more severe cocaine-induced paranoia than individuals with high DBH activity, while
anxiety measures are unaffected by DBH genotype (Cubells et al., 2000; Kalayasiri et al.,
2007). It is unclear whether rodents can experience anything resembling human
paranoia, but an analogous negative state may perhaps contribute to cocaine aversion in
Dbh −/− mice.

Dbh −/− mice are also profoundly hypersensitive to the locomotor activating
effects of cocaine, which appears to be mediated by the amplified DA receptor signaling
that develops during chronic NE deficiency, rather than a specific lack of NE at the time
of cocaine administration (Schank et al., 2006). In contrast, the insensitivity of Dbh −/−
mice to the anxiogenic effects of cocaine is due to an “acute” lack of NE and is unrelated
to excessive DA signaling.

Another dissociation appears to apply to adrenergic receptor systems as well.
While the α1-AR has been identified as the key modulator of the locomotor activating
and rewarding effects of cocaine (Darracq et al., 1998; Drouin et al., 2002a; Drouin et al.,
2002b), the α1-AR antagonist prazosin was without effect in our EPM experiments; only
the β-AR antagonist propranolol led to a reduction in cocaine-induced anxiety behavior
in control mice. These results demonstrate the involvement of distinct receptor systems
in the effects of NE on drug-induced hyperactivity/reward and anxiety behaviors in mice.

We found that acute restoration of NE to Dbh −/− mice using DOPS restored
cocaine-induced anxiety in the EPM. However, DOPS also tended to decrease percent
open arm time in saline treated Dbh −/− mice, complicating the interpretation of these
results. An important consideration that might explain this result is that *Dbh* -/- mice lack NE from birth and have a compensatory increase in brain β-ARs (Sanders et al., 2006). Thus, it is possible that the acute introduction of NE produces a surge in β-AR signaling in *Dbh* -/- mice analogous to that produced by cocaine in normal animals, and thereby increases anxiety-like behavior. Although this effect makes it difficult to draw conclusions about cocaine-induced anxiety in this particular experiment, our results with disulfiram and propranolol in control mice support the conclusion that the anxiogenic effects of cocaine require NE and β-AR signaling.

Our results are pertinent to the clinical treatment of drug dependence because the DBH inhibitor disulfiram is capable of decreasing cocaine intake in human addicts (Carroll et al., 2004). We found that disulfiram abolishes the anxiogenic effects of cocaine in the EPM, which is counterintuitive because one would expect a treatment that reduces one of the aversive properties of cocaine to encourage drug use, rather than attenuate it. Notably, however, cocaine withdrawal also produces anxiety, which often precipitates relapse. While our study does not specifically address chronic cocaine use and subsequent withdrawal and relapse effects, the β-AR also appears to play a vital role in the expression of cocaine withdrawal-induced anxiety (Harris and Aston-Jones 1993; McDougle et al., 1994; Kampman et al., 2001; Rudoy et al., 2007) and stress-induced reinstatement (Leri et al., 2002). Combined with the data presented here, these results suggest that the acute anxiogenic effects of cocaine and the anxiety associated with cocaine withdrawal depend on a similar β-AR mediated signaling pathway. Thus, we hypothesize that the ability of disulfiram to reduce cocaine intake in addicts is due, in part, to a reduction in NE synthesis and subsequent attenuation of withdrawal-associated
anxiety and stress-induced relapse. Therefore, β-AR antagonists may also be useful as cocaine addiction pharmacotherapy by interfering with these anxiety responses.

Acknowledgments

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Figure 3.1

[Graph showing time in open arms (%) for different treatments in Dbh +/- and Dbh -/- mice.]

- Vehicle
- Cocaine 5 mg/kg
- Cocaine 10 mg/kg
- Cocaine 20 mg/kg

Significance levels: **p < 0.01, ***p < 0.001
Figure 3.1 Effects of cocaine on performance in the elevated plus maze in Dbh +/- and Dbh +/- mice. Cocaine was administered to mice 20 minutes prior to testing in the EPM. Shown is percent open arm time during the 5 minute test. **p<0.01 compared to vehicle control for that genotype. ##p<0.01, ###p<0.001, compared to opposite genotype for that dose. N=8 mice per group.
Figure 3.2

![Bar chart showing time in open arms (%) for Vehicle and DOPS with Saline and Cocaine conditions. The chart indicates a significant difference (*) between the conditions.](image-url)
Figure 3.2 NE restoration alters anxiety behavior in *Dbh* -/- mice. DOPS + benserazide or saline was administered approximately 5 hours prior to EPM testing, when brain restoration of NE peaks in *Dbh* -/- mice treated with DOPS. Shown is percent open arm time of *Dbh* -/- mice given vehicle or cocaine (10 mg/kg, i.p.) 20 minutes before the 5 minute EPM test. *p*<0.05 compared to vehicle pretreatment control for same pre-test drug treatment. (N=6-8 per group)
Figure 3.3

![Chart showing time in open arms for Saline and Cocaine with Vehicle and Disulfiram conditions.](chart.png)
Figure 3.3 Disulfiram attenuates cocaine-induced anxiety in $Dbh^{+/-}$ mice. $Dbh^{+/-}$ mice were injected with disulfiram (3 x 200 mg/kg, i.p., two hours between each injection) or vehicle. Two hours following the last disulfiram treatment, mice were injected with saline or cocaine (10 mg/kg, i.p.), and tested in the EPM 20 minutes later. Shown is percent open arm time during the 5 minute EPM test. *$p<0.05$ compared to disulfiram pretreatment control for same pre-test drug treatment (N=8 per group).
Figure 3.4

Control  Praz     Yoh     Prop

Time in Open Arms (%)
Figure 3.4 The β-adrenergic antagonist propranolol attenuates cocaine-induced anxiety in Dbh +/- mice. Dbh +/- mice were treated with vehicle, the α1-adrenergic antagonist prazosin, the α2-adrenergic antagonist yohimbine, or the β-adrenergic antagonist propranolol 10 minutes prior to cocaine injection (10 mg/kg, i.p.). Mice were tested in the EPM 20 minutes following injection of cocaine. Shown is percent open arm time during the 5 minute EPM test. **p<0.01 compared to vehicle control. (N=10-17 per group)
Figure 3.5

![Bar graph showing time in open arms (%) for Vehicle and Propranolol groups. The graph compares Saline and Cocaine conditions.](image-url)
Figure 3.5 The β-adrenergic antagonist propranolol attenuates cocaine-induced anxiety in wild type C57BL/6J mice. Wild type mice were treated with either propranolol or saline 10 minutes prior to cocaine injection. Mice were tested on the EPM 20 minutes following injection of cocaine. Shown is percent open arm time during the 5 minute EPM test. *p<0.05 compared to saline treated animals for same pretreatment (N=7 per group).
CHAPTER 4:
DISULFIRAM INHIBITS COCAINE-INDUCED REINSTATEMENT OF DRUG SEEKING BEHAVIOR IN RATS
4.1 Abstract

Disulfiram has historically been prescribed to alcoholics to prevent alcohol abuse, and achieves this effect by causing a severe aversive reaction to alcohol consumption due to inhibition of the enzyme aldehyde dehydrogenase. Inhibition of this enzyme leads to the buildup of acetaldehyde, a toxic intermediate in the alcohol metabolic pathway. Recently, it has been shown that disulfiram is also effective for the treatment of cocaine dependence. Although the mechanism of action for the therapeutic effect of disulfiram in cocaine abusers is not known, it is unlikely to involve aldehyde dehydrogenase inhibition. Disulfiram inhibits a wide range of endogenous enzymes, and there is good evidence that disulfiram may be mediating its effects on cocaine use via inhibition of the enzyme dopamine β-hydroxylase (DBH), which is responsible for converting dopamine (DA) to norepinephrine (NE) in noradrenergic neurons. In the first set of experiments we explored the effect of disulfiram on tissue catecholamine levels in rat brain. We found that treatment with 100 mg/kg disulfiram decreased the ratio of NE to DA in frontal cortex tissue by approximately 75%, similar to previously published data. Next, we used the rat self-administration paradigm to assess whether pretreatment with disulfiram alters cocaine-seeking behavior. Administration of disulfiram (100 mg/kg, i.p. 2 hours prior to session) had no effect on the maintenance phase of cocaine self-administration behavior (FR1, 0.5 mg/kg per infusion), nor did it significantly alter self-administration of food pellets. In contrast, disulfiram pretreatment completely blocked the expression of cocaine-induced reinstatement of drug-seeking following extinction. We have previously shown that disulfiram and adrenergic receptor antagonists attenuate the expression of cocaine-induced anxiety in mice. Taken together, these results suggest that disulfiram
treatment may be reducing cocaine use in addicts, at least in part, by preventing withdrawal-induced anxiety and drug induced relapse via inhibition of NE synthesis.
4.2 Introduction

Disulfiram (Antabuse) has historically been prescribed to alcoholics to prevent alcohol abuse, and achieves this effect by inhibiting the enzyme aldehyde dehydrogenase (Eneanya et al., 1981; Mays et al., 1995). Inhibition of this enzyme leads to the buildup of acetaldehyde, a toxic intermediate in the alcohol metabolic pathway, and induces a severely aversive reaction that includes intense nausea, flushing, and hypotension. This intensely negative physiological response helps to prevent future consumption of alcohol.

Recently, it has been shown that disulfiram is also effective for the treatment of cocaine dependence. Initial studies concerning disulfiram’s potential as a pharmacotheraphy for cocaine abuse were first undertaken because there is a high rate of alcohol and cocaine co-abuse. The prediction was that a decrease in alcohol consumption would in turn decrease cocaine intake, and disulfiram was indeed effective at decreasing cocaine use in alcoholics (Carroll et al., 1998). However, disulfiram treatment also decreased cocaine intake in subjects who were not alcohol dependent and abstained from alcohol for the duration of the study. Further, recent research by Carroll and colleagues (2004) has shown an even greater effectiveness of disulfiram in cocaine addicts who are not alcohol abusers. These observations suggest that disulfiram’s clinical efficacy on cocaine addiction is independent of its effect on alcohol metabolism. The primary goal of these experiments was to explore the mechanism of action for the therapeutic effect of disulfiram through the use of a preclinical animal model.

Disulfiram inhibits a wide range of endogenous enzymes in addition to aldehyde dehydrogenase, and there is good evidence that disulfiram may be mediating its effects on cocaine use via inhibition of the enzyme dopamine β-hydroxylase (DBH), which is
responsible for converting dopamine (DA) to norepinephrine (NE) in noradrenergic neurons. It has been known for decades that disulfiram decreases DBH activity, leading to a decrease in NE, an increase in DA, and a corresponding decrease in the NE to DA ratio in brain tissue of rodents (Maj et al., 1968; Musacchio et al., 1966; Karamanakos et al., 2001). We have previously shown this effect in our laboratory in studies using mice (Bourdelat-Parks et al., 2005), and present similar results in rats in this report.

In humans, serum DBH enzymatic activity varies within the general population, with a single base polymorphism in the promoter region of the gene accounting for a large portion of this variability (reviewed in Cubells and Zabetian, 2004). A cytosine nucleotide at this position corresponds to the high activity allele (“C allele”), and a thymine nucleotide indicates the low activity allele (“T allele”). Homozygotes for the T allele express about 10% of the DBH enzymatic activity of people with two high activity alleles (CC homozygotes), and CT heterozygotes exhibit an intermediate level of activity.

Variations in DBH activity interact with cocaine-induced behavioral responses as well as the efficacy of disulfiram to decrease cocaine intake. For example, cocaine-dependent individuals with genetically low DBH function are more responsive to disulfiram pharmacotherapy, specifically implicating this enzyme in disulfiram’s effect (R. Schottenfeld and J. Cubells, personal communication). Further, low levels of DBH activity in humans associates with an increased sensitivity to cocaine-induced paranoia (Cubells et al., 2000; Kalayasiri et al., 2007). This is consistent with our finding that Dbh knockout (Dbh -/-) mice exhibit an increased sensitivity to the aversive properties of cocaine (Schank et al., 2006).
An increased response to the aversive effects of cocaine represents a potential behavioral target for disulfiram’s clinical efficacy. Another potential mechanism of disulfiram action is attenuation of the rewarding properties of cocaine. NE signaling, primarily through the α1-adrenergic receptor (α1-AR), facilitates DA release, locomotor activity, and place preference in response to psychostimulants (Darracq et al., 1998; Drouin et al., 2002a; Drouin et al., 2002b; Ventura et al., 2003; Auclair et al., 2004). Alternatively, disulfiram could be attenuating cocaine use by preventing relapse. NE plays a critical role in the reinstatement of drug seeking behavior induced by both stressors and priming injection of cocaine via activation of the β-AR and α1-AR, respectively (Erb et al., 2000; Leri et al., 2002; Zhang and Kosten, 2005).

Intravenous self-administration in rats is a commonly used preclinical model to study the reinforcing effects of abused drugs. There are several phases of drug taking behavior that can be analyzed using this paradigm. Acquisition refers to when the rat first learns to press the active lever to receive drug infusions at a reliable rate, and will consistently select the active lever at a greater frequency than an inactive lever. Maintenance responding refers to when the animal expresses a consistently high frequency of active lever selection that is stable over several days. Extinction describes a phase where drug is no longer delivered following active lever responses and instead vehicle infusion, or no infusion at all, results from active lever presses. After an initial increase in responding, active lever responses will gradually decrease and reach a stable, low level after several extinction sessions. Responding on the active lever can be reinstated by certain stimuli, including priming injection of the drug, stressors such as intermittent footshock, or the presentation of cues or environments that were previously paired with drug availability.
In this reinstatement phase, which is thought to model relapse, active lever responding is reactivated by these particular stimuli, and occurs even though the active lever continues to deliver no drug reward.

As mentioned above, NE signaling impacts a wide range of cocaine-induced behaviors including certain behavioral phases of rat self-administration. Disulfiram, which inhibits DBH function, decreases NE levels in brain and can impair signaling at adrenergic receptors. The primary objective of the experiments outlined below was to analyze the effect of disulfiram on cocaine self-administration and cocaine-induced reinstatement as a means to further understand the potential mechanism of disulfiram’s effect on cocaine intake in the clinic.

4.3 Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River, MA), weighing approximately 200-250 grams at time of arrival were used in these experiments. Rats were left untreated for 1 to 4 weeks prior to use. During this time rats were handled several times per week but were not used in any experiments. Animals were housed on reversed light cycle (lights on 20:00, lights off 08:00), and all behavioral testing took place during the dark phase. Unless otherwise noted, food and water were available ad libitum during the course of these experiments except during self-administration, extinction, and reinstatement sessions. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.
Assessment of the effect of disulfiram on catecholamine levels

To determine the effect of disulfiram on catecholamine levels in rat brain tissue, we conducted a series of experiments using High Performance Liquid Chromatography (HPLC). In HPLC experiments, rats were housed on normal light cycle (lights on 7:00, lights off 19:00), and were injected and sacrificed during the light phase.

First, a dose-response experiment was performed, with rats (N=2-4) receiving either drinking water (2 ml/kg), or disulfiram (100 or 200 mg/kg, sonicated and suspended in drinking water) by intragastric gavage. These treatments were given either 1 or 3 times. When multiple injections were given, they were separated by 2 hours. Animals were sacrificed by CO$_2$ asphyxiation 2 hours following the final injection and frontal cortex tissue was removed and immediately frozen. Samples were stored at -80°C until preparation for HPLC analysis.

Because intragastric injection of disulfiram did not alter catecholamine levels (see Results section), this experiment was repeated with intraparitoneal (i.p.) injection of disulfiram (N=2-4). Dosing regimens and timecourse were the same as the experiment outlined above, with the only difference being route of administration and vehicle used (0.9% saline).

Following the pilot dose-response experiment, a separate group of rats (N=5-6) was injected with either saline or 100 mg/kg disulfiram (i.p at 1.5 ml/kg, sonicated and suspended in saline), and sacrificed by CO$_2$ asphyxiation 2 hours later. Frontal cortex tissue was then removed and immediately frozen. All samples were stored at -80°C until preparation for HPLC analysis.
Analytical samples 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 tissue was completely homogenized. The samples were centrifuged at 13,200 rpm for 30 minutes at 4°C, and the supernatant was removed from the tubes. The supernatant was then centrifuged again at 13,200 rpm for 30 minutes at 4°C, this time using a 22-micron filter column. The resulting fluid was the final product used for analysis. The sample was injected using an ESA 542 Autosampler (ESA Biosciences Inc., Chelmsford, MA) and injected onto Synergi Max-RP 4u (150 x 4.6mm) with Security Guard pre column filter with Max-RP cartridges (Phenomenex, Inc., Torrance, CA). Samples were run at a constant rate of 1 ml/min maintained by ESA 584 pumps. An ESA CoulArray 5600A detector with a potential set at -150mV, 200 mV was utilized 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.).

**Testing Chambers**

Chambers used for testing (Med Associates, Georgia, VT) were equipped with two levers, one that was designated “active” and resulted in drug infusions when pressed, and one that was “inactive” and had no programmed consequences following lever depression. A cue light was located on the panel containing the levers and was illuminated following reward delivery. This panel also contained a fitting where a food tray could be inserted during food training sessions and a lever arm could be inserted during drug self-administration sessions. Each testing chamber consisted of a plexiglass compartment with wire bar floor that fit inside of a larger, sound-attenuating chamber.
The infusion system consisted of a syringe pump connected via silastic tubing (Dow-Corning, Midland, MI) to a swivel (Instech, Plymouth Meeting, PA) and metal leash (Med Associates) system. The leash was used to secure the animal during testing sessions, and the infusion line was threaded through this leash and connected to the jugular catheter via a cannula back mount (Med Associates). All programs were run with MED-PC IV software (Med Associates) and statistics were performed using Prism 4.0 for Macintosh.

Food Training

To increase the level of responding when animals were first introduced to the chambers in sessions with cocaine availability, rats were first trained to press the active lever for 45 mg food pellets (Bio-Serve). In these sessions rats were placed in the chambers in the morning and allowed several hours to reach training criteria. Water was available ad libitum in the test chambers. These sessions were started between 9:00 and 12:00, and lasted for 8 hours, or until the animal met criteria, which was defined as at least 70% selection of the active lever and at least 100 food pellets obtained. These sessions were repeated on consecutive days until the animal reached criteria, with all animals reaching training criteria in 1 to 3 days. During these sessions no timeout was included following food pellet delivery, and the cue light was not illuminated after reward presentation. Once 100 food pellet rewards had been obtained, the levers were retracted and the program terminated.

Surgery

After animals were trained to lever press for food pellets, jugular catheters were implanted under isoflurane anesthesia. Briefly, catheters were constructed by attaching
silastic tubing (Dow-Corning, ID 0.64 mm, OD 1.19 mm) to a cannula back mount (Med Associates). The cannula back mount consisted of a circular mesh base with a threaded plastic mount housing a 22-gauge cannula. Following blunt dissection, the catheter tubing was inserted 3.0 cm into the jugular vein and passed subcutaneously to the back of the animal, where it attached to the cannula mount and was secured using 4-0 silk suture material (Ethicon, Johnson and Johnson, Somerville, NJ). Following surgery all animals received an injection of 2.5 mg/kg banamine (Fort Dodge, Overland Park, KS). Catheters were flushed with approximately 0.08 ml of heparin sodium solution (300 IU/ml, Abraxis Pharmaceuticals, Los Angeles, CA) daily. When in question, catheter patency was confirmed by infusion of the short acting intravenous anesthetic brevital (5 mg/kg; King Pharmaceuticals, Bristol, TN), which causes immediate immobility following intravenous infusion.

**Self-Administration**

Self-administration sessions were run between 12:00 and 18:00 each day. Sessions were 2 hours in duration and lasted until this time had elapsed or until the animal exceeded 40 reward infusions. At this time the levers were retracted and the program terminated. Cocaine solution was prepared at 3 mg/ml in sterile saline (0.9 % NaCl) with 10 U/ml heparin added. Cocaine infusions were delivered on an FR1 schedule, with each active lever press resulting in a drug infusion. Infusion volume, which was adjusted based on the weight of each rat, was approximately 50 µl, and was delivered over 4 to 5 seconds for a dose of 0.5 mg/kg. After each cocaine infusion a cue light was illuminated on the panel above the levers for 20 seconds. During this time, responses on the active lever were recorded but did not result in drug infusion. At the
beginning of each session animals received one unit dose cocaine infusion prior to presentation of the response levers.

**Maintenance**

In maintenance experiments, rats (N=8) were first trained to press the active lever for food pellet delivery and then implanted with jugular catheters. After 5 to 7 days of recovery, cocaine self-administration sessions were begun and testing was conducted as described above. These conditions remained in place until maintenance criteria were met. This was defined as greater than 20 active lever presses and greater than 75% selection of the active lever for 3 consecutive days. Further, to meet maintenance levels for stable responding, both active lever presses and infusions obtained were required to stay relatively constant, with no greater than 20% variation from the mean on any of the 3 days. The minimum number of days allowed for the maintenance phase was 5 days. The average number of days required for rats included in all experiments to reach maintenance criteria was 8.54 ± 0.798 (mean ± SEM).

After criteria were met, rats were pretreated on the following day with either saline (0.9% NaCl, i.p. at 1.5 ml/kg) or disulfiram (100 mg/kg, i.p at 1.5 ml/kg, sonicated and suspended in saline) 2 hours prior to self-administration testing. Following this pretreatment day, rats were allowed 1 to 2 days of self-administration sessions with no pretreatment. On the final day, rats were pretreated again with either saline or disulfiram (100 mg/kg). The pretreatment received on day 2 was the opposite of that received on day 1, and the order in which each rat received these pretreatments was randomized. The general design of this experiment is a counterbalanced, repeated measures design, with
each animal receiving both pretreatments in a random order (see figure 4.1 for diagrammatic depiction of experimental timeline)

**Reinstatement**

Reinstatement experiments followed the same timeline as the maintenance experiments up to the point of achievement of maintenance criteria (figure 4.1). At this point, animals in the reinstatement experiment (N=7) began extinction sessions. These sessions were identical to self-administration sessions (two hour sessions, one session per day), except that presses on the active lever did not result in drug infusion or cue presentation. Extinction sessions were continued until animals reached extinction criteria, which was defined as no greater than 15 active lever presses in a session for 3 consecutive days. Further, the average of active lever responses over the 3 days of extinction was required to be less than 30% of the average of active lever responses on the final 3 days of maintenance. The average number of days required to reach this criteria was 16.29 ± 2.01 (mean ± SEM).

After these criteria were met, rats were given a cocaine-primed reinstatement test on the following day. In the reinstatement test procedure, animals were pretreated with either saline (0.9% NaCl, i.p at 1.5 ml/kg) or disulfiram (100 mg/kg, i.p at 1.5 ml/kg, sonicated and suspended in saline), and 2 hours later were injected with cocaine (10 mg/kg, i.p. at 1 ml/kg, dissolved in saline) and placed immediately into the self-administration chambers. The reinstatement session was identical to extinction sessions, with active lever responses having no programmed consequences. After this reinstatement test, animals were re-extinguished until they again met extinction criteria. Animals reached extinction criteria in this second extinction phase in 7.57 ± 1.67 days.
(mean ± SEM). After animals met extinction criteria a second time, they were given a second reinstatement test, with the opposite pretreatment (saline or disulfiram) given 2 hours prior to the cocaine-induced reinstatement test in a random, counterbalanced design.

Because disulfiram did not affect maintenance responding (see Results section), a subset of rats that were used in the maintenance experiment were given 1 to 2 days of normal self-administration sessions following the second pretreatment day, and then extinction sessions were begun. After this point they followed the same timeline as rats included in the reinstatement experiment that had not received pretreatments prior to maintenance sessions.

**Lever Responding for Food Pellets**

To explore possible non-specific effects of disulfiram on general responding, the maintenance experiment described above was performed in a separate group of rats (N=4) with food pellets provided instead of cocaine infusions. Animals were first trained to press the lever for food pellet delivery as described above, and food pellet sessions were given once per day, as in cocaine self-administration experiments. During the course of the experiment, rats were maintained on a restricted diet of 16 g of normal rat chow per day, given in the evening, at least 1 hour after self-administration sessions had ended. Parameters of food self-administration sessions were identical to cocaine self-administration experiments except that sessions were 1 hour in duration and the session was terminated if the rewards obtained exceeded 60. Animals were given food administration sessions until they met maintenance criteria, as defined above. On the following day after maintenance criteria had been achieved, rats were treated with either
saline (1.5 ml/kg, i.p) or disulfiram (100 mg/kg, i.p. at 1.5 ml/kg, sonicated and suspended in saline) 2 hours prior to food pellet self-administration sessions. Following 2 days of self-administration sessions with no pretreatment, rats were treated with the opposite pretreatment. As in the maintenance experiment for cocaine self-administration, this is a repeated measures, counterbalanced design, with each animal receiving both pretreatments in a random order.

4.4 Results

**Intragastric injection of disulfiram has no effect on catecholamine levels**

We initially performed tissue catecholamine HPLC experiments with intragastric injection because preliminary reports suggested that disulfiram given via intragastric gavage decreased maintenance of cocaine self-administration behavior (Muntoni et al., 2004), but we were unable to replicate this result (data not shown). Therefore, prior to continuing our analysis of the effects of this drug on cocaine intake, we assessed the effect of this route of administration on catecholamine levels. Rats (N=2-4 per group) were injected via intragastric gavage with drinking water or disulfiram (100 mg/kg or 200 mg/kg, given 1 or 3 times, each injection spaced 2 hours apart), and sacrificed 2 hours following the final injection. Animals injected with drinking water 1 or 3 times were combined to form a single “control” group. Disulfiram administered by this route had no effect on catecholamine levels. One way ANOVA revealed no significant effect of intragastric disulfiram on NE tissue content (F(4,16)=0.06565, p=0.9910), DA tissue content (F(4,16)=0.4845, p=0.7471), or NE to DA ratio (F(4,16)=1.690, p=0.2166; see Figure 4.2).
Intraparitoneal disulfiram injection decreases NE to DA ratio in rat brain tissue

Next, inhibition of DBH function by i.p. injection of disulfiram was assessed because intragastric disulfiram had no effect on catecholamine content in brain tissue, and because multiple groups have reported an effect of i.p. disulfiram administration on DBH function (Mussacchio et al., 1966; Maj et al., 1968; Karamanakos et al., 2001; Bourdelat-Parks et al., 2005). In this experiment, rats (N=2-4 per group) were given i.p injections of saline or disulfiram (100 mg/kg or 200 mg/kg, given 1 or 3 times, each injection spaced 2 hours apart), and sacrificed 2 hours following the final injection. Rats injected with saline 1 or 3 times were combined to form a single “control” group. One way ANOVA revealed that disulfiram significantly affected NE to DA ratio (F(4,13)=14.29, p=0.0006; Figure 4.3). Post-hoc Dunnet’s tests comparing each treatment to saline control revealed a significant difference between saline treated rats and rats treated with 1 x 100 mg/kg (q=4.248, p<0.01), 3 x 100 mg/kg (q=6.028, p<0.01), 1 x 200 mg/kg (q=4.997, p<0.01), or 3 x 200 mg/kg disulfiram (q=6.017, p<0.01). One way ANOVA also indicated a significant main effect of disulfiram treatment on NE tissue content (F(4, 13)=4.308, p=0.0321) and DA tissue content (F(4,13)=4.156, p=0.0353). However, post-hoc Dunnet tests revealed a significant difference between only saline and 3 x 200 disulfiram for DA tissue content (q=3.907, p<0.05), and between saline and 1 x 200 mg/kg disulfiram (q=3.219, p<0.05) and 3 x 200 mg/kg disulfiram (q=3.361, p<0.05) for NE tissue content.

In the previous experiment, rats treated with 200 mg/kg appeared extremely lethargic, and a separate group treated with this dose did not administer food pellets during food training sessions (data not shown); therefore a dose of 100 mg/kg injected 2
hours prior to testing was used in the remainder of experiments. First, we reassessed the effect of this dose of disulfiram on catecholamine levels in rat brain tissue in an experiment including larger group numbers. Rats (N=5-6) were sacrificed 2 hours following a single i.p. injection of saline or disulfiram (100 mg/kg). This dose of disulfiram decreased tissue content of NE while increasing tissue DA levels (Figure 4.4). Unpaired t-tests indicated a significant difference between groups treated with saline and disulfiram for measures of NE tissue content (t(9)=3.638, p=0.0054), DA tissue content (t(9)=2.324=0.0453), and NE:DA ratio (t(9)=4.831, p=0.0009), similar to previously published results (Mussachio et al., 1966; Maj et al., 1968; Karamakos et al., 2001; Bourdelat-Parks et al., 2005).

**Disulfiram has no effect on maintenance of cocaine self administration**

Next, we explored the effect of disulfiram pretreatment on maintenance of cocaine self-administration. After rats (N=8) met predetermined criteria for maintenance levels of responding, they were treated on subsequent days with either saline or disulfiram (100 mg/kg) 2 hours prior to self-administration sessions. We found that disulfiram had no effect on maintenance responding for cocaine (Figure 4.5). For statistical analysis, the frequency of lever presses and number of rewards obtained over the last 3 days of maintenance were averaged for each rat, and this was used as the dependent variable for “maintenance” responding. When maintenance responding was compared to sessions where pretreatments were administered, one way repeated measures ANOVA revealed no significant change in active lever presses (F(2,23)=3.277, p=0.4823) or drug infusions obtained (F(2,23)=0.9685, p=0.4037). Further, disulfiram...
(100 mg/kg) did not affect the number of food pellets administered during food pellet self-administration sessions. In this experiment, all rats (N=4) administered the maximum number of food pellets following both disulfiram and saline injection.

**Disulfiram blocks the expression of cocaine-induced reinstatement**

Finally, we assessed the effect of disulfiram on cocaine-induced reinstatement. In this experiment, rats were extinguished following the maintenance phase of cocaine self-administration until they reached a low, stable level of responding. Once animals reached predetermined criteria for extinction of active lever pressing, they were given a cocaine primed (10 mg/kg, i.p) reinstatement test on the following day. For maintenance responding, an average of the last 3 days of maintenance was used as the dependent variable, as described above. Also, the number of responses over the last 3 days of extinction were averaged to obtain the dependent variable for extinction responding. Paired t-tests indicated no difference between rate of responding in extinction sessions prior to each reinstatement test, so these values were averaged for each animal to obtain a singular statistic for extinction responding.

Cocaine priming significantly enhanced responding on the previously active lever, and this effect was completely blocked by pretreatment with disulfiram (Figure 4.6). When active lever responding was compared using one way repeated measures ANOVA, analysis revealed a significant main effect of treatment phase (F(3,27)=6.157, p=0.0046). Bonferroni post-hoc analysis revealed a significant difference between extinction and reinstatement with saline pretreatment (t=3.075, p<0.05), and between reinstatement with saline pretreatment and reinstatement with disulfiram pretreatment (t=2.739, P<0.05), but
not between extinction sessions and reinstatement with disulfiram pretreatment (t=0.3368, p>0.05). These results indicate that cocaine-priming injections reinstated responding on the active lever in animals pretreated with saline prior to cocaine, but not those pretreated with disulfiram.

One way repeated measures ANOVA revealed a main effect for the influence of treatment phase on inactive lever responding (F(3,27)=4.286, p=0.0190). This is likely a result of the fact that once maintenance criteria were reached, inactive lever responding rarely exceeded 1 or 2 presses in any maintenance session, but increased slightly in extinction and reinstatement sessions. Post-hoc Bonferroni tests for specific comparisons revealed no significant difference in inactive lever responding between extinction and cocaine-induced reinstatement tests with either saline (t=0.1681, p>0.05) or disulfiram (t=2.118, p>0.05) pretreatment. Most importantly, there was no significant difference in inactive lever responding between cocaine-induced reinstatement test with saline pretreatment and cocaine-induced reinstatement test with disulfiram pretreatment (t=1.950, p>0.05).

4.5 Discussion

The most important finding from the current set of experiments is that disulfiram blocks the expression of cocaine-induced reinstatement following extinction, but has no effect on maintenance of cocaine self-administration. The disulfiram dose used in these experiments significantly decreased NE to DA ratio in rat frontal cortex tissue, and did not significantly alter self-administration of food pellets. This is one of few reports of
the effect of disulfiram on cocaine-induced behaviors in rodents (Maj et al., 1968; Muntoni et al., 2004 Haile et al., 2003; Schank et al., 2008).

It has recently been reported that disulfiram pretreatment decreases responding during the maintenance phase of cocaine self-administration in rats (Muntoni et al., 2004). In that experiment, disulfiram was given by intragastric gavage, so we initially used this route of administration. In pilot experiments, we found that disulfiram failed to alter cocaine self-administration and had no effect on catecholamine levels when given by this method. We then repeated these experiments using the more common intraperitoneal method and found that disulfiram, at all doses tested, decreased NE to DA ratio in rat brain. This agrees with previous reports from our lab and others showing that disulfiram can decrease DBH function and alter catecholamine levels in brain tissue from mice and rats (Musacchio et al., 1966; Maj et al, 1968; Karamanakos et al., 2001; Bourdelat-Parks et al., 2005). Therefore, we used a single i.p. injection of 100 mg/kg disulfiram in the remainder of our experiments.

In our self-administration experiments, we observed no effect of disulfiram pretreatment on maintenance responding for cocaine. Previous reports have failed to demonstrate a significant role of NE signaling in the maintenance of cocaine self-administration (for review see Weinshenker and Schroeder, 2007), and our results agree with this concept. However, NE has been shown to play a significant role in the reinstatement of drug seeking behavior following a foot shock stressor (Erb et al., 2000) or cocaine priming injection (Zhang and Kosten, 2005). In these reports it was found that NE signaling through the β-AR in the bed nucleus of the stria terminalis and amygdala appears to be critical for the expression of stress-induced reinstatement (Leri et al., 2002),
while the $\alpha_1$-AR at an undetermined site is most important for the expression of cocaine-induced reinstatement (Zhang and Kosten, 2005). The latter finding suggests that disulfiram may be attenuating cocaine-induced reinstatement by inhibiting DBH, and thus decreasing $\alpha_1$-AR signaling.

While NE may not play a critical role in the maintenance of cocaine self-administration, NE signaling has been shown to influence escalated cocaine intake in rats following certain treatment regimens. Specifically, $\alpha_1$-AR antagonists prevent escalated cocaine intake induced by non-contingent cocaine injections prior to self-administration sessions (Zhang and Kosten, 2007) and by cocaine self-administration sessions with long access of drug availability (Wee et al., 2008). Disulfiram decreases NE levels in brain tissue, and can presumably attenuate signaling at all subtypes of adrenergic receptor. Therefore, it is of great interest to assess the effect of disulfiram on escalated intake under these conditions.

In humans, disulfiram decreases cocaine intake in cocaine addicts, but the mechanism of action for this effect is unknown. Given that variations in DBH function within the human population influence sensitivity to certain effects of cocaine, and given that level of DBH function interacts with the ability of disulfiram to decrease cocaine intake, it is likely that DBH is a significant biochemical target of disulfiram pharmacotherapy for cocaine addiction. A major question that remains is the behavioral target for disulfiram-mediated attenuation of cocaine intake. For example, disulfiram could be increasing the aversive properties of cocaine, as might be expected given the fact that $Dbh^{-/-}$ mice and people with low DBH activity are more sensitive to some aversive effects of the drug (Cubells et al., 2000; Schank et al., 2006; Kalayasiri et al.,
Alternatively, because NE is critical for the function of DA neurons in the mesolimbic reward pathway (Weinshenker and Schroeder, 2007), disulfiram could be decreasing the subjectively positive properties of cocaine, thereby blunting the rewarding effects of the drug. Finally, disulfiram could be attenuating cocaine use in cocaine dependent people by preventing relapse. Relapse to compulsive drug seeking behavior can be induced by certain stimuli such as severe life stresses, exposure to drug related cues that illicit craving, and lapses in abstinence behavior. Of those three possibilities, our results suggest that disulfiram is most likely attenuating cocaine intake in dependent people by preventing relapse.

In our experiments, we analyzed the effect of disulfiram on cocaine-primed reinstatement. Given the involvement of NE in stress-induced reinstatement, it is likely that disulfiram would also be effective at preventing this phenomenon as well. Stress- and drug-induced relapse present a particularly challenging behavioral process to disrupt, and treatments that target this behavior would be valuable medications.

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Figure 4.1

Maintenance:

Reinstatement:
Figure 4.1 Experimental Timeline for Maintenance and Reinstatement Experiments
Figure 4.2

A

![Bar chart showing NE content (ng/g tissue) for control and Dis treated groups.]

B

![Bar chart showing DA content (ng/g tissue) for control and Dis treated groups.]

C

![Bar chart showing NE:DA ratio for control and Dis treated groups.]

Legend:
- Control
- Dis 100
- Dis 200
- Dis 100 x 3
- Dis 200 x 3
Figure 4.2 Intragastric administration of disulfiram has no effect on catecholamine levels in rat frontal cortex. Saline or disulfiram (Dis; 100 mg/kg and 200 mg/kg) was administered 1 or 3 times via intragastric gavage in male rats. When multiple injections were given, each dose was separated by 2 hours. Rats were sacrificed 2 hours following the last injection. Shown are measures for NE tissue content (A), DA tissue content (B), and NE to DA ratio (C).
Figure 4.3

A

![Graph A showing NE content (ng/g tissue) for Control, Dis 100, Dis 200, Dis 100 x 3, Dis 200 x 3]

B

![Graph B showing DA content (ng/g tissue) for Control, Dis 100, Dis 200, Dis 100 x 3, Dis 200 x 3]

C

![Graph C showing NE:DA ratio for Control, Dis 100, Dis 200, Dis 100 x 3, Dis 200 x 3]
Figure 4.3 Intraperitoneal disulfiram alters catecholamine levels in rat frontal cortex. Saline or disulfiram (Dis; 100 mg/kg and 200 mg/kg) was administered 1 or 3 times via i.p. injection in male rats. When multiple injections were given, each dose was separated by 2 hours. Rats were sacrificed 2 hours following the last injection. Shown are measures for NE tissue content (A), DA tissue content (B), and NE to DA ratio (C). *p<0.05 compared to control, **p<0.001 compared to control.
Figure 4.4

A

B

C
Figure 4.4 Disulfiram (100 mg/kg, i.p) alters catecholamine levels in rat frontal cortex. Saline or disulfiram (100 mg/kg) was administered via i.p. injection, and rats were sacrificed 2 hours later. Shown are measures for NE tissue content (A), DA tissue content (B), and NE to DA ratio (C). *p<0.05, **p<0.01, ***p<0.001 compared to control.
Figure 4.5
Figure 4.5 Disulfiram does not affect maintenance of cocaine self-administration.

After reaching maintenance levels, rats were pretreated with saline or disulfiram (100 mg/kg, i.p.) 2 hours prior to cocaine self-administration sessions. Shown are active lever responses and number of rewards obtained over the 2 hour session. Maintenance values reflect an average number of responses and rewards obtained over the last 3 days of maintenance.
Figure 4.6 Disulfiram blocks the expression of cocaine-induced reinstatement. After rats met extinction criteria they were given 2 reinstatement tests with an intervening re-extinction phase. In this test rats were injected with cocaine (10 mg/kg, i.p.), placed directly into self-administration chambers, and responses were measured over the 2 hour test session. Rats were pretreated with saline or disulfiram (100 mg/kg, i.p.) 2 hours prior to cocaine injection. Shown are active lever responses and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect a combined average of the last 3 days of extinction prior to each reinstatement test. *p<0.05 compared to active lever responses during extinction, #p<0.05 compared to active lever responses during cocaine induced reinstatement tests with disulfiram pretreatment.
CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS
5.1 The Role of Norepinephrine in Cocaine-Induced Reward, Anxiety, and Relapse

In this report, we have demonstrated a significant role of NE in a variety of cocaine-induced behaviors. We utilized a diverse set of tools including genetic deletion of DBH enzyme, pretreatment with pharmacological DBH inhibitors, and administration of specific adrenergic antagonists to assess the role that NE plays in cocaine-induced neurochemistry, locomotion, reward, aversion, anxiety, drug administration, and reinstatement. To accomplish this analysis we used widely accepted behavioral and neurochemical paradigms such as conditioned place preference, elevated plus maze testing for anxiety, rat self-administration, in vivo microdialysis, receptor radioligand binding, and HPLC analysis.

First, we demonstrated that NE influences DA signaling in brain regions that are pertinent to the rewarding effects of cocaine. Complete lack of NE, as is seen in Dbh-/- mice, leads to a decreased level of baseline extracellular DA in the NAC and CP, and a compensatory postsynaptic DA receptor hypersensitivity. Also, amphetamine-induced DA release was blunted in the CP and PFC, and completely abolished in the NAC. These results agree with previous reports concerning the effect of adrenergic antagonists and NE lesions on dopaminergic function, and indicate that NE exerts a net activating effect on DA signaling in the brain. The DA receptor hypersensitivity observed in Dbh-/- mice in turn leads to enhanced locomotor response to drugs that influence the dopaminergic system, including cocaine. Hypersensitivity to amphetamine-induced locomotion has been previously reported (Weinshenker et al., 2002a), and one goal of the current experiments was to assess whether this increased sensitivity to psychostimulants extended to cocaine.
We proceeded to show that this dopaminergic hypersensitivity and increased locomotor response to cocaine also associates with an increased sensitivity to the rewarding and aversive effects of cocaine. Specifically, Dbh-/- mice exhibited a place preference to a low dose of cocaine that did not support place preference in control animals, and expressed a novel place aversion at a high dose of cocaine that is typically rewarding in controls. To further explore this alteration in cocaine-induced reward/aversion, we conditioned control mice with extremely high doses of cocaine and were not able to induce a place aversion, suggesting that the changes seen in Dbh-/- mice are not the result of a simple leftward shift in the dose-response curve. To explore the role of distinct adrenergic receptors subtypes in place preference to cocaine, we administered specific antagonists to control animals and found that only the α1-AR antagonist prazosin blocked the development of cocaine-induced place preference.

The fact that Dbh-/- mice display a hypersensitivity to the aversive properties of cocaine led us to ask which aversive properties are specifically enhanced to underlie this effect. To address this topic we first assessed the cocaine-induced anxiety response in Dbh-/- mice and found that, contrary to our hypothesis, these animals are completely insensitive to the anxiogenic properties of cocaine. This was a highly unexpected result in light of enhanced cocaine aversion in these animals, but agrees with the generally accepted notion that NE is involved in stress and anxiety responses. We were able to recapitulate this behavioral phenotype in control mice by inhibiting NE synthesis via acute injections of disulfiram. Further, we demonstrated that the expression of cocaine-induced anxiety specifically requires β-AR signaling.
Given the fact that disulfiram treatment decreases cocaine intake in human cocaine addicts, we explored the impact of disulfiram on drug taking and drug seeking behaviors in rats. We found that while disulfiram pretreatment had no effect on maintenance of cocaine self-administration, it completely blocked the expression of cocaine-induced reinstatement of drug seeking.

Taken together, these results confirm previous findings that NE activity exerts an activating effect on DA signaling, and that a chronic lack of NE leads to an enhancement of postsynaptic DA receptor sensitivity. We further expanded results demonstrating that \(Dbh^{-/-}\) animals are hypersensitive to the effects of amphetamine by showing that these animals are also hypersensitive to cocaine-induced locomotion, reward, and aversion but exhibit a paradoxical insensitivity to the anxiogenic effects of the drug. We also demonstrate that disulfiram can attenuate a specific phase of drug seeking behavior, cocaine-induced reinstatement. For a schematic representation of the proposed pathways involved in behavioral alterations following acute and chronic inhibition of NE signaling, see Figure 5.1.

5.2 Implications for Disulfiram Mechanism of Action on Cocaine Abuse

At the beginning of this report, we introduced three possible behavioral targets that may explain disulfiram’s effect on cocaine intake in the clinic. Disulfiram could be acting to attenuate cocaine intake by enhancing aversive properties of the drug, blunting the rewarding effects of the drug, or by preventing relapse to compulsive drug seeking behavior. Our preclinical findings shed light on these three hypotheses, and their significance will be discussed below.
Our initial data concerning the effect of genetic knockout of Dbh suggest that a chronic lack of NE can enhance some aversive properties of cocaine. Specifically, Dbh -/- mice displayed a novel place aversion to cocaine at a dose that was typically rewarding in control animals. Disulfiram, which inhibits DBH and decreases NE levels, could be decreasing cocaine intake in cocaine-dependent patients by enhancing cocaine’s aversive effects. Surprisingly however, Dbh -/- animals show an insensitivity to the anxiogenic properties of acute administration of cocaine. This suggests that cocaine-induced place aversion in Dbh -/- mice is mediated by an enhancement of some aversive property of the drug other than anxiety. Humans with low DBH activity are more sensitive to cocaine-induced paranoia (Cubells et al., 2000; Kalayasiri et al., 2007) and disulfiram-induced psychosis (Ewing et al, 1977; Major et al., 1979). While it is unclear whether rodents experience complex aversive properties such as these, it is possible that some analogous aversive response could be accentuated in these animals, although no validated behavioral paradigms currently exist for their measurement.

Our results provide the least support for the hypothesis that disulfiram is acting by blunting the rewarding properties of cocaine. Dbh -/- mice, which have a complete and chronic lack of NE, actually show an enhanced cocaine-induced reward, as evidenced by their expression of a place preference at a low dose of cocaine that does not support place preference in control animals. Further, disulfiram had no effect on maintenance of cocaine self-administration. A pretreatment that decreases the acute rewarding effects of cocaine would be expected to alter self-administration of the drug. However, we did show an inhibition in the expression of cocaine-induced place preference in animals that were pretreated with prazosin prior to cocaine conditioning sessions. Disulfiram
decreases NE levels globally, and could presumably decrease NE signaling at all major subtypes of adrenergic receptors. Therefore, it is possible that disulfiram could be blunting the rewarding properties of cocaine by attenuating NE signaling at the α1-AR.

This concept agrees with previous reports that acute administration of prazosin blunts DA release in the NAC (Auclair et al., 2002), psychostimulant-induced locomotion (Drouin et al., 2002a), and the development of sensitization to psychostimulant-induced locomotor activity (Weinshenker et al., 2002a). Taken together, these results suggest that the effects of disulfiram on the rewarding properties of cocaine are still largely undetermined and warrant further exploration (for extensive review of this topic see Weinshenker and Schroeder, 2007).

Another potential way in which disulfiram could be acting is by preventing relapse following a period of abstinence. Our data strongly support this possibility. Relapse can be elegantly modeled in rodents using reinstatement of drug seeking behavior, which is a permutation of the rat self-administration technique (reviewed in Shaham et al., 2000; Shaham et al., 2003). In this procedure, a rat is given access to cocaine during self-administration sessions and, after reaching maintenance levels for stable responding, is extinguished until responding on the active lever drops to a very low level. Responding on this lever can be reactivated by certain stimuli, such as a non-contingent injection of the test drug, presentation of cues associated with drug availability, or a stressor, despite no drug being available. Each of these stimuli has an analogous stimulus in humans, and can serve as cues for relapse into drug seeking. For example, patient reports often suggest that stimuli that can precipitate relapse into
compulsive drug taking behavior include brief lapses in abstinence, exposure to environmental cues that induce drug conditioned craving, and significant life stresses.

We found that disulfiram completely blocked the expression of cocaine-induced reinstatement, but had no effect on maintenance of cocaine self-administration. This suggests that disulfiram could be particularly effective at decreasing the risk of relapse in cocaine dependent people if they use drug while trying to remain abstinent. As mentioned above, this brief lapse in abstinence from cocaine use often triggers a cycle of relapse into compulsive drug seeking. Prazosin, which blocks the α1-AR, attenuates cocaine-primed reinstatement in rats (Zhang and Kosten, 2005), and suggests a target receptor for disulfiram’s effect in this case.

Further, another common cause of relapse to drug use is a desire to alleviate negative affective states that result from withdrawal (Koob and LeMoal, 2008). In the case of cocaine, withdrawal from chronic administration can cause such symptoms as depression, anxiety, and anhedonia (Perrine et al., 2008; Weiss et al., 2001). In our experiments, we show that pretreatment with disulfiram or a β-AR antagonist attenuates cocaine-induced anxiety. While this was an assessment of cocaine’s acute anxiogenic properties, it is known that NE signaling specifically through β-ARs is also involved in the expression of cocaine withdrawal-induced anxiety (Rudoy et al., 2007). Therefore, disulfiram could be acting by blunting anxiety associated with cocaine withdrawal, which would in turn decrease the risk of relapse into compulsive drug use.
5.3 Future Directions

There are a number of directions that this line of research could take. It is important to fully understand the mechanism of disulfiram action on cocaine abuse because it is one of the only medications to show clinical efficacy as a pharmacotherapy for cocaine addiction. Understanding the mechanism of disulfiram’s effect will provide useful information about effective behavioral and biochemical targets in medications for addiction, and will aid in the development of more potent and specific treatments.

First, in our self-administration experiments we utilized acute DBH inhibition by administration of disulfiram. We found that this had no effect on maintenance of cocaine self-administration, which was unexpected in light of consistent reports that disulfiram decreases cocaine intake in cocaine dependent people. However, when people are treated with disulfiram in clinical trials, it is often during a 12-week trial (George et al., 2000; Petrakis et al., 2000; Carroll et al., 2004). In our animal models, it is possible that a more chronic disulfiram treatment regimen may be required to observe any effect on maintenance of cocaine self-administration. This is especially likely if disulfiram’s action on cocaine dependence relies on the development of DA receptor supersensitivity. For example, we observe DA receptor hypersensitivity and enhanced cocaine-induced behaviors in Dbh -/- animals that chronically lack NE. However, acute administration of adrenergic antagonists such as prazosin have the opposite effect in that they decrease DA signaling and blunt behavioral responses to cocaine. Disulfiram itself has such an effect in rodent models. Specifically, acute disulfiram decreases psychostimulant-induced locomotion while repeated disulfiram treatment enhances sensitization to the locomotor activating effects of cocaine (Maj et al., 1968; Haile et al., 2003). A powerful addition to
these studies would be to assess DA receptor sensitivity following acute or chronic disulfiram, and assess how this measure correlates with changes in behavioral responses to psychostimulants.

Another consideration pertaining to our maintenance experiments is that we only utilized one dose of cocaine and one schedule for reward delivery. To truly make the claim that disulfiram has no effect on maintenance of cocaine self-administration, it will be necessary to assess the effect of this drug on self-administration of a range of unit doses of cocaine. For example, if disulfiram is enhancing an acute aversive property of cocaine, we may only see an effect at higher unit doses of the drug, where aversive properties may begin to override the rewarding effects in some cases. Further, we used a Fixed Ratio 1 (FR1) schedule in our experiments. In many cases, animals received the maximum number of rewards allowed during the session. One way to interpret these data is that the behavioral expenditure for obtaining the maximum rewards available is rather low. If the response requirement was elevated, to FR5 for example, disulfiram may begin to have an effect on maintenance of self-administration. Another possibility here is to simply increase the number of reward infusions allowed in a session. This may address both concerns in that more responses would be required to achieve maximum reward delivery, and higher levels of cocaine may be on board at the end of the session. An interesting and powerful addition to this analysis would be to perform progressive ratio (PR) experiments. In a PR schedule, the number of responses required to obtain a reward infusion is increased throughout the session. The total number of responses that will be performed to achieve a reward infusion is known as the “breaking point,” and this
measure can be used as an index of motivation for drug delivery. It would be very interesting to explore the effect of disulfiram on this type of responding.

Also, in our reinstatement experiments we explored only reinstatement induced by a priming injection of cocaine. We found that disulfiram completely blocks this behavior, and this is likely a result of decreased NE availability at the \( \alpha_1 \)-AR. It is important for us to also assess the effects of disulfiram on stress-induced reinstatement. Intermittent footshock can serve as a potent stimulus for reinstatement of drug seeking behavior (Erb and Stewart, 1998), and this effect has been shown to rely on NE signaling at \( \beta \)-ARs in the bed nucleus of the stria terminalis and central amygdala (Leri et al., 2002). It would be surprising if disulfiram treatment, which decreases brain NE content, has no impact on stress-induced reinstatement of cocaine seeking behavior.

Finally, sex differences exist in the clinical efficacy of disulfiram as a pharmacotherapy for cocaine addiction with females being less sensitive to the effects of this treatment than males (Nich et al., 2004). This is interesting considering the fact that DBH enzyme expression is partially influenced by sex hormones (Serova et al., 2002). In our self-administration experiments we used only male animals. It would be interesting to explore whether there is a gender difference in effectiveness of disulfiram to alter behavioral responses to cocaine in rats.

5.4 Specific DBH inhibition

Throughout this report we hypothesize that disulfiram is affecting cocaine responses via inhibition of the enzyme DBH. However, because disulfiram is a non-specific inhibitor of this enzyme, it will be important to assess many of the behaviors...
studied here following pretreatment with specific DBH inhibitors. If these drugs mimic the effects of disulfiram in our animal models, it will lend further support to the interpretation that disulfiram’s action on cocaine use depends on its inhibition of DBH.

Disulfiram impacts the function of a number of endogenous enzymes in addition to DBH. While it is unlikely that many of these targets play a role in behavioral responses to cocaine, there are a few that could potentially confound our interpretations due to their involvement in catecholamine signaling and cocaine metabolism (for review see Eneanya et al., 1981). For example, disulfiram inhibits aldehyde dehydrogenase, and its inhibition of this enzyme underlies its effect on alcohol consumption. In addition to its central role in alcohol metabolism, aldehyde dehydrogenase is also involved in the breakdown of DA (Cooper et al., 2003), a neurotransmitter that, as described in great detail above, impacts cocaine-related behaviors. Disulfiram also inhibits the function of cytochrome P450s (Zemaitis and Greene, 1975), which are liver enzymes involved in the metabolism of a wide range of drugs. However, disulfiram specifically inhibits the CYP2E1 isoform of cytochrome P450 (Kharasch et al., 1999), while the CYP3A4 isoform is primarily involved in cocaine metabolism (Boelsterli et al., 1991; LeDuc et al., 1993; Pellinen et al., 1994). Therefore, disulfiram’s effect on cytochrome P450 enzymes provides more useful information about possible interactions with medications taken by disulfiram-treated individuals than it does about the impact of disulfiram on cocaine-induced behaviors in rodents and humans. Further, disulfiram itself can cause DA release from synaptosomes (Vaccari et al., 1996), and can impair the metabolism of cocaine via inhibition of carboxylesterases (Stewart et al., 1979; Nousiainen and Torronen, 1984; McCance-Katz et al., 1998). Whether disulfiram’s activity at any of these targets
underlies its effect on cocaine-induced behaviors remains unclear. Regardless, these issues lend further importance to the analysis of more specific DBH inhibiting agents for preclinical exploration of cocaine-induced behaviors and in medication development.

A highly specific DBH inhibitor known as nepicastat is available (Stanley et al., 1995), and the effect of this drug on cocaine-induced behaviors will be studied closely in our laboratory. Nepicastat, unlike disulfiram, binds directly to DBH enzyme to inhibit its activity, and lacks appreciable activity at a wide range of proteins, receptors, and enzymes (Stanley et al., 1995). In addition, nepicastat is a more potent inhibitor of DBH than disulfiram (IC$_{50}$ for disulfiram = 1 µM; IC$_{50}$ for nepicastat = 9 nM). We have explored the effect of this inhibitor on cocaine-induced behaviors in mice, and have found that in some cases chronic nepicastat treatment can induce behavioral alterations that mimic those seen in Dbh -/- animals (see Appendix 1). It will be of great interest to assess the impact of nepicastat pretreatment on cocaine responses in rats. Our hypothesis is that, given the fact that disulfiram may be acting primarily via inhibition of DBH, nepicastat will induce the same behavioral alterations as those seen following treatment with disulfiram. As noted above, there may be an important distinction between acute and chronic inhibition of DBH with this drug, and therefore both courses of treatment will be utilized.

Further, by administering disulfiram to Dbh -/- mice, we can test the prediction that if disulfiram is acting via inhibition of DBH specifically, it will have no effect in these animals. Because Dbh -/- mice lack DBH enzyme completely, delivery of inhibitors of this enzyme should have no further effect on the unique behavioral phenotypes of these animals. In addition, because Dbh genotype and disulfiram have
additive effects on catecholamine levels, we predict that \( Dbh^{+/-} \) mice will be more sensitive to the effects of disulfiram than \( Dbh^{++} \) mice (Bourdelat-Parks et al., 2005). This analysis would lend strong support to the assertion that disulfiram is acting via DBH inhibition specifically, but could be confounded by interactions between compensatory changes in nervous system development in knockout mice and any off target effects of disulfiram.

Analyzing the effects of nepicastat in these behavioral paradigms is of great interest because this drug represents a very promising potential treatment for cocaine addiction. As outlined above, there is a well-established interaction between DBH function and responses to cocaine in the human population, and disulfiram has shown significant clinical efficacy as a treatment for cocaine addiction. Potent and specific inhibition of DBH enzyme with nepicastat would likely result in a more targeted pharmacological treatment with fewer side effects.
Figure 5.1. Neurochemical alterations following acute and chronic NE inhibition.
Figure 5.1 Neurochemical alterations following acute and chronic NE inhibition.

(A) In the central nervous system there are extensive anatomical and functional connections between the NE and DA systems. The LC, A1, and A2 project to the VTA, NAC, and PFC, where they regulate release in DA terminal regions located in the cortex and striatum by exerting an activating influence on DA transmission. The NE system also densely innervates regions of the limbic system involved in the expression of anxiety behavior, such as the central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST). (B) Following acute inhibition of DBH function, levels of NE in the synapse decrease, thereby removing the activating influence of NE on DA release, which leads to decreased baseline extracellular DA levels. This likely results from impaired NE signaling at the $\alpha_1$-AR (solid black chevron), as treatment with prazosin can induce similar effects on DA function. Extracellular NE levels also decrease in the BNST and amygdala. Altered anxiety behavior following acute NE inhibition is likely the result of inhibited NE signaling at the $\beta$-AR (open black chevron) in these regions. (C) Following chronic inhibition of DBH, there is a sustained decrease in NE levels, which leads to a chronic decrease in baseline extracellular DA and upregulation of DA receptors in the striatum (open gray chevron). Interestingly, DA levels and receptors in the PFC are largely unaffected. Differences in the neurochemical alterations following acute and chronic NE inhibition are believed to underlie qualitative differences in cocaine-induced behaviors following such treatments.
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APPENDIX 1:

CONTINUOUS MINIPUMP DELIVERY OF DOPAMINE β-HYDROXYLASE INHIBITORS ALTERS COCAINE-INDUCED LOCOMOTION
A1.1 Abstract

The catecholamines dopamine (DA) and norepinephrine (NE) mediate many behavioral responses to psychostimulant drugs, including cocaine and amphetamine. Dopamine β-hydroxylase (DBH) is the enzyme that converts DA to NE in the catecholamine biosynthetic pathway, and previous data from our laboratory have shown that mice with genetic deletion of the Dbh gene (Dbh -/- mice) exhibit altered neurochemical and behavioral responses to psychostimulants. Disulfiram, a copper-chelating agent that inhibits DBH activity, alters catecholamine tissue content as well as psychostimulant-induced locomotion in rodents. Using osmotic minipumps to deliver disulfiram, we exposed mice to a dose of 50 mg/kg/day for 2 to 3 weeks. Similar to the effects of acute disulfiram administration, we found that locomotion induced by a dose of 10 mg/kg cocaine was decreased in animals implanted with disulfiram minipumps. Because disulfiram inhibits many enzymes besides DBH, we also tested the effect of the highly specific DBH inhibitor, nepicastat, on cocaine-induced behavior. We found that treatment with nepicastat (50 mg/kg/day) significantly increased locomotion induced by 10 mg/kg cocaine, an effect that recapitulates the cocaine hypersensitivity previously observed in Dbh -/- mice. We also assessed the effects of chronic delivery of these drugs on cocaine-induced anxiety and found that neither disulfiram nor nepicastat minipumps altered this behavior. Taken together, these results suggest that chronic pharmacological DBH inhibition can alter certain drug-induced behaviors in rodents; however, there may be some distinction between the effects of specific versus non-specific inhibition of this enzyme.
A1.2 Introduction

In the preceding chapters, we have presented findings that demonstrate an integral role of norepinephrine (NE) signaling in cocaine-induced behaviors. NE is synthesized by the enzyme dopamine β-hydroxylase (DBH), and mice that have a genetic deletion of this gene (Dbh −/− mice) express a wide range of unique behavioral phenotypes in response to psychostimulant administration. For example, these mice are more sensitive to the locomotor activating effects of both cocaine and amphetamine (Weinshenker et al., 2002; Schank et al., 2006). NE normally provides excitatory drive onto midbrain dopamine (DA) neurons, and this increased sensitivity to psychostimulants is associated with a compensatory increase in postsynaptic DA receptor sensitivity following chronically decreased extracellular DA levels (Schank et al., 2006). Lesions of the locus coeruleus, which is a major NE nucleus in the brainstem, also produce a general hypersensitivity of the dopaminergic system and an enhanced response to amphetamine (Harro et al., 2000; Haidkind et al., 2002). These results suggest that chronic lack of NE leads to a hypersensitive dopaminergic system that imparts enhanced responsiveness to agents that influence DA signaling, including psychostimulant drugs.

The behavioral alterations observed in Dbh −/− mice also extend to the rewarding, aversive, and anxiogenic properties of cocaine. For example, Dbh −/− mice exhibit an increased sensitivity to both the rewarding and aversive properties of cocaine (Schank et al., 2006). Specifically, these mice express a place preference to a low dose of cocaine that does not support place preference in control animals, and a novel place aversion to a high dose of cocaine that is typically rewarding in controls. To determine if increased sensitivity to the anxiogenic properties of cocaine underlies this enhanced drug-induced
aversion, we have also assessed the cocaine-induced anxiety behavior of Dbh -/- mice in the elevated plus maze paradigm. Surprisingly, these animals exhibited an insensitivity to the anxiogenic properties of cocaine (Schank et al., 2008).

A major goal of the experiments outlined here was to determine if we could model the behavioral phenotypes that are observed in Dbh -/- mice by chronic administration of DBH inhibitors. Non-specific DBH inhibition was achieved with disulfiram (Bourdelat-Parks et al., 2005), and highly specific DBH inhibition with nepicastat (Stanley et al., 1995). We delivered these drugs via osmotic minipump for 2 to 3 weeks and assessed their effect on a variety of cocaine-induced behaviors including drug-induced locomotion, anxiety, and reward.

Studying the role of DBH in behavioral responses to psychostimulants is of great interest because disulfiram has shown clinical efficacy in the treatment of cocaine addiction (Carroll et al., 1998; George et al., 2000; Petrakis et al., 2000; Carroll et al., 2004). However, the mechanism of disulfiram’s efficacy in cocaine abusers is largely unknown. Understanding the way in which disulfiram can alter cocaine-induced behaviors will provide useful information for the development of more potent and specific medications for the treatment of psychostimulant addiction.

A1.3 Materials and Methods

Animals

Male and female Dbh +/- mice (aged 2 to 5 months), which have normal NE levels (Thomas et al., 1998), were used in these experiments. Unless otherwise noted, animals were housed on a normal light cycle (lights on 7:00, lights off 19:00), and
behavioral testing took place during the light phase. In all cases, food and water were available ad libitum, except during behavioral testing. Animals were treated in accordance with the NIH Intramural Animal Care and Use Program guidelines. The experiments described followed the Emory University Division of Animal Resources’ Guide for the Care and Use of Laboratory Animals and were approved by the Emory IACUC committee.

**Minipump construction**

*Minipumps.* Osmotic minipumps (Alzet) dispensed 6 µl per day, and held a total volume of approximately 0.2 ml. The total useable life of the minipump was 4 weeks. In all experiments we performed behavioral tests 2 to 3 weeks following minipump implant.

*Disulfiram minipumps.* Disulfiram is insoluble in saline vehicle, but will dissolve in 100% DMSO (Sigma-Aldrich). Because 100% DMSO is incompatible with proper minipump function, a length of PE60 tubing was coiled and attached to the flow moderator of the minipump. As the inert solution inside the minipump (50% DMSO in saline) diffuses out of the pump and into the attached tubing, it forces the disulfiram/100% DMSO mixture (which is separated from the inert solution by an air bubble) out of the end of the tubing coil. To produce coils for drug delivery, PE tubing was fixed in a coil shape, heated in boiling water for 1 to 2 minutes, and then placed in ice cold water for 30 seconds. Disulfiram (50 mg/kg/day) was dissolved in 100% DMSO, and loaded into preconstructed coils, which were inserted into minipumps filled with 50% DMSO in saline. The concentration of disulfiram solution, which was adjusted based on mouse weights, was approximately 250 µg/ml. After filling, minipumps were submerged in saline (0.9% NaCl) solution and incubated at 37°C for 48 hours.
**Nepicastat minipumps.** Nepicastat (Roche) will dissolve in saline with 50% DMSO, and this vehicle is compatible with the osmotic minipump interior. Therefore, the coiled tubing required for disulfiram delivery is unnecessary for administration of this drug. Nepicastat (50 mg/kg/day) was first added to 100% DMSO and vortexed until thoroughly dissolved. Next, an equal volume of saline (0.9% NaCl) was added and this mixture was heated in a water bath at 42°C for 15 minutes. The concentration of nepicastat solution, which was adjusted based on mouse weights, was approximately 250 mg/ml. This solution was then injected directly into osmotic minipumps. Minipumps were incubated in saline solution at 37°C for 48 hours prior to implantation, as described above.

**Surgery**

Mice were anesthetized with inhalational isoflurane, and minipumps were implanted into the intraperitoneal cavity by opening an incision through the skin and muscle layers. After implantation of minipumps the muscle layer was sutured using vicryl suture material (Ethicon, Johnson and Johnson) and the skin incision was stapled with 9 mm stainless steel wound clips (Clay Adams, Becton Dickinson). Each animal was given a subcutaneous injection of analgesic (2.5 mg/kg banamine; Fort Dodge) immediately following surgery.

**Weight Monitoring**

As an index of general health in animals implanted with disulfiram minipumps, mouse weights were recorded over the first 3 weeks following surgery. Animals used to obtain this data were included in a separate behavioral experiment from those reported here.
**Locomotor Activity**

Animals were tested 2 or 3 weeks following disulfiram minipump implant. Mice were placed in activity chambers at approximately 11:00, and were allowed 4 hours to habituate to the chamber. Ambulations (consecutive beam breaks) were measured in transparent plexiglass cages (40 x 20 x 20 cm) placed into a rack with 7 infrared photobeams spaced 5 cm apart, with each end beam 5 cm from the cage wall (San Diego Instruments Inc., La Jolla, CA). Cocaine was then injected (10 mg/kg, i.p.), and locomotor activity was measured for 2 more hours. Data were analyzed by ANOVA followed by Bonferroni post-hoc tests. No differences were found between 2 or 3 week minipump duration, and data were combined. Mice in the disulfiram minipump experiment were treated with cocaine for five consecutive days. Locomotor activity in response to cocaine did not change between days, and only data from the first cocaine treatment day is shown. For the nepicastat experiment, all animals received 3 week minipump duration and were treated with cocaine on one day only.

**High Performance Liquid Chromatography (HPLC)**

Following the completion of locomotor activity experiments mice implanted with disulfiram or vehicle minipumps were sacrificed by CO₂ asphyxiation and frontal cortex tissue was removed and immediately frozen. Samples were stored at -80°C until preparation for HPLC analysis. A separate group of mice were implanted with disulfiram (50 mg/kg/day) minipumps and sacrificed 3 weeks later. These animals (N=4-6 per group) did not receive cocaine treatment.

Samples were prepared by adding 20 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 tissue was completely homogenized. The samples were centrifuged at 13,200 rpm for 30 minutes at 4°C, and the supernatant was removed from the tubes. The supernatant was then centrifuged again at 13,200 rpm for 30 minutes at 4°C, using a 22-micron filter column. The resulting fluid was the final product used for analysis. The analytical sample was injected using a Waters 717 autoinjector (Whatman, Milford, MA) and run through a C-14 μM max reverse phase column (Penomenix 150 x 4.6 mm; Torrace, CA) and electrochemical detector (potential maintained at 0.5 nA). Samples were delivered at a constant rate of 1 ml/min (retention times: NE, 5.08 min; DA, 12.12 min). The position and height of NE and DA peaks were compared with reference standard solutions (Sigma). Peak areas were quantified by Millenium\textsuperscript{32} Software (Waters). In some samples, catecholamine levels could not be effectively measured, and these samples were removed from data analysis.

**Elevated Plus Maze (EPM)**

Male and female \textit{Dbh} +/− mice (aged 2 to 5 months) were implanted with disulfiram, nepicastat, or vehicle minipumps and individually housed in a reverse light cycle (lights on at 19:00, lights off at 7:00). These mice were allowed a minimum of 2 weeks to habituate to the new lighting conditions, after moving from normal light cycle (lights on at 7:00, lights off at 19:00), prior to behavioral testing.

The EPM apparatus consisted of two open arms and two enclosed arms arranged in a plus orientation. The arms were elevated 30 inches above the floor, with each arm projecting 12 inches from the center. Because rodents naturally prefer dark, enclosed compartments, a greater willingness to explore the open, well-lit arms is believed to represent a decrease in the animal’s anxiety. This interpretation has been validated by the
In all experiments, cocaine (10 mg/kg, i.p. at 10 ml/kg, dissolved in saline) was injected 20 minutes prior to behavioral testing as described (Yang et al., 1992; Schank et al., 2008). To begin each test, mice were placed in the EPM facing one of the open arms and allowed to freely explore the apparatus for 5 minutes, during which time their behavior was videotaped. The measure used for analysis is the percentage of time spent exploring the open arms, which was calculated by dividing the time spent in the open arms by the combined time spent in open and closed arms. Because some drug treatments alter locomotor activity in the EPM, it is important to use this percentage measurement as the dependent variable for analysis (Pellow et al., 1985). Entry into an arm of the plus maze was defined as the animal placing all four paws in that particular compartment of the apparatus. All tests were run during the dark cycle, between 14:00 and 18:00. Data were analyzed by unpaired t-tests using Prism 4.0 for Macintosh.

After implantation of minipumps containing vehicle (100% DMSO, N=11) or disulfiram (50 mg/kg/day, N=16), mice were allowed to recover for 3 weeks, during which time they were periodically weighed to monitor general health. After this 3 week period, cocaine-induced anxiety (10 mg/kg, i.p. at 10 ml/kg, dissolved in saline) was assessed using the EPM, as described above. Mice implanted with nepicstatin (50 mg/kg/day, N=8) or vehicle (50% DMSO in saline, N=4) minipumps were treated in the same fashion with EPM testing being conducted 3 weeks following minipump implant.
Conditioned Place Preference (CPP)

Experiments were conducted in an isolated behavior room between 10:00 and 16:00. Mice were placed in the “neutral” middle compartment of a three compartment conditioned place preference chamber (San Diego Instruments, La Jolla, CA) and allowed to freely explore the other two compartments that were distinguishable by floor texture and wall pattern for 20 min, and time spent in each compartment was recorded (“pretest”). One day later, mice were subjected to “conditioning” sessions for three consecutive days. Mice were given an injection of saline (10 ml/kg, i.p.) and restricted to one compartment for 30 min in the morning, then given an injection of cocaine (20 mg/kg, i.p. at 10 ml/kg, dissolved in saline) and restricted to the other compartment for 30 min in the afternoon (approximately 4 hr after the morning conditioning session). Mice were designated to receive cocaine on either the “A” side or the “B” side using an unbiased design (i.e. for each group, equal numbers of mice received cocaine on each side, and equal numbers of mice received cocaine on the “preferred” side and “non-preferred” side based on pretest results). On the day following the last conditioning session, mice were placed in the neutral middle compartment in a drug-free state, allowed to freely explore all compartments for 20 min, and time spent in each compartment was recorded. Testing sessions were begun roughly halfway between the morning and afternoon conditioning sessions of the third day of conditioning. The preference score was calculated by subtracting the amount of time spent on the saline-paired side from the amount of time spent on the cocaine-paired side. Data were analyzed by paired t-tests (pretest preference vs. post-test preference for each group). CPP tests were performed 2
weeks after implantation of minipumps containing vehicle (50% DMSO in saline, N=4) or nepicastat (50 mg/kg/day, N=4).

A1.4 Results

Chronic disulfiram does not lead to significant weight change

Post-surgery weights were monitored daily for 3 weeks after disulfiram minipump implantation (Figure A1.1). Two way ANOVA revealed a main effect of day (F(19,123)=10.76, p<0.0001) and a day x treatment interaction (F(19,123)=1.831, p=0.0253); however, there was no significant main effect of treatment (vehicle vs. disulfiram, F(1,123)=2.819, p=0.1371). Bonferroni post-hoc tests indicated that there were no significant differences between the disulfiram group and vehicle group on any day.

Chronic disulfiram attenuates cocaine-induced locomotion

Two to three weeks following minipump implantation, mice were injected with cocaine and locomotor activity was assessed. Locomotor activity was quantified as total number of ambulations, and was recorded in 30 minute bins. Repeated measures two way ANOVA revealed a main effect of time (F(12,144)=11.68, p<0.0001) on locomotor activity (Figure A1.2). Bonferroni post-hoc analysis, which compared each 30 minute interval to the last interval prior to cocaine injection, revealed a significant difference between the last interval prior to cocaine injection and the first (vehicle: t=5.800, p<0.001; disulfiram: t=5.601, p<0.001) and second (vehicle: t=2.683, p<0.05; disulfiram: t=2.490, p<0.05) intervals after initial introduction to the activity chambers for both
treatment groups. Novel environment-induced locomotion is a behavior that is commonly expressed in rodents, and our results indicate that chronic disulfiram had no effect on this behavior. Post-hoc analysis also revealed a significant difference between the last interval prior to cocaine injection and both the first and second intervals following cocaine administration for the vehicle treated group only (first interval post-cocaine: $t=3.554, p<0.01$; second interval post-cocaine: $t=3.376, p<0.01$). This indicates that cocaine increases locomotor activity in control mice, but not in mice treated with chronic disulfiram.

**Chronic nepicastat increases cocaine-induced locomotion**

Three weeks following implantation of minipumps that delivered vehicle (50% DMSO in saline, $N=5$) or nepicastat (50 mg/kg/day in 50% DMSO, $N=6$), mice were injected with cocaine (10 mg/kg) and locomotor activity was assessed, as above. Repeated measures two way ANOVA revealed a main effect of time ($F(12,120)=18.62$, $p<0.0001$) and a time x treatment interaction ($F(12,120)=2.633$, $p=0.0037$; Figure A1.3). Bonferroni post-hoc tests were used to compare each interval to the last interval prior to cocaine injection. This analysis indicated a significant difference between the last interval prior to cocaine and the first (vehicle: $t=5.109$, $p<0.001$; nepicastat: $t=5.793$, $p<0.001$) and second (vehicle: $t=3.340$, $p<0.01$; nepicastat: $t=2.922$, $p<0.01$) intervals following introduction to the activity chambers for both treatment groups. This analysis also revealed an increase in locomotor activity during the first interval following cocaine injection for both groups (vehicle: $t=2.337$, $p<0.05$; nepicastat: $t=7.781$, $p<0.001$). However, in the first interval following cocaine injection, nepicastat (50 mg/kg/day)
treated animals exhibited greater locomotor activity when compared to vehicle treated animals ($t=4.586, p<0.05$). We also performed this experiment in a separate group of mice implanted with minipumps containing a 10 mg/kg/day dose of nepicastat, and we obtained identical results to those outlined above for 50 mg/kg/day nepicastat (data not shown).

**Chronic disulfiram and nepicastat do not alter cocaine-induced anxiety**

Cocaine-induced (10 mg/kg) anxiety was assessed using the EPM 3 weeks after minipump implants. When animals receiving chronic disulfiram (50 mg/kg/day) were compared to vehicle treated animals, unpaired t tests indicated no significant difference between these two groups ($t(25)=0.1207, p=0.9049$, Figure A1.4). Also, no difference was detected between groups treated with vehicle or nepicastat (50 mg/kg/day; $t(10)=0.9914, p=0.3449$; Figure A1.5).

**The effect of chronic nepicastat on cocaine-induced place preference**

Cocaine (20 mg/kg) CPP experiments were performed 2 weeks after implantation of vehicle or nepicastat (50 mg/kg/day) minipumps. Pretest and posttest preference scores were compared using paired t tests for each treatment group. This analysis revealed a significant difference between pretest and posttest preference for the vehicle treated group ($t(3)=3.961, p=0.0287$) but not the nepicastat treated group ($t(3)=0.9071, p=0.4035$; Figure A1.6).
**Chronic disulfiram does not alter catecholamine levels**

Catecholamine levels were assessed in mice following chronic disulfiram treatment. In mice that were implanted with minipumps and then tested for cocaine-induced locomotion 2 or 3 weeks later, disulfiram had no effect on NE to DA ratio when compared to vehicle treated mice \( t(5)=0.7991, p=0.4605 \); Figure A1.7A). To determine if cocaine exposure had an effect on this measurement, catecholamine levels were measured in mice that were implanted with vehicle or disulfiram (50 mg/kg/day) minipumps, but received no cocaine injections. In these mice chronic disulfiram had no effect on NE to DA ratio when compared to vehicle treatment \( t(8)=0.2845; p=0.7832 \); Figure A1.7B).

**A1.5 Discussion**

In the set of experiments outlined above, we assessed the influence of chronic delivery of DBH inhibiting agents on a variety of cocaine-induced behaviors. We used both the non-specific inhibitor disulfiram as well as the highly specific and potent inhibitor nepicastat. A major goal of these experiments was to attempt to pharmacologically recapitulate behavioral phenotypes observed in mice with a deletion of the *Dbh* gene. We found that while these drugs had no impact on cocaine-induced anxiety, they had very interesting effects on cocaine-induced locomotion.

Perhaps the most interesting result from this set of experiments was our finding that disulfiram and nepicastat modulate the expression of cocaine-induced locomotion in opposite directions. *Dbh* +/- mice exhibit an increased sensitivity to the locomotor activating effects of cocaine, and we hypothesized that mice treated with chronic
inhibitors of this enzyme would express a similar behavioral phenotype. Nepicastat treated animals, like Dbh -/- mice, show an enhanced behavioral response to cocaine; however, disulfiram treatment induced an unexpected decrease in locomotor activity. This contradictory result could be explained in part by the fact that nepicastat is a more potent inhibitor of DBH, and the dose of disulfiram administered may not have been high enough to generate a significant inhibition of this enzyme. This interpretation is bolstered by our finding that chronic disulfiram, at the dose that we administered, did not alter catecholamine levels in the prefrontal cortex. By this reasoning, some other target that disulfiram inhibits more potently could be mediating its effect on cocaine-induced locomotion at this dose. Another interesting observation was that novel environment induced-locomotion was unaffected by chronic DBH inhibition with either drug. Dbh -/- mice show a decreased amount of locomotor activity when first introduced to the activity chambers, but this behavior was not affected in our minipump mice. It is possible that a more complete inhibition of DBH is required to achieve this effect.

In our experiments, we measured catecholamine content in brain tissue from mice implanted with minipumps delivering disulfiram and found that there were no significant changes in catecholamine levels. This could be a technical issue concerning HPLC analysis, or could be a reliable assessment of the fact that disulfiram at the dose provided was not high enough to potently decrease DBH function (see discussion above). This lack of effect on catecholamine levels was observed following chronic disulfiram administration in mice that were treated with cocaine, as well as a separate group of animals that were cocaine-naïve. There was a slight difference in NE to DA ratio between cocaine naïve and cocaine exposed mice, but in both experiments, chronic
disulfiram did not affect this measure. It would be of interest to explore the effects of an increased dose of chronic disulfiram on catecholamine levels and cocaine-induced behavior; however, there are significant obstacles to the chronic administration of higher doses of this drug due to solubility issues. Also, it is of great importance to reliably assess the effect of nepicastat on catecholamine levels.

The fact that disulfiram is a non-specific inhibitor of DBH may explain differences observed between its effects and that of nepicastat, which is highly specific. The primary metabolite of disulfiram is a copper chelator (Johansson 1989), and will thereby inhibit many enzymes that use copper as a cofactor (for review see Petersen, 1992; Haley, 1989). Nepicastat, however, binds directly to DBH enzyme to inhibit its activity. While nepicastat lacks appreciable activity at a wide range of proteins, receptors, and enzymes (Stanley et al., 1995), disulfiram can inhibit a variety of endogenous targets, some of which are involved in catecholamine signaling and cocaine metabolism (see Section 5.4). For example, disulfiram inhibits aldehyde dehydrogenase, and its inhibition of this enzyme underlies its effect on alcohol consumption. In addition to impacting alcohol metabolism, aldehyde dehydrogenase is also involved in the breakdown of DA (for review see Eneanya et al., 1981), which may affect cocaine-related behaviors. Further, disulfiram itself can cause DA release from synaptosomes (Vaccari et al., 1996), and can impair the metabolism of cocaine (McCance-Katz, 1998). Whether disulfiram’s activity at these targets underlies its effect on cocaine-induced locomotion remains unclear.

In addition to cocaine-induced locomotion, we also explored the effect of chronic administration of DBH inhibitors on cocaine-induced reward and anxiety. Dbh −/− mice
exhibit an increased sensitivity to both the rewarding and aversive effects of cocaine (Schank et al., 2006), but a paradoxical insensitivity to the anxiogenic properties of this drug (Schank et al., 2008). We failed to replicate this phenotype in our mice treated with either disulfiram or nepicastat. Chronic administration of these inhibitors had no statistically significant effect on cocaine-induced anxiety as measured by the EPM. However, the effects of nepicastat in this test appeared to show a trend in the hypothesized direction, with animals treated with chronic nepicastat showing slightly lower levels of cocaine-induced anxiety, as is observed in Dbh -/- mice (Schank et al., 2008). Therefore, it would be wise to repeat this particular experiment.

To assess the effects of chronic DBH inhibition on cocaine reward, we implanted mice with nepicastat minipumps and assessed cocaine-induced place preference two weeks later. Interestingly, we found that while vehicle treated animals expressed a strong place preference to cocaine, the place preference exhibited by nepicastat treated animals did not reach statistical significance. This suggests that, like Dbh -/-, nepicastat may prevent the expression of cocaine-induced place preference at a high dose of cocaine. A caveat to this interpretation is that the experiment in question included a small number of animals, and drawing definitive conclusions from these results would require inclusion of a larger number of subjects. Further, preference scores for nepicastat treated animals appeared to show a trend toward cocaine-induced place preference, and the variability in this group was rather large. Regardless, this interesting effect warrants further attention.

Taken together, these results suggest that chronic DBH inhibition can alter certain drug-induced behaviors in rodents. However, there appears to be some distinction between the effects of specific versus non-specific inhibition of this enzyme. The most
significant unanswered question at this point is the effect of chronic administration of these drugs on catecholamine levels. A more potent inhibition of DBH by nepicastat may explain the differences observed in the effects of these two inhibitors, and these concerns seem to be validated by our failure to detect an effect of chronic disulfiram on catecholamine levels in mouse brain tissue. Effectively modeling the phenotypes observed in \textit{Dbh} \text{-/-} mice by delivering chronic DBH inhibitors is of great interest given that responses to certain behavioral effects of cocaine are altered in people with low DBH activity, and that disulfiram can attenuate cocaine use in cocaine dependent people. Based on the data presented here, and the technical obstacles that exist, chronic DBH inhibition with nepicastat might present the best promise for use in our mice. Due to its potent inhibition of DBH, nepicastat, like disulfiram, may show clinical efficacy in decreasing cocaine intake. To model this phenomenon in rodents would provide an avenue for exploring the mechanism of this effect.

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Figure A1.1

![Graph showing percent post-op weight over post-op days for VEH and DIS groups.]
Figure A1.1 Chronic disulfiram does not lead to significant weight change. Post-surgery weights were monitored daily for 3 weeks after disulfiram minipump implantation. Weight is expressed as percent of post-operation weight. Two way ANOVA revealed an effect of day; however, there were no differences between the disulfiram group and vehicle group.
Figure A1.2
Figure A1.2 Chronic disulfiram attenuates cocaine-induced locomotion. When compared to the last interval prior to cocaine injection, both treatment groups showed a significantly greater level of locomotion following introduction to the activity chambers, however, only mice implanted with vehicle minipumps showed significantly increased locomotion following cocaine injection. *p<0.05 for both groups, **p<0.001 for both groups, #p<0.01 for vehicle group only.
Figure A1.3

Ambulations vs. Time (min)

- **Vehicle**
- **Nep 50**

Key:
- *** (P < 0.001)
- ** (P < 0.01)
- # (P < 0.05)
- COC
- @
- ### (P < 0.001)
**Figure A1.3 Chronic nepicastat enhances cocaine-induced locomotion.** When compared to the last interval prior to cocaine (10 mg/kg) injection, both treatment groups showed increased locomotor activity during the first and second intervals after introduction to the activity chambers, and during the first interval following cocaine injection. However, in the first interval following cocaine injection, nepicastat (50 mg/kg/day) treated animals exhibited greater locomotor activity when compared to vehicle treated animals. **p<0.01 compared to last interval before cocaine for both treatment groups, ***p<0.001 compared to last interval before cocaine for both treatment groups, #p<0.05 compared to last interval before cocaine for vehicle group only, ###p<0.001 compared to last interval before cocaine for nepicastat group only, @p<0.05 compared to vehicle treated group for that interval
Figure A1.4

![Bar graph showing Open Arm Time (%) for Disulfiram and Vehicle groups.](image)
Figure A1.4 Chronic disulfiram does not affect cocaine-induced anxiety. Three weeks after mice were implanted with osmotic minipumps that delivered vehicle (100% DMSO) or disulfiram (50 mg/kg/day in 100% DMSO), cocaine-induced (10 mg/kg) anxiety was assessed using the elevated plus maze. This analysis revealed no significant differences between vehicle and disulfiram treated groups.
Figure A1.5

![Graph showing the comparison between Vehicle and Nepicastat in terms of Open Arm Time (%). The graph indicates a significant increase in Open Arm Time for Nepicastat compared to Vehicle.]
Figure A1.5 Chronic nepicastat does not affect cocaine-induced anxiety. Three weeks after mice were implanted with osmotic minipumps that delivered vehicle (50% DMSO in saline) or nepicastat (50 mg/kg/day in 50% DMSO), cocaine-induced (10 mg/kg) anxiety was assessed using the elevated plus maze. This analysis revealed no significant differences between vehicle and nepicstat treated groups.
Figure A1.6

![Graph showing preference (sec) for Vehicle and Nepicastat during Pretest and Posttest.](image)
Figure A1.6 The effects of chronic nepicastat on cocaine-induced place preference.

Two weeks after mice were implanted with minipumps that delivered vehicle (50% DMSO in saline) or nepicastat (50 mg/kg/day in 50% DMSO), cocaine (20 mg/kg) reward was assessed using the conditioned place preference paradigm. In this experiment, mice that were treated with vehicle showed a strong place preference for the cocaine-paried side, but mice treated with nepicastat did not. *p<0.05 compared to pretest preference.
Figure A1.7

A

B

Vehicle
Disulfiram

Vehicle
Disulfiram

NE/DA Ratio

NE/DA Ratio
Figure A1.7 Chronic disulfiram does not alter catecholamine levels. Chronic disulfiram treatment does not effect NE to DA ratio in cocaine treated (A), or untreated (B) mice.
A1.6 References


APPENDIX 2:

REDUCED ANTICONVULSANT EFFICACY OF VALPROIC ACID IN
DOPAMINE β-HYDROXYLASE KNOCKOUT MICE

Adapted from:
A2.1 Abstract

Valproic acid (VPA) is a widely used treatment for both epilepsy and bipolar disorder, although its therapeutic mechanism of action is not fully understood. Because norepinephrine (NE) is implicated in seizure susceptibility and affective disorders, and given previous findings indicating that VPA can act on the NE system, it is possible that NE may mediate some of the therapeutic actions of VPA. To test this hypothesis, we measured flurothyl-induced seizure susceptibility and severity parameters after both acute and chronic VPA treatment in dopamine β-hydroxylase knockout (Dbh −/−) mice that lack NE. We found that the protective effects of acute VPA on seizure susceptibility, as measured by latency to first myoclonic jerk, were attenuated in Dbh −/− mice. Further, while acute VPA reduced the number of control mice that progressed to tonic extension, Dbh −/− mice completely lacked the benefit of VPA on seizure severity. The carryover anticonvulsant effects following cessation of chronic VPA treatment were similar in both genotypes. Therefore, we conclude that NE is necessary for some of the anticonvulsant effects of VPA, especially the effect of acute VPA on seizure severity.
A2.2 Introduction

Over the last several decades, considerable evidence has accumulated indicating a role of norepinephrine (NE) in the development, expression, and susceptibility to seizures (reviewed by Weinshenker and Szot, 2002). Generally, it has been observed that an activation of the NE system has anticonvulsant effects while an impairment of the NE system has proconvulsant consequences. Multiple animal models exist which lend support to the notion that endogenous NE is anticonvulsant, including the genetically epilepsy-prone rat (Browning et al., 1989; Dailey et al., 1991; Yan et al., 1993; Jobe et al., 1994), the E1 mouse line (Tsuda et al., 1990; Tsuda et al., 1993), and NE deficient mice (Szot et al., 1999).

Of particular interest are findings indicating that an intact NE system is necessary for the action of multiple anticonvulsant therapies. For example, lesions of the locus coeruleus (LC), the major NE nucleus in the brain, attenuate the effects of multiple anticonvulsant drugs, including carbemazepine, phenytoin, and phenobarbital (Quattrone and Samanin, 1977; Quattrone et al., 1978; Crunelli et al., 1981; Waller and Buterbaugh, 1985). An intact noradrenergic system is also required for the efficacy of less traditional antiepileptic therapies such as vagal nerve stimulation and the ketogenic diet (Krahl et al., 1998; Szot et al., 2001).

Valproic Acid (VPA) is a first-line treatment for epilepsy and bipolar disorder, although its therapeutic mechanism of action is not fully understood. Considerable evidence suggests that VPA can act through the GABAergic system, NMDA receptors, and/or sodium channels (for review see Löscher, 1999; Rogawski and Löscher, 2004). However, VPA can also exert neurochemical effects on the NE system. For example,
Baf and colleagues (1994) observed an increase in NE tissue levels in the hippocampus and brainstem following chronic VPA treatment. The expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in NE biosynthesis, is also increased in the LC of rats by acute and chronic VPA treatment (Sands et al., 2000). Furthermore, tricyclic antidepressants, which block the reuptake of NE and increase extracellular NE levels, have been shown to enhance the anticonvulsant effects of VPA (Kleinrok et al., 1991).

Given the numerous clinical effects of VPA, its suggested influence on the NE system, and the purported anticonvulsant effects of NE, it is of interest to determine if disruption of NE signaling might alter the anticonvulsant properties of VPA. VPA acts on multiple targets involved in neurotransmission, and NE may act in concert with these targets to modulate the anticonvulsant efficacy of VPA.

The model used in the current study is a mouse with a genetic knockout of the dopamine β-hydroxylase (Dbh) gene (Dbh−/− mice). DBH is the enzyme that converts dopamine (DA) to NE; thus Dbh−/− mice completely lack NE (Thomas et al., 1995). This model is especially relevant in light of previous findings showing that serum DBH activity is lower in patients with some types of epilepsy (Miras-Portugal et al., 1975). Dbh−/− mice show increased susceptibility to numerous seizure-inducing stimuli including flurothyl (Szot et al., 1999), the stimulus used for seizure induction in the present study. Interestingly, various subtype specific NE receptor agonists are capable of attenuating seizure hypersensitivity in these mice (Weinshenker et al., 2001; Weinshenker and Szot, 2002; Szot et al., 2004).

In the current study we evaluate the effectiveness of VPA in NE-deficient Dbh−/− mice. To date, few studies have examined the interaction of VPA and NE in the
modulation of seizure sensitivity. Given the observations outlined above, we hypothesized that the anticonvulsant effects of VPA would be reduced in Dbh-/- mice. Because chronic VPA enhances TH and NE in the brain, we also examined the role of NE in the “carryover effect” observed after chronic administration of VPA (Lockhard and Levy, 1976; Löscher and Nau, 1982). The carryover effect is defined as the persistence of anticonvulsant activity after the disappearance of VPA from the bloodstream following cessation of chronic VPA treatment. Lastly, we evaluated whether enhancement of NE transmission by the NE reuptake inhibitor reboxetine could enhance the effectiveness of low dose, acute VPA treatment.

A2.3 Materials and Methods

Animals

Dbh-/- mice, maintained on a mixed 129/SvEv and C57BL/6J background, were developed and generated as described (Thomas et al., 1995; Thomas et al., 1998). Dbh +/- mice have normal catecholamine levels and are indistinguishable from wild type littermates for all previously tested phenotypes, including flurothyl seizure susceptibility (Thomas et al., 1995; Thomas et al., 1998; Thomas and Palmiter, 1997, Szot et al., 1999). Therefore, Dbh +/- littermates were used as controls. Adult male and female mice (3 to 7 months old at time of seizure testing) were used in these experiments. No sex differences in seizure susceptibility or VPA efficacy were observed, and results were combined. Mice used in experimentation weighed roughly 20 to 40 g. Throughout the course of the experiment the colony room was maintained at 22°C with lights on from 7:00 to 19:00. Food and water were available ad libitum, and animals were maintained according to guidelines outlined in the NIH Guide for Care and Use of Laboratory Animals. All
experiments were approved by the Emory University Institutional Animal Care and Use Committee.

**Seizure susceptibility**

Flurothyl seizure thresholds were determined for *Dbh* +/- and *Dbh* -/- mice as described previously (Szot et al., 1999). Mice were placed in an air-tight, clear plexiglass chamber, and the volatile convulsant flurothyl (2,2,2-trifluoroethylether; Sigma-Aldrich, St. Louis MO) was infused via syringe pump at a rate of 20 µl/min onto filter paper from which it vaporized. The latency in seconds to the first myoclonic jerk (MJ) and to clonic/tonic seizure (CT) were measured. MJ, the first behavioral sign of seizure, is evidenced as a brief, large-scale muscle twitch, and is commonly thought of as an index of seizure induction. CT, which appears later as repetitive, full-body convulsions with loss of posture, can be thought of as an index of seizure generalization. Also recorded was the number of mice progressing to tonic extension of the hindlimbs. Mice that appeared sedated after VPA administration were not included in statistical analysis, as sedation itself is anticonvulsant and could mask seizure-inhibiting effects of VPA treatment. Sedation was described as severe depression of locomotor activity, ataxia, and loss of immediate righting reflex. It is of note that the only mice lacking seizure activity in this study were those that exhibited behavioral sedation. Each mouse was tested individually, removed immediately from the chamber after completion of seizure behavior, and received only one exposure to flurothyl. During seizure testing, mice from different groups were tested in an alternating fashion to prevent any confounding effect of time of testing and circadian differences in seizure susceptibility.
In the experiment using acute drug treatment, VPA (100, 200, and 300 mg/kg) or saline was injected i.p. in a volume of 4 ml/kg, 30 minutes prior to seizure induction by flurothyl (N=7-8 per group, except for \(D_{bh}\)-/- at VPA dose of 300, where 8 mice were tested, but 4 appeared sedated and were eliminated from the study, therefore N=4). For chronic studies, VPA (200 mg/kg) or saline was injected i.p. twice daily in a volume of 4 ml/kg for 4 or 6 weeks (N=10-16 per group). The last VPA injection was given at least 14 hours before seizure testing was carried out. In experiments where VPA was given in conjunction with reboxetine (NE reuptake inhibitor), reboxetine (20 mg/kg) was mixed with VPA (100 mg/kg) and injected i.p. in a volume of 4 ml/kg, 30 minutes prior to seizure induction (N=7-8 per group).

**Serum VPA levels**

Serum VPA was measured to determine how levels obtained in mice in these experiments compared to levels found in humans during clinical VPA treatment. Valproic Acid Reagent was used to measure the VPA concentration by a particle enhanced turbidimetric inhibition immunoassay method on the Beckman Synchron LX-20 system. Binding of a specific antibody to particle bound VPA causes an increase in turbidity in the sample. Non-particle bound VPA competes for binding with the antibody. The Synchron LX system automatically adds one part of sample to 104 parts reagent and analyzes aggregate formation by measuring the change in absorbance at 600 nanometers.
Drugs

The anticonvulsant VPA (Sigma-Aldrich, St. Louis MO) and NE reuptake inhibitor reboxetine were dissolved in sterile 0.9% saline. Reboxetine was provided graciously by Pfizer, Sandwich Kent, England.

Statistics

Measures of latency to MJ and CT are presented as mean ± standard error of the mean. These values are compared in the VPA acute and chronic studies by two way ANOVA with the factors being genotype (Dbh +/− vs. Dbh −/−) and dose of drug (saline, 100, 200, 300 mg/kg for acute; saline vs. 200 mg/kg for chronic), and post-hoc Bonferroni tests were performed. Comparisons of latencies to MJ and CT for Dbh +/− mice receiving VPA (100 mg/kg) alone or VPA (100 mg/kg) with reboxetine (20 mg/kg) were performed using independent samples t-tests. For numbers of mice progressing to tonic extension, treatment groups were compared to controls using Fisher’s Exact Test. Fisher’s Exact Test is similar to the $\chi^2$ test, and is primarily used when observed values are very low, as in the present experiment.

A2.4 Results

Effects of acute VPA administration of flurothyl-induced seizures in Dbh +/− and Dbh −/− mice

Latency to MJ, latency to CT, and number of mice reaching tonic extension were measured following seizure induction by flurothyl after injection of saline or VPA (100, 200, or 300 mg/kg) in Dbh +/− and Dbh −/− mice and are shown in figure A2.1. Statistical analysis of latency to MJ using two way ANOVA revealed significant main effects for
dose (F(3,56)=62.27, p<0.0001) and genotype (F(1,56)=135.4, p<0.0001), as well as a
dose x genotype interaction (F(3,56)=5.057, p=0.0036; figure. A2.1A). For saline treated
groups, the Dbh +/- mice had a significantly reduced latency to MJ (p<0.05; figure
A2.1A), as described previously (Szot et al., 1999). Although VPA dose dependently
increased latency to MJ in both genotypes, the latency to MJ was significantly shorter in
the Dbh +/- mice compared to the Dbh +/- mice for all doses of VPA (p<0.05; figure
A2.1A). In addition, the anticonvulsant efficacy of VPA was reduced in Dbh +/- mice.
For a dose of 100 mg/kg, which lacked anticonvulsant activity, no considerable genotype
difference from saline for latency to MJ was observed (Dbh +/- 3.375 sec, Dbh +/- 11.13
sec). However, the magnitude of change from saline is attenuated in Dbh +/- mice for
doses of 200 mg/kg (Dbh +/- 129.3 sec, Dbh +/- 78.13 sec) and 300 mg/kg (Dbh +/-
263.1 sec, Dbh +/- 150.9 sec).

Statistical analysis of latency to CT using two way ANOVA revealed a significant
main effect for dose (F(3,52)=58.28, p<0.0001; figure A2.1B). Bonferroni post-hoc tests
indicated that for saline treated mice, the latency to CT was significantly shorter for the
Dbh +/- mice than for the Dbh +/- mice (p<0.05; figure A2.1B) as previously described
(Szot et al., 1999). For both genotypes, the latency to CT significantly increased with
doses of 200 and 300 mg/kg VPA (p<0.001; figure A2.1B), with Dbh +/- mice reaching a
similar latency as Dbh +/- mice for all doses of VPA.

The number of mice progressing to tonic extension of the hindlimbs after CT was
recorded as a measure of maximal seizure severity and is shown in Table A2.1. VPA
dose-dependently suppressed progression to tonic extension in Dbh +/- mice, but had no
effect in Dbh +/- mice. After comparing all VPA doses to saline control within genotype
using Fisher’s Exact Test, the number of Dbh +/- mice reaching tonic extension was significantly different at a VPA dose of 300 mg/kg (p=0.01), and a protective trend was seen at 200 mg/kg (p=0.12). When combining the two doses of VPA (200 and 300 mg/kg) that had an anticonvulsant effect on latencies to MJ and CT, the difference from saline controls for the Dbh +/- mice was highly significant (p=0.0078). However, there were no significant differences between VPA groups and saline for the Dbh -/- mice at any dose of VPA.

Therapeutic serum levels of VPA in humans is normally in the range of 40-100 µg/ml (Tietz et al., 1994). With acute VPA treatment we observed serum levels ranging from 151-247 µg/ml 30 minutes after injection of 200 mg/kg VPA, indicating that the mice in our experiments have VPA levels close to human therapeutic range at the time of seizure induction. Because no differences in VPA serum levels between genotypes were observed, the reduction of VPA efficacy in Dbh -/- mice cannot be due to differences in VPA metabolism.

**The effects of reboxetine/VPA coadministration on flurothyl-induced seizures**

To determine whether enhancement of NE transmission could increase the efficacy of VPA in Dbh +/- mice, we coadministered VPA (100 mg/kg) with the NE reuptake inhibitor reboxetine (20 mg/kg). Statistical analysis revealed a trend toward increased VPA efficacy for latency to MJ, but it was not significant (VPA/saline: 408±14 sec, N=8; VPA/reboxetine: 440±10 sec, N=7; p=0.09). No significant differences were found between the two groups for latency to CT (VPA/saline: 526±18 sec, N=8; VPA/reboxetine: 502±13 sec. N=7; p=0.30).
The effects of chronic VPA administration on flurothyl-induced seizures in \( Dbh^{+/-} \) and \( Dbh^{-/-} \) mice

To test whether NE is important for the carryover anticonvulsant effect of VPA, we administered VPA or saline twice daily for 4 or 6 weeks, and results are shown in figure A2.2. The last injection of VPA was given on the evening before the day of seizure testing, so that VPA was undetectable in serum at the time of flurothyl administration (data not shown). There were no differences between the measurements for 4 or 6 week treatment, so the results for these groups were combined.

Statistical analysis of latency to MJ revealed significant main effects for both genotype (\( F(1,46)=135.9, p<0.0001 \)) and drug treatment (\( F(1,46)=23.57, p<0.0001 \)), but no significant interaction (figure A2.2A). A carryover effect was evident; VPA treatment induced a small but significant increase in latency to MJ that was similar for both genotypes (\( p<0.05 \); figure A2.2A). Latency to MJ for the \( Dbh^{-/-} \) mice was significantly shorter than \( Dbh^{+/-} \) mice for both saline and VPA treated groups (\( p<0.05 \); figure A2.2A).

For CT, statistical analysis revealed significant main effects for both genotype (\( F(1,46)=7.425, p=0.0091 \)) and drug treatment (\( F(1,46)=20.65, p<0.0001 \); figure A2.2B), but no significant interaction. Chronic VPA treatment increased the latency to CT in both genotypes, but the effect only reached significance for the \( Dbh^{-/-} \) mice (\( p<0.05 \); figure A2.2B). Bonferroni post-hoc tests showed that \( Dbh^{-/-} \) mice had a shorter latency to CT than \( Dbh^{+/-} \) mice when treated chronically with saline (\( p<0.05 \); figure A2.2B).

Almost every mouse in the carryover effect experiments reached tonic extension, and there were no differences between genotypes (data not shown). Also, chronic VPA
administration did not alter the anticonvulsant effect of a subsequent acute injection of VPA in Dbh +/- or Dbh +/- mice (data not shown).

A2.5 Discussion

In the current experiments, we utilized a mouse that lacks NE to investigate the role of NE in the anticonvulsant effects of VPA. We observed flurothyl-induced seizure behavior following both acute and chronic VPA treatment. Given the purported anticonvulsant activity of NE itself, and data suggesting that VPA can act on the NE system, we hypothesized that the Dbh +/- mice would be less responsive to the anticonvulsant effects of VPA.

It appears from our experimentation that NE may be necessary for some aspects of the anticonvulsant activity of acute VPA administration. First, we examined the effect of acute VPA administration on seizure susceptibility. As previously reported (Szot et al., 1999), we found that Dbh +/- mice had a shorter latency to MJ. A novel finding from our experiment is that VPA treatment was more effective at increasing the latency to MJ in Dbh +/- mice than in Dbh +/- mice. This was evidenced by a shorter latency to MJ in the Dbh +/- mice for every dose of VPA used, and an attenuated change from saline controls in VPA treated Dbh +/- mice compared to Dbh +/- mice. In contrast, both Dbh +/- and Dbh +/- mice benefited equally from VPA treatment in terms of latency to CT.

NE also appears to be important for the acute VPA effects on maximal seizure severity. VPA significantly decreased the number of Dbh +/- mice progressing to tonic extension, but had no protective effect in Dbh +/- mice. Taken together, these results demonstrate that Dbh +/- mice have increased seizure susceptibility and are resistant to
some of the anticonvulsant effects of VPA. NE appears to be most important for the effects of VPA on seizure induction and on maximal seizure severity, but may play a lesser role in VPA effects on seizure generalization.

The persistence of an anticonvulsant effect after cessation of chronic VPA treatment, termed the carryover effect, was first observed and described by Löscher and others (Lockhard and Levy, 1976; Löscher and Nau, 1982), and may be a result of remaining VPA metabolites. Because chronic VPA treatment increases TH expression in the LC as well as NE levels in the brainstem and hippocampus, we tested the hypothesis that these changes to the NE system contribute to the carryover effects of chronic VPA. However, we found that $Dbh^{-/-}$ mice still benefited from the anticonvulsant carryover effects of VPA. After chronic VPA treatment, $Dbh^{-/-}$ and $Dbh^{+/-}$ mice showed a similar level of improvement on latency to MJ compared to saline controls, but the $Dbh^{-/-}$ mice did not reach the same latency as the $Dbh^{+/-}$ mice. This shortened latency to MJ seen in the $Dbh^{-/-}$ mice is likely a result of differences in baseline seizure susceptibility between the two genotypes. Unexpectedly, the carryover effect was slightly enhanced in the $Dbh^{-/-}$ mice for latency to CT. The basis for this finding is unclear, but may be due to a greater effect of VPA in an inherently seizure susceptible animal.

Kleinrok and colleagues (1991) reported that NE reuptake inhibitors could potentiate the anticonvulsant effect of VPA against maximal electroshock seizures. To determine whether increasing extracellular NE could enhance the anticonvulsant effect of VPA in our model, we coadministered the NE reuptake inhibitor reboxetine and a subthreshold dose of VPA (100 mg/kg). We found little effect of reboxetine on the efficacy of low dose, acute VPA treatment. A trend was observed for latency to MJ,
however, this difference failed to reach significance. This suggests that increasing extracellular NE by use of a reuptake inhibitor may not be capable of increasing the effectiveness of a dose of VPA that usually has no anticonvulsant efficacy against flurothyl-induced seizures. It is possible that under seizure-inducing conditions, the noradrenergic system is already maximally activated, so that a further increase in extracellular NE has no additional effect. In support of this interpretation are findings indicating that $Dbh^{+/-}$ mice experience no anticonvulsant effects when administered drugs that increase NE signaling (Weinshenker et al., 2001). Other factors could have explained the inconsistency between the results of our experiments and those of Kleinrok and colleagues, including differences in strain background, seizure inducing procedure, and dose of VPA.

As with any knockout model, there are concerns relating to developmental abnormalities that may influence later neurochemistry and pharmacology. In the $Dbh^{-/-}$ mouse it is doubtful that such effects confound the results of our experiments. First, NE is required for fetal development, so these mice must receive adrenergic agonists and DOPS (a precursor that can be converted to NE by aromatic amino acid decarboxylase, thus bypassing DBH) during gestation through the mother’s drinking water. Therefore, they lack NE only postnatally. A recent study by Jin and colleagues (2004) shows that $Dbh^{-/-}$ mice display normal development of the adrenergic system in the CNS and cerebellum when compared to control mice. Further, acute DOPS is capable of restoring normal seizure sensitivity to adult $Dbh^{-/-}$ mice (Szot et al., 1999). Finally, adrenergic agonists eliminate differences in seizure sensitivity between adult $Dbh^{-/-}$ and $Dbh^{+/-}$ mice, and adrenergic antagonists induce a seizure sensitive state in adult $Dbh^{+/-}$ mice.
that resembles that of $Dbh^{-/-}$ (Weinshenker et al., 2001). Taken together, these findings suggest that the NE system in the $Dbh^{-/-}$ mouse develops normally, and can be activated by acute NE replacement in the adult mouse. Therefore, any differences seen in seizure susceptibility are most likely a result of an acute lack of NE and not due to developmental abnormalities.

Presently, it is unclear exactly how the NE system is involved in the anticonvulsant action of VPA. It is possible that an intact NE system is required for a general inhibition of seizure activity, regardless of the anticonvulsant treatment. Therefore, VPA would not necessarily be acting on the NE system directly, but a lack of NE would preclude some anticonvulsant effects of VPA treatment. Along these lines, $Dbh^{-/-}$ mice are also unresponsive to the anticonvulsant effects of the ketogenic diet (Szot et al., 2001).

Alternatively, VPA increases tissue NE levels (Baf et al., 1994), and could do so directly through its ability to induce expression of TH (Sands et al., 2000), the rate limiting enzyme in NE synthesis. VPA has been shown to increase gene expression mediated by the activator protein-1 family of transcription factors (Chen et al., 1999), and TH expression can be induced by the stimulation of activator protein-1 binding sites (Chae et al., 1996). Therefore, the stimulation of this site represents a potential mechanism by which VPA could increase NE levels.

In summary, these findings indicate that a functional NE system may be necessary for the full anticonvulsant effect of VPA on seizure susceptibility and seizure severity in mice. Other anticonvulsant therapies also depend on NE activity, including vagal nerve stimulation (Krahl et al., 1998), the ketogenic diet (Szot et al., 2001), and drug treatment
with phenytoin, carbamazepine, or phenobarbital (Quattrone and Samanin, 1977; Quattrone et al., 1978; Crunelli et al., 1981; Waller and Buterbaugh, 1985). It appears as though several systems are involved in the anticonvulsant effects of VPA (for review see Löscher, 1999), and we suggest here that NE contributes to the complex mechanism of action of this widely used drug. It is important to consider such results in the clinical treatment of epilepsy, as genetic variation in DBH activity exists in the human population (reviewed by Cubells and Zabetian, 2004). Analysis of DBH function through genetic screening could indicate portions of the population that may have decreased responsiveness to certain anticonvulsant treatments. Therefore, knowledge of genotype for the DBH enzyme could be used as a tool for directing clinical treatment of epilepsy.

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Figure A2.1

A

B

![Graph A](image1)

![Graph B](image2)
Figure A2.1 Seizure susceptibility of Dbh +/- and Dbh -/- mice after exposure to flurothyl following acute VPA treatment. Mice were injected with saline or VPA (100, 200, or 300 mg/kg, i.p.) 30 minutes prior to induction of seizures with flurothyl. Shown is the latency in seconds to (A) the first myoclonic jerk and (B) clonic/tonic seizure (B). N=7-8 per group, except for Dbh -/- at VPA dose of 300, where 8 mice were tested, but 4 appeared sedated and were eliminated from the study. *p<0.05 compared to saline control for that genotype. #p<0.05 compared to Dbh +/- for that dose.
Figure A2.2

A

B
Figure A2.2 Seizure susceptibility of *Dbh* +/- and *Dbh* -/- mice after exposure to flurothyl following chronic treatment with VPA or saline. Mice were treated with saline or VPA (200 mg/kg, i.p.) 2 times per day for 4-6 weeks, and seizures were induced by flurothyl 14 hours following the last injection. Shown is latency in seconds to (A) first myoclonic jerk and (B) clonic/tonic seizure. N=10-16 per group. *p<0.05 compared to saline control for that genotype, #p<0.05 compared to *Dbh* +/- for that treatment.
Table A2.1. Number of mice reaching tonic extension of the hindlimbs following seizure induced by flurothyl after acute VPA treatment.

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<th>Genotype</th>
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<th>VPA 300 mg/kg</th>
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<td>6/8</td>
<td>3/8</td>
<td>1/8</td>
</tr>
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<td>Dbh -/-</td>
<td>7/8</td>
<td>7/8</td>
<td>7/7</td>
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</table>
Table A2.1. Number of mice reaching tonic extension of the hindlimbs following seizure induced by flurothyl after acute VPA treatment. After acute treatment with VPA at doses ranging from 0 to 300 mg/kg, VPA dose-dependently suppressed progression to tonic extension in $Dbh$ +/- mice, but had no effect in $Dbh$ -/- mice. The number of $Dbh$ +/- mice reaching tonic extension was significantly different from saline treated mice at a VPA dose of 300 mg/kg ($p=0.01$), and a protective trend was seen at 200 mg/kg ($p=0.12$). However, there were no significant differences between VPA groups and the saline group for the $Dbh$ -/- mice at any dose of VPA.
A2.6 References


