Anatomy and Pharmacology of Dopamine Transporter-Mediated Reward and Locomotor Responses to Psychostimulants

DISSERTATION

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By

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Abstract

Psychostimulants are addictive drugs that exert their effects mainly through inhibition of the dopamine, norepinephrine, and serotonin transporter reuptake activities. Previous studies have shown that dopamine transporter (DAT) blockade is necessary for expression of cocaine-induced reward and locomotor behaviors — both of which are potentially upstream of the development of addiction. However, there is little neuroanatomical specificity in many of these studies, and it is unclear how the norepinephrine (NE) and serotonin (5-HT) systems may contribute to the development of addiction. We hypothesized that DAT in the dorsal and ventral striatum are important targets for cocaine-induced hyperlocomotion and reward, respectively. We also hypothesized that NET and SERT inhibition play a modulatory role in these behaviors.

Therefore in these studies, the effects of DAT inhibition are studied in a brain region-specific manner and the contribution of the NE and 5-HT systems are probed either directly or indirectly. In the first experiments (chapter 2), the anatomical questions are investigated by using viral vectors for restoration of the wild-type DAT gene to certain brain regions of a cocaine-insensitive, mutant DAT knock-in mouse (DAT-CI mice). From this, we found that DAT blockade in the rostrolateral striatum is involved in cocaine-induced locomotion, but not reward. We did not find a discrete brain region where the viral injection restored cocaine-induced reward behaviors.
For the second set of experiments, we show that blockade of non-DAT targets, such as the norepinephrine transporter (NET) and/or the serotonin transporter (SERT), are sufficient for inducing cocaine conditioned place aversion (chapter 3). We also show that the genetic background of DAT-CI mice is critical in determining whether or not cocaine is aversive – and whether amphetamine is a stimulant of locomotion (chapter 4) – effects that are generally thought to be compulsory. Several projects that yielded few or equivocal results, regarding also the contribution of non-DAT targets, will be presented briefly (chapter 5).

In summary of the findings, the anatomical experiments show that DAT-inhibition in the rostrolateral striatum is involved in cocaine-induced hyperlocomotion. They also show that DAT-inhibition in the dorsal striatum, or the ventral striatum are effectively insufficient to produce either cocaine-induced reward or hyperlocomotion. The second set of experiments highlight the importance of genetic background in the modulation of reward and locomotor behaviors – even in inbred mouse models – as well as the involvement of non-DAT targets in aversive behaviors that counteract reward. These results bring us closer to understanding the interaction of reward and motor-related brain regions in addiction, as well as to understanding the genetic and neurochemical origins of their outputs.
Dedication

In loving memory of Brian O’Neill
Acknowledgments

I would like to acknowledge all of those who have helped me throughout my time as a graduate student. Foremost my advisor, Dr. Howard Gu, has been a great mentor who has taught me nearly everything I know about being a good person and a good scientist simultaneously. Thanks also to all past and present members of the Gu lab with whom I worked, especially Dawn Han and Bradley Martin. I would also like to thank Dr. Georgia Bishop, who has taught me much in the ways of neuroscience and graciously assisted in my project. I must also thank the faculty who have taken time out to serve on my committee: Drs. Randy Nelson, Karl Obrietan, Cosmin Roman, and Lane Wallace.

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I have not dedicated this work to myself. (This is hardly my work.) There are many more people unaddressed here, or who I cannot bring myself to address, who are extremely important – and unbounded by the page, I thank them.

…..still it was not a happy night for me that follow’d./..../[but when] the one I love most lay (...) by me..../.that night I was happy. (Whitman 1900)
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List of Abbreviations

5-HT – Serotonin

AAV – Adeno-Associated Virus

ADHD – Attention Deficit/Hyperactive Disorder

ANOVA – Analysis of Variance

BNST – Bed Nucleus of the Stria Terminalis

BGH (pA) – Bovine Growth Hormone poly-Adenylation Signal

CBA - Chicken Beta-Actin (promotor)

CPP, CPA – Conditioned Place-Preference, Conditioned Place-Aversion

DA – Dopamine

DAT – Dopamine Transporter

DAT$_{wt}$, DAT$_{vcv}$ – wild-type DAT, cocaine-insensitive (mutant) DAT

DAT-CI (mice) – Cocaine-insensitive Dopamine Transporter Knock-in Mice

dCPu, lCPu – Dorsal Striatum, Lateral Striatum

FSCV – Fast Scanning Cyclic Voltammetry

HA – Hemagglutinin (influenza)

ITR – Inverted Terminal Repeat

mNAc – Medial Nucleus Accumbens

NE – Norepinephrine

NET – Norepinephrine Transporter
NET-CI (mice) – Cocaine-insensitive Norepinephrine Transporter Knock-in Mice

SNC – Substantia Nigra Compacta

SERT – Serotonin Tranporter

SERT-CI (mice) – Cocaine-insensitive Serotonin Transporter Knock-in Mice

VTA – Ventral Tegmental Area

WPRE – Woodchuck Hepatitis Virus Post-Transcriptional Regulatory Element
Chapter 1: Introduction

Drugs of abuse are often categorized into classes including psychostimulants, opiates, ethanol, cannabinoids, hallucinogens, and nicotine. This dissertation concerns addiction to the psychostimulants in general, and cocaine in particular. This introduction, however, will be a review of the literature on several theories of addiction, on the dopamine motor system, and on the reward system – all of which pertain to addiction across all of the classes of drugs. An integrated understanding of the preexisting literature on these three topics will lay the groundwork for accepting that the study of reward and locomotor behaviors is important – namely, for the reason that reward and locomotor behaviors likely converge or integrate on the behavioral level to produce drug-directed motor habits. The introduction is then followed by chapters detailing three original studies of psychostimulant effects with respect to anatomical, pharmacological, and genetic mechanisms modulating reward and locomotion.

Theories of Addiction

As mentioned above, the psychostimulants are just one of at least five classes of drugs. Many of the laity classify drugs by a simpler two-category system, where the psychostimulants are “uppers” and opiates, for example, are among the “downers.” The psychomotor stimulant theory of addiction proposed by Wise and Bozarth (1987) states
that across all class of drugs, addictive drugs have a psychomotor stimulant property in common. It is important to take notice of the difference between the phrases, because the distinction is important in the studies that follow: psycho-motor stimulation is a phenomenon whereby a psychic, and motor system is activated; on the other hand, the word “psychostimulant” refers directly to the substances that have this effect most robustly (Post et al. 1974). Importantly, even the so-called “downers” – which certainly may have psychological and motor depressive effects at high doses (Schnur et al. 1983) – are known to have psychomotor stimulant effects at low doses. Furthermore, they are known to have psychomotor stimulant effects at some point during timecourse of any dosing, when the drugs are at low doses in the brain (Babbini et al. 1979). Thus, both the psychostimulants and the most archetypical depressants have a psychomotor stimulant effect.

Interestingly, it is known that addictive drugs also have in common their effect of elevating dopamine levels in the nucleus accumbens (Di Chiara & Imperato 1988). Therefore, it seems reasonable to hypothesize that both the motor and psychological stimulant effects are subserved by dopamine in the nucleus accumbens. Indeed, this was the prevailing hypothesis during the inception of the psychomotor stimulant theory (Gold et al. 1988).

However, as will be discussed in the next sub-section, the dopamine system is naturally subdivided into mesoaccumbal and nigrostriatal projections. Perhaps because the natural role of signaling within these systems has (more recently) been mapped separately to psychic and motor effects, a more common hypothesis is that drugs of abuse
cause “hijacking” neuroadaptations in these systems simultaneously (Wolf 2002) – or otherwise cause an unnatural convergence and focusing of motor and psychological functions on drugs. Importantly, this new hypothesis must either explain how the (now separate) drug effects and involved neural systems interact to form addiction – or else it must altogether abandon the idea that motor stimulation has anything to do with the development of addiction.

The incentive sensitization theory attempts to do the former, and can safely be viewed as a contemporary synthesis of the “psycho” and “motor” components of the previous theories into a single new concept “wanting” (for review, see Robinson & Berridge 1993). Specifically, the theory elaborates on the basic “hijacking” hypothesis by describing the result of these neuroadaptations as a hypersensitization of “wanting,” but not “liking.” In this framework, liking plays a surprisingly minor role (if any) in the development of addiction. Liking is a positive hedonic state, and can be observed as an evolutionarily conserved hedonic reaction (smiling) to a “likeable” stimulus (Mahler et al. 2007). Wanting is reward-seeking behavior not necessarily associated with liking. The concept of “wanting” is somewhat difficult to clearly understand in its true technical sense, and as a result almost always appears in quotation marks in the original work of the authors – K.C. Berridge and T.E. Robinson – but confusion is avoided here by never using the colloquial meaning of “wanting.”

The theory’s experimental backing points to dopamine in the nucleus accumbens as a mediator of incentive salience attribution (Wyvell & Berridge 2000). For natural rewards, dopamine is released in the nucleus accumbens when the organism is learning
that a stimulus is rewarding (Horvitz 2002; Bayer & Glimcher 2005). This dopamine signal is apparently responsible for encoding the value of the reward and the increased importance (salience) of the contextual cues that may act as predictors of reward in the future (Enomoto et al. 2011). Thus, these cues may become conditioned reinforcers of a developing reward-seeking behavioral strategy (Wyvell & Berridge 2001). If sufficiently conditioned, these cues may trigger wanting – with the cooperation of a motor-related brain region for the behavioral output. At this point the theory grants the possible involvement of motor-related regions such as the dorsal striatum (as hypothesized in this research) and the prefrontal cortex (Flagel et al. 2010; Paulson & Robinson 1995).

Data extending the theory to drugs of abuse are fairly lacking, other than those that indicate the theory is, at face, applicable to addiction. Many drug addictions, especially those to the psychostimulants, are driven by external cue-triggered cravings (Epstein et al. 2009) rather than by avoidance of an internal experience of withdrawal (Heilig et al. 2010). Drugs of abuse cause various drug-induced behaviors to pharmacologically “sensitize,” in addition to becoming cue-triggered (Robinson et al. 1998). This is in line with the idea that supraphysiological increases in dopamine may cause addiction-related neuroadaptations as well as condition strong incentive salience attribution to drug-related cues. Psychologically, these effects could underlie the increasingly potent drug cravings in addicts – due to the ability of cues to recruit motivational systems more efficiently, or for motivational systems to be chronically more active and more sensitive to the influence of drugs.
These sensitization processes are evident on the molecular level, with dopamine receptor supersensitivity, AMPA receptor redistribution, and dendritic spine density increases developing in the accumbens during drug exposure (Brown et al. 2011; Anderson & Pierce 2005; Schmidt & Pierce 2010) - however their exact significance in the addiction process is unknown. They are most commonly correlated, on the behavioral level, with sensitized locomotor responses to the drug. It is likely that the neuroadaptive changes would either need to be reversed, or their behavioral expression prevented, in order to successfully treat addiction.

As you may already see, the incentive salience framework makes many more distinctions than psychomotor stimulant theory. Our hypothesis is more in line with the modern theory, where dopamine within the nucleus accumbens (ventral striatum) would subserve psychological wanting, and dopamine within the dorsal striatum would subserve locomotion. These two components would have to work together in order to generate drug-seeking behavior. A drug such as cocaine, which inhibits the dopamine transporter throughout the brain, could exert a neuroadaptive influence on both of these systems simultaneously, and generate addiction. The main aim of this dissertation research was to test whether cocaine inhibition of DAT – in each of these two brain regions – is actually involved in producing locomotor and reward behaviors separately. The following subsections review the preexisting knowledge of each of these two phenomena, in turn.
The Dopamine Motor System

There is no satisfactory framework summarizing dopamine function on a systems level, however if forced to pick the best one it might be a “psychomotor system” (see above). Dopamine was discovered during studies of a motor disease (Parkinson’s disease) rather than a psychiatric disease such as schizophrenia or addiction (Carlsson et al. 1958). While dopamine transmission is certainly involved in psychiatric conditions, its role in these conditions tends to be more nebulous than its role in locomotion. Therefore, discussion of the “psycho” component of the construct (and the mesolimbic branch of the dopamine system) is reserved for the final introductory section, and we will currently discuss the details of the dopamine system in the context of locomotion.

In Parkinson’s disease, the aforementioned nigrostriatal branch of the dopamine system is selectively affected. These neurons progressively degenerate, and the main deficit is in voluntary movements related to some stimulus of weak emotional value (Drago et al. 2008). In contexts of weak motivational power or value, patients will display unsmooth movements or have difficult initiating the movements (Wu et al. 2011). In cases where the movement demands less self-derived motivation, such as initiation of a previously learned habit or in response to some unambiguous motivator (ie. a physical threat), Parkinson’s patients will display relatively smoother and more swiftly-initiated movements (de Wit et al. 2011). This may be because motion comes at an energetic cost, and would therefore be initiated only when motivated by something of sufficient value or power.
From this one can see, before even looking at the current studies, the difficulty in separating reward and locomotion subserved by dopamine. The basal ganglia circuit is a semi-theoretical system that may be helpful in this aim. The circuit consists of cortico-striato-thalamic loops, and is depicted below. All three of the major areas involved receive dopaminergic innervation, and all three are topographically subdivided – such that there are actually many functionally related cortico-striato-thalamic loops (Carlsson 1988). With this said, the basal ganglia is traditionally (and helpfully) categorized into three basic loops: a motor loop, a cognitive loop, and a limbic loop (Ena et al. 2011). We will categorize the cognitive (not depicted) and limbic loops as the “non-motor” parts, and revisit those topics and anatomy in the next section.
This diagram depicts the classical “direct” and “indirect” pathways. The direct pathway is composed of D1-like receptor expressing striatonigral neurons whereas the indirect pathway is composed of D2-like receptor expressing striatopallidal neurons. Both populations are resident neurons throughout the striatum, but this figure implicates the dorsal portion specifically – since the dopamine source is the substantia nigra (SNC). The experiments that follow test whether this is an oversimplification, and whether ventral tegmental (VTA) dopamine should be implicated as well (in motor functions). The experiments also test the converse situation, where the SNC-modulated circuit may be involved in reward functions (see discussion below on the “reward pathway”).
Within the motor loop, there are two pathways – termed the “direct” and “indirect” pathways – that involve either a striatonigral or striatopallidal projections, respectively. The striatonigral neurons have primarily D1-like receptors while the striatopallidal neurons have primarily D2-like receptors (Bertran-Gonzalez et al. 2010) – and both regions receive dopamine from the substantia nigra.

The motor loop is thought to play a more complex role in motion than can perhaps be said of more traditional motor systems (motor cortex to spinal systems). As described above, and as might be realized from the repetitive pattern of tremors in Parkinson’s disease, the motor loop seems to be involved in the initiation and patterning of motions (Desmurget & Turner 2010). This seems to be equally true of dopamine’s involvement in the motor loop, however the exact mechanism by which dopamine may act to pattern movements is very unclear. For example, although agonism at D1-like receptors causes an opposite molecular response to agonism at D2-like receptors (Nishi et al. 1997), dopamine agonism in both the direct and indirect pathways causes disinhibition of thalamic premotor neurons as its final output (Sil'kis 2002).

The Reward System

The reward system is, on a basic level, the most important neural construct in describing every behavior of an organism – although the main reason for this may be semantic: When a reward is defined as “a stimulus for which an organism will work to attain or to maintain contact,” then the reward system is defined as “the neural mechanisms involved in registering this stimulus as a reward.” Once defined in this way,
the reward system is at the heart of every single behavior, other than certain reflexes perhaps. This can even be generalized to include certain reflexes, such as a pain-induced withdrawal reflex, once a “negative reward” (or punishment) is defined as “a stimulus for which an organism will work to avoid contact” (Leknes et al. 2011). Furthermore, one can easily see that the designation of a reward would often change for a given organism, depending on its goals at the time. The question then becomes: what exactly is this reward system – if it is not the entire brain – which is involved in guiding the widest possible range of behaviors?

By way of counterexample, clues on the answer to this question come from the condition known as addiction. In the state of addiction, the organism loses control over its behavior. Addiction is best defined as a particular type of non-control, where there is a very narrowed behavioral repertoire (Koob et al. 1998). Drug-directed behaviors persist to the exclusion of other behaviors, despite known (or predictable) negative consequences of taking the drug (Pelloux et al. 2007).

Of course, drugs of abuse and other stimuli would not be addictive if they were not positively rewarding at some earlier point. Clinically, it has been observed that addictive drugs produce an intense euphoria upon an acute dose (Grigson 2008). At more chronic time-points, the drug can lose its euphorogenic properties and acquire negative or aversive properties (Koob & Le Moal 2008), however the addict continues to seek the drug. These observations collectively support the basic hypothesis that drug addiction occurs through a usurpation of the reward and motor system (or critical parts of it, more likely). Not surprisingly, it is thought that this “hijacking” is caused by neural plasticity
or other neuroadaptive changes induced by the drug itself. Unfortunately, the affected system, and the particular neuroadaptive changes in that system, are unknown.

An important finding in the history of addiction research is that all addictive drugs studied elevate the neurotransmitter dopamine in the nucleus accumbens, and that these elevations are correlated with the positive experience of the drug (Wise & Bozarth 1985). Reward is often equated with this pleasurable experience, however it would more accurately be described as “wanting” (see above). Reward as defined here – and certainly the “reward system” which guides actual responses – is likely more than just dopamine, the accumbens, and the conditioning of responses. In fact, there is obviously a motor component necessary to express any response. Therefore, the reward system is commonly defined as a broader network of accumbens-related loci such as the amygdala, hippocampus, prefrontal cortex, and thalamus – all of which also receive dopaminergic modulation and are apparently critical in reward processing and/or motor responses (Ikemoto 2010).
This diagram depicts the limbic portion of the striato-thalamo-cortical loops (non-basal ganglia). The structure appears to be similar to that in figure 1.1, in that it is a similarly composed loop - with a dopamine source impinging on the “striato” portion of the loop. However, notice that the amygdala and hippocampus nodes are not in series (as all nodes in the basal ganglia are). Therefore, in this loop there are two additional nodes which enter the circuit only by convening on the nucleus accumbens. This structure likely gives rise to additional complexity in reward functions compared to motor functions, since there is an apparent opportunity for integration of multiple signals and/or functions within the nucleus accumbens. Importantly, the nucleus accumbens is the main target of the dopaminergic modulation (PFC et al are included, but are in fact less densely innervated).
Considering once again that cocaine is an inhibitor of the dopamine transporter (DAT) throughout the brain, it seems incumbent to determine whether these specific attributions of function are true for cocaine’s effects. We hypothesized that inhibition of DAT in the dorsal striatum (nigrostriatal system) is involved in cocaine’s hyperlocomotive effect, but not its rewarding effect. Conversely, DAT inhibition in the ventral striatum (mesoaccumbal system) would be involved in cocaine’s rewarding, but not hyperlocomotive effect. Understanding the molecular and anatomical bases of these phenomena would lay the groundwork for future studies aimed at determining how these systems may become intertwined over the course of drug exposure, to produce compulsive drug-taking comprising addiction.
Chapter 2: Anatomy of cocaine-induced reward and locomotor behaviors

2.1 Abstract

Cocaine’s main pharmacological actions are the inhibition of the dopamine, serotonin, and norepinephrine transporters. Its main behavioral effects are reward and locomotor stimulation, potentially leading to addiction-related habits. Using knock-in mice with a cocaine-insensitive dopamine transporter (DAT-CI mice) we have shown that inhibition of dopamine transporter (DAT) is necessary for both of these behaviors. In this study, we sought to determine brain regions in which DAT inhibition by cocaine stimulates locomotor activity and/or produces reward. We used adeno-associated viral vectors to re-introduce the cocaine-sensitive wild type DAT in specific brain regions of DAT-CI mice, which otherwise express only a cocaine-insensitive DAT globally.

Viral-mediated expression of wild-type DAT in the rostrolateral striatum restored cocaine-induced locomotor stimulation and sensitization, but not reward as measured by conditioned place-preference (CPP). In contrast, the expression of wild-type DAT in the dorsal striatum – or in the medial nucleus accumbens in DAT-CI mice – did not restore cocaine-induced reward or locomotor stimulation. These data help to precisely determine the locus of cocaine’s molecular actions that induce hyperlocomotion. The locus or mechanisms underlying cocaine-induced reward remain undetermined. It is possible that
multiple dopamine-related brain regions are involved in cocaine-induced reward behavior.

2.2 Introduction

Cocaine is an inhibitor of the dopamine, norepinephrine, and serotonin transporters (Han & Gu 2006; Ritz et al. 1987). It is simultaneously an addictive drug with euphorogenic effects, and adverse cardiovascular and psychiatric effects (Rotheram-Fuller et al. 2007). While inhibition of each of the monoamine transporters is likely to contribute to each of cocaine’s effects in some way, there has been much effort to determine the specific role of each target in producing a specific effect. Using knock-in mice with a cocaine-insensitive dopamine transporter (DAT-CI mice), we determined that DAT inhibition is necessary for cocaine’s rewarding and hyperlocomotive effects (Chen et al. 2006). This finding was a significant step forward, because it was previously thought that several of the monoamines were mutually or redundantly involved in producing cocaine reward (Uhl et al. 2002).

We set out to extend this finding further and determine specifically where DAT inhibition in the brain is involved in producing cocaine reward and hyperlocomotion. Since the dopamine system is anatomically divided into two functional sets of neurons (Amalric & Koob 1993) – projecting to the dorsal and ventral parts of the striatum, separately – our hypothesis followed this natural division.

Therefore, we hypothesized that cocaine inhibition of DAT in the ventral striatum is involved in its rewarding effects; whereas DAT inhibition in the dorsal striatum is
involved in its hyperlocomotive effects. Using the DAT-CI mice – which lack these responses to cocaine – we tested these hypotheses by expressing the wild-type dopamine transporter (DAT\textsubscript{wt}) in these brain regions in isolation. We microinjected adeno-associated viruses containing DAT\textsubscript{wt} coding regions (AAV-DAT\textsubscript{wt}) to specific brain regions. Then we tested for the restoration of locomotor and reward responses to cocaine in the injected DAT-CI mice, in a conditioned place-preference (CPP) paradigm.

Since we measured reward and locomotor behaviors simultaneously – in the course of the CPP paradigm – we determined the role of DAT inhibition in a given region, in producing either of the behaviors. This design is experimentally efficient and is effective, even without specific hypotheses, in determining whether or not there are dissociated substrates for the two behaviors. We hypothesized in this specific way due to the large body of evidence from Parkinson’s disease research implicating dorsostriatal dopamine transmission in motor functions (for review, see Blandini \textit{et al.} 2000).

Interestingly however, there is emerging evidence that nigrostriatal function may be involved in drug-induced reward (Quinlan \textit{et al.} 2004; Wise 2009) and that mesoaccumbal function may be involved in drug-induced hyperlocomotion (Heusner \textit{et al.} 2003).

These new data have renewed the need to understand the circuitry involved in drug-induced reward and locomotion. Using the system of AAV-mediated expression of DAT\textsubscript{wt} in DAT-CI mice, we can directly test these questions with respect to cocaine and DAT-inhibition within specific nodes of the circuit. This is important, because there are likely critical details that are very specific to cocaine addiction. For example, there are
known differences in the neural mechanisms of drugs closely related to cocaine, such as amphetamine (Pum et al. 2007; Tzschtente & Schmidt 2000). Also, in contrast to seemingly more potent addictions such as those to opiates, there are no approved therapies for cocaine addiction. This study was aimed toward identifying anatomical substrates of cocaine’s DAT-mediated effects, which could also lead to new therapeutic targets.

2.3 Results

In three experiments, DAT-CI mice – which globally express a cocaine-insensitive dopamine transporter – were intracerebrally injected with AAV containing the wild-type DAT coding regions. Since DAT-CI mice normally do not display cocaine-induced reward or hyperlocomotive behaviors, we tested whether this injection would restore either of these behaviors, in each of three regions (see subsections below). A combined paradigm was used for all behavioral experiments, in which conditioned place-preference (CPP) and locomotion were measured – with locomotor data being collected on all of the 10 daily sessions, and with conditioned place-preference (reward) data being collected during the pretest and posttest (on days 1 and 10). Days 2 – 8 are four pairs of environment:treatment conditioning – with saline and cocaine treatments occurring in different environments, on alternating days. These data allow us to detect a locomotor sensitization effect of repeated drug administration, in addition to acute stimulation from cocaine (relative to saline).
For locomotion, a two way ANOVA (drug x group) was performed on the average locomotion (of all four pairings) on saline days versus on cocaine days, for the injected and uninjected DAT-CI groups. For CPP, a two way ANOVA (phase x group) was performed on the CPP score in the pre-test versus post-test.

Experiment 1: Validation of the DAT-CI and AAV System

First, behavioral characterization of uninjected wild-type and DAT-CI littermates was performed at the doses of 10 mg/kg and 20 mg/kg cocaine. Only the 10 mg/kg dose is shown and analyzed here, in depth. Both doses are included in a different analysis of the AAV-injected DAT-CI experiments to follow. Therefore, the 10 mg/kg data is redundant after the first instance.

For locomotion (figure 2.1A), the two way ANOVA showed a significant main effect of genotype (F1, 46 = 8.898; p < 0.01), but not of drug (F1,46 = 1.363; p = 0.249). There was a significant genotype x drug interaction (F1, 46 = 18.159; p < 0.001). Bonferonni-Dunn post hoc tests determined that DAT-CI mice had significantly higher saline-induced “baseline” locomotion (p < 0.001). Wild-type mice had locomotor stimulation by cocaine, relative to saline (p < 0.001). Notably, mutant DAT-CI mice did not have locomotor stimulation by cocaine (p = 0.075). In fact, DAT-CI mice tend to exhibit a locomotor suppressive effect of cocaine, relative to saline.

For reward (figure 2.1B), the two way ANOVA of the conditioned place preference data showed a significant main effect of genotype (F1, 46 = 6.396; p < 0.05), and of phase (F1, 46 = 5.081; p < 0.05), as well as a significant genotype x phase
interaction ($F_{1, 46} = 4.584; p < 0.05$). Bonferonni-Dunn post hoc tests determined that wild-type mice had significantly higher preference for the drug-paired compartment in the post-test relative to the pretest ($p < 0.001$). On the other hand, DAT-CI mice did not have significant CPP ($p = 0.947$). These behavioral assays of the genotypes are intended to serve as a baseline for comparison with the AAV-injected groups to follow. The uninjected wild-type and DAT-CI data in figure 2.3 are from the same cohorts as those presented here (for 10 mg/kg).
Figure 2.1 – Cocaine-induced locomotion and reward in wild-type and DAT-CI mice

All data are from a single experiment at the 10 mg/kg dose. Locomotor data (A) are horizontal distances travelled (in 30 minute sessions) as an average over the four administrations of either drug or saline. Reward data (B) are represented as time spent in the drug-paired minus saline-paired compartments in the pre-conditioning test versus post-conditioning test. For both behaviors wild-type mice, but not DAT-CI mice, exhibit a significant cocaine effect. Data are means ± SEM.
Next, an ex-vivo characterization of the functional effects of the AAV injection was carried out. Mutant DAT-CI mice with a unilateral injection of AAV-DAT\textsubscript{wt} in the striatum (ICPu; similar to figure 2.3A) were used in FSCV measurements in the presence and absence of cocaine (figure 2.2). The voltammetry was configured to measure electrically stimulated dopamine release and reuptake. No statistical analyses were performed, because the number of mice tested was low. Qualitatively, there appeared to be an increased peak dopamine concentration in the presence of cocaine (relative to absence of cocaine) on the AAV-DAT\textsubscript{wt} injected side, but not in the uninjected striatum. Furthermore, there was a protracted descending phase of the curve on the AAV-DAT\textsubscript{wt} injected side that was not protracted on the uninjected side. In fact, the uninjected striatum generated an essentially superimposable pair of curves in the presence and absence of cocaine. These data indicated that the AAV system could produce functionally relevant effects on dopamine reuptake in DAT-CI mice – sufficiently enough for confidence in proceeding to behavioral experiments, despite the lack of statistical significance.
Figure 2.2 – Voltammetric determination of cocaine induced dopamine outflow

Fast scanning cyclic voltammetry (FSCV) measurements of electrically-stimulated dopamine outflow in striatal slices from AAV-DAT\textsubscript{wt} injected DAT-CI mice. Mice were injected unilaterally, and measurements were taken on both sides, in the presence and absence of cocaine. There were no apparent differences between the dopamine reuptake curves in the presence and absence of cocaine in the uninjected striatum of DAT-CI mice – as expected since DAT-CI mice express a cocaine-insensitive DAT. However on the AAV-DAT\textsubscript{wt} injected side, there was an apparent increase in [DA]\textsubscript{0} and a slower decay-phase of the curve in the presence of cocaine – both indicating a restored inhibition-like effect of dopamine reuptake by cocaine, on the AAV-injected side.
Experiment 2: Injection to the lateral striatum (lCPu)

Experiments 2 – 4 were attempts at AAV-mediated “restoration” of cocaine’s behavioral effects to DAT-CI mice. All three experiments, each being an injection in a different brain region, are presented in figure 2.4 (data at 10 mg/kg are on the left and 20 mg/kg on the right). The first behavioral experiment in AAV-injected mice was targeted to the lateral striatum (lCPu). In this experiment, separate cohorts of mutant DAT-CI mice injected bilaterally with AAV-DAT<sub>wt</sub> were subjected to the behavioral paradigm at doses of 10 mg/kg and 20 mg/kg cocaine. This injection was intended for both dorsal and ventral subregions of the rostral striatum (see figure 2.3A) in order to test both the locomotor and reward aspects of the hypothesis simultaneously.

For locomotion (figure 2.4A, 2.4B), a two way (group x doses) ANOVA between AAV-injected and uninjected DAT-CI mice was performed. There was a significant main effect of group (F<sub>1, 28</sub> = 5.594; p = 0.05), of cocaine doses (F<sub>1, 28</sub> = 19.249; p < 0.001), and a significant group x doses interaction (F<sub>1, 28</sub> = 31.651; p < 0.001). These results indicate that injection of AAV-DAT<sub>wt</sub> to the lCPu region of DAT-CI mice restores both baseline and cocaine induced locomotion toward normal levels. Bonferroni-Dunn post hoc tests show that there is a significant difference in locomotion on the “saline” dose between AAV-injected and uninjected DAT-CI mice (p < 0.01) Post hoc tests also show that the AAV injected DAT-CI mice have significant locomotor stimulation, relative to saline (p < 0.001) at 20 mg/kg. There are ongoing experiments at 10 mg/kg, since there are currently only 4 mice in this group. There is a trend toward locomotor stimulation that may become significant as the subject “n” is increased from 4 to 8.
For reward (figure 2.4C, 2.4D), a two way (phase x group) ANOVA of the CPP data found no significant conditioning (“phase”) effect in DAT-CI mice injected with AAV-DAT_{wt} in the lCPu – (F_{1, 28} = 0.048; p = 0.828). There was also no main effect of AAV injection (F_{1, 28} = 0.055; p = 0.816) nor an interaction (F_{1, 28} = 0.005; p = 0.946). These results suggest that the manipulation does not restore cocaine’s rewarding effect to DAT-CI mice.

After the experiments were completed, we performed immunohistochemical staining of the HA tag on the N-terminus of the exogenous DAT. We found that the injections were placed consistently in the rostrolateral striatum, with DAT_{wt} expression spanning both the dorsal and ventral portions. In the ventral area, the lateral shell of the nucleus accumbens was always infected, whereas the core was infected in approximately 50% of the replicates.

**Experiment 3: Injection to the dorsal striatum (dCPu)**

The next experiment involving DAT-CI mice was in a cohort injected with AAV-DAT_{wt} in the dorsal striatum (dCPu; figure 2.3B). This experiment was intended to test the hypothesis that DAT inhibition in this region is involved in cocaine-induced hyperlocomotion, but not reward. The experiment in this region was the only one (of three) that was performed at the 10 mg/kg dose only.

For locomotion (figure 2.4A, 2.4B), the two way (dose x group) ANOVA between AAV-injected and uninjected DAT-CI mice (“groups”) showed no significant main effect of AAV injection (F_{1, 38} = 0.492; p = 0.488) and no dose x group interaction.
There was a trend toward a significant main effect of dose ($F_{1, 38} = 3.558; p = 0.067$), which resembles the unusual tendency toward cocaine-induced locomotor suppression in uninjected DAT-CI mice discussed earlier. These results suggest that injection of AAV-DAT\textsubscript{wt} to the dCPu of DAT-CI mice has no restorative effect on cocaine-induced hyperlocomotion.

For reward (figure 2.4C, 2.4D), a two way (phase x group) ANOVA of the CPP data found no significant conditioning (“phase”) effect in DAT-CI mice injected with AAV-DAT\textsubscript{wt} in the dCPu at the 10 mg/kg dose ($F_{1, 38} = 0.564; p = 0.457$). There was also no main effect of AAV injection ($F_{1, 38} = 1.395; p = 0.245$) nor an interaction ($F_{1, 38} = 0.383; p = 0.540$). These results suggest that the manipulation does not restore cocaine’s rewarding effect to DAT-CI mice.

As in the previous experiment, we performed immunohistochemical staining after the behavioral tests, in order to localize the site of injection. We found that the injections were placed consistently in the caudal part of the dorsomedial striatum (dCPu). There was a discrete bolus of DAT\textsubscript{wt} expression that spanned the dCPu and supracapsular BNST – with placement centered at the border of these two regions in nearly 100% of the replicates.

**Experiment 4: Injection to the ventral striatum (mNAc)**

The next experiment was in a cohort of DAT-CI mice injected with AAV-DAT\textsubscript{wt} in the ventral striatum only – specifically in the medial nucleus accumbens (mNAc;
This experiment was intended to test the hypothesis that DAT inhibition in this region is involved in cocaine reward, but not hyperlocomotion.

For locomotion (figure 2.4A, 2.4B), the two way ANOVA (group x doses) between AAV-injected and uninjected DAT-CI mice (“groups”) showed a significant main effect of group ($F_{1, 70} = 6.146; p < 0.05$) but not of cocaine doses ($F_{2, 70} = 2.843; p = 0.065$) and no interaction ($F_{2, 70} = 1.623; p = 0.205$). Bonferroni-Dunn post hoc tests show that there is a significant difference in locomotion on the “saline” dose between AAV-injected and uninjected DAT-CI mice ($p < 0.01$). Furthermore, there is the typical locomotor suppression to 20mg/kg cocaine in uninjected, but not AAV-DAT$_{wt}$ injected DAT-CI mice ($p < 0.05$).

These results suggest that injection of AAV-DAT$_{wt}$ to the mNAc of DAT-CI mice restores their basal hyperlocomotion (relative to wild-type; see figure 2.1A) back toward normal levels, but has no restorative effect on cocaine-induced locomotor stimulation. This is an interesting result, given that the localization of the mNAc injection (see below) is very near to the ventral part of the lCPu injection presented above (see figure 2.3A). This seems to suggest a great deal of specificity in the effect of the lCPu injection of AAV-DAT$_{wt}$ – with either the dorsal or ventrolateral extents of the injection being fully sufficient for the restoration of cocaine-induced locomotor stimulation observed.

For reward (figure 2.4C, 2.4D), a repeated measures ANOVA was used in this case only, since this experiment is the only one with multiple doses analyzed. Thus, there is an additional factor and a three-way interaction – with “phase” as the repeated factor. The two way (doses x group) repeated measures ANOVA yielded the following
results. There was no significant main effect of phase ($F_{1, 34} = 0.030; p = 0.864$) of group ($F_{1, 34} = 0.022; p = 0.882$) or of doses ($F_{1, 34} = 0.094; p = 0.761$). There were also no significant interactions. These results suggest that the manipulation does not restore cocaine’s rewarding effect to DAT-CI mice – at either the 10 mg/kg or 20 mg/kg doses. The apparent trend toward cocaine aversion in the 20 mg/kg group is not significant, even in an unadjusted pairwise comparison of the confidence intervals (pre-test vs post-test; CL 95%, $p = 0.123$).

We performed immunohistochemical staining after the behavioral tests as before, in order to localize the injection site. We found that the injections were placed in the medial portion of the nucleus accumbens (mNAc) – as well as in the septum. The regions were consistently co-infected when septal infection occurred – and the notable variance between replicates was the absence of bilateral injection in approximately 25% of the cases. Almost all of these cases consisted of unilateral infection of the accumbens, without expression in the septum – presumably due to unintentional dilution in the ventricle, or trans-ventricular injection of the nucleus accumbens.
Figure 2.3 – Localization of DAT<sub>wt</sub> expression in AAV-injected DAT-CI mice

Depicted are micrographs showing the localization of viral-expressed DAT<sub>wt</sub> performed after completion of the behavioral tests. The coronal sections have dark black areas which is the immunohistochemical staining of the HA tag on the DAT<sub>wt</sub>. All micrographs are on a scale of 1.25 M.

The lateral straitum (lCPu) injection is shown in (A), and was found to be in the very rostral parts of the dorsal and ventral striatum. The dorsal striatum (dCPu) injection is shown in (B), and was found to be in the very caudal parts of the dorsomedial striatum. The ventral striatum is shown in (C) and was found to be in the medial part of the nucleus accumbens (mNAc).
Figure 2.4 – Behavioral Characterization of AAV-DAT<sub>wt</sub> injected DAT-CI mice

The locomotor data from controls, plus these groups of AAV-injected DAT-CI mice (in order, left to right) are presented in panels A (10 mg/kg) and B (20 mg/kg). Similarly, the reward data of the same groups (from left to right) are presented in panels C (10 mg/kg) and D (20 mg/kg). These are arranged such that similar groups and cocaine doses are stacked, and the different behaviors appear in rows. As in figure 2.1, data are means ± SEM of the average locomotion over the 4 conditioned pairings (for locomotion) – or are of the time spent in the drug-paired minus saline-paired compartments (for reward).

We found that AAV-DAT<sub>wt</sub> injection to the lCPu of DAT-CI mice restored cocaine-induced locomotion (B), but not reward (D), from 20 mg/kg cocaine. Similar injections to the dCPu and mNAc of DAT-CI mice did not restore either cocaine-induced reward or locomotor stimulation. There is no apparent restoration of either behavior, from any injection, at the 10 mg/kg dose.
Figure 2.4
2.4 Discussion

Previously, we observed that mice globally expressing a cocaine-insensitive dopamine transporter (DAT-CI mice) do not exhibit cocaine-induced locomotion or reward-related behaviors (Chen et al. 2006). DAT is expressed in many brain areas, as well as even peripheral areas. Therefore this result leads to the conclusion that DAT inhibition is necessary for these behavioral effects – either somewhere, but potentially everywhere it is expressed. In this study, we used AAV-mediated gene transfer in DAT-CI mice, in order to determine if there is a minimal brain region where expression of DAT\textsubscript{wt} would phenocopy wild-type mice. Importantly, this manipulation tests a sort of ‘sufficiency’ which is different in nature and significance to the previous finding of ‘necessity.’

We found that injection of AAV containing the DAT\textsubscript{wt} coding regions (AAV-DAT\textsubscript{wt}) into the rostrolateral striatum restored the hyperlocomotor response to 10 mg/kg and 20 mg/kg cocaine. Since the cocaine was administered in the context of a conditioned place preference (CPP) paradigm, the mice received repeated doses of cocaine during which locomotor sensitization could also be observed. The AAV-DAT\textsubscript{wt} injection in this region of the striatum also restored locomotor sensitization to cocaine (data not shown). On the 10\textsuperscript{th} day of the paradigm, the CPP of the mice was measured in the drug free state. Mice injected in this region (ICPu) did not exhibit a significant CPP for 10 mg/kg or 20 mg/kg cocaine.

After the 10\textsuperscript{th} day of the paradigm additional sessions could be conducted, since the training and measurement of CPP would have been completed. In the cohort of DAT-
CI mice injected with AAV-DAT\textsubscript{wt} in the ICPu, administration of 5 mg/kg RTI-113 on the 11\textsuperscript{th} day induced a robust locomotor stimulation – similar to 20 mg/kg cocaine (data not shown). Since RTI-113 is a DAT-specific inhibitor, this result suggests that DAT inhibition in the ICPu is sufficient to produce hyperlocomotion. On the other hand, cocaine inhibition of DAT in this region (in combination with global inhibition of NET and SERT), is not sufficient to induce cocaine reward.

It is important to realize that in this system, the exogenous DAT is expressed constitutively and somewhat indiscriminately, since the chick beta-actin (CBA) promoter was used. Since AAV1 have tropism directed predominantly toward neurons several weeks after infection (Wang \textit{et al.} 2003), the final outcome of injection into the striatum (for example) could be ectopic expression of DAT in the resident neurons (medium spiny neurons, cholinergic interneurons) and glia. Studies also suggest that there may be expression in the presynaptic terminals and retrograde transport to afferent brain regions (Boulis \textit{et al.} 2003; Ch tarto \textit{et al.} 2007).

We found, by FSCV, that injection of AAV-DAT\textsubscript{wt} in the striatum of DAT-CI mice restored an inhibition-like effect of cocaine with respect to dopamine reuptake (Fig. 2.2). Based on this functional result, we felt prepared to proceed to the behavioral experiments. It appears that, given the predictable behavioral results, the involvement of DAT inhibition in the striatum in producing these behaviors is dominated by its influence on volume transmission and dopamine tone – rather than on any finer effect that might be expected to be disrupted by the ectopic nature of the system. This result seems informative in itself, considering that the role of tonic versus phasic firing of dopamine
neurons is an important topic of drug abuse research (Grieder et al. 2012). We believe this result may be specific to drugs of abuse such as cocaine, where there are superstimulus elevations of dopamine that cause volume transmission to predominate. It is possible that dopamine-dependent physiological responses, such as responses to natural rewards, may be disrupted in both DAT-CI mice and mice microinjected in this system – specifically because DAT inhibition does not occur in response to those rewards.

The results from the other two regions tested – the dorsal striatum (dCPu) and the medial accumbens (mNAc) – suggest that DAT-inhibition in these regions is not sufficient to restore either cocaine reward or locomotion. This is not to say that the regions are uninvolved in either behavior. In fact, both of these regions may well be areas where DAT-inhibition is necessary for a given behavioral effect – where cocaine-insensitivity in these regions alone might cause a loss of behavior similar to those observed in DAT-CI mice. One can imagine future studies where a site-specific knock-in (knock-on) of the cocaine-insensitive DAT would test this type of dependency, however the technical demands of this type of study are very high.

Relevant to our result showing the “insufficiency” of these DAT blockade in these regions, anatomical and behavioral studies have already suggested that dorsal and ventral subregions of the striatum may interact to produce certain behaviors. It has been shown that in the striatum, a shift in involvement of ventral to more dorsal subregions develops over time, during the formation of self-administration habits (Belin & Everitt 2008). Considering that drug-taking seems to be motivated by its rewarding effect initially, but is later a more compulsory habit, this finding seemed to fit with clinical observations. It
also supported the longstanding hypothesis (as well as our original hypothesis) that dopamine in the ventral striatum is involved in reward, as opposed to in the dorsal striatum where it is involved in motor functions. Our results did not show that this dorsal/ventral dissociation of reward and locomotion is true in the case of cocaine inhibition of DAT. Furthermore, we cannot rule out an interaction of both dorsal and ventral regions of the striatum in producing cocaine-induced hyperlocomotion in itself.

In conclusion, these results strengthen the original conclusion derived from DAT-CI mice that DAT blockade is necessary for cocaine-induced reward and locomotion – since the loss of the locomotor phenotype is fully reversible. Secondly, the results demonstrate that DAT-inhibition in the rostrolateral striatum is largely responsible for cocaine’s locomotor inducing effect. Lastly, the results suggest that multiple dopamine-related brain regions may interact to produce cocaine-induced reward. Future studies may investigate the role of DAT inhibition in regions like the amygdala or the midbrain origins of dopamine itself, since these are known to express DAT and be involved in drug action (Yap & Miczek 2008).

2.5 Materials and Methods

Animal Subjects

In this study, knock-in mice with a cocaine-insensitive dopamine transporter (DAT-CI mice) were used, which were generated as described previously (Chen et al. 2006). These mice contain a triply mutated dopamine transporter (DAT) which is composed of the following substitutions: L104V/F105C/A109V (termed DAT_{vcv}).
congenic DAT-CI and wild-type littermates were generated from sibling pairings of heterozygous mice.

The animal subjects then underwent a series of procedures overviewed in this timeline, each of which procedures are detailed further below:

Figure 2.5 – Design and Timeline of Virogenetic Experiments
During the course of these experiments, all mice were kept in standard housing conditions, which include ad libitum access to food/water and 12 hours each of dark/light. Only male mice were used, and all mice were between 2 – 6 months of age at the time of behavioral testing. Experimental groups compared to one another were age matches. All animal procedures were approved by The Ohio State University Internal Laboratory Animal Care and Use Committee (ILACUC).

Packaging and Purification of Viral Vectors

Recombinant adeno-associated viral vectors (AAV1) containing either wild-type dopamine transporter (AAV-DAT_{wt}) or the mutant transporter (AAV-DAT_{mut}) were used in this study. The vectors were packaged and purified similar to procedures described elsewhere (Clark et al. 1999). Briefly, HEK293 cells were triple co-transfected via the calcium phosphate method with the following plasmids: a capsidation plasmid, a helper plasmid, and the recombinant genome plasmid. In this system, only the portions between the inverted terminal repeats (ITR) of the genome plasmid become packaged in the virus. Therefore, a map of this plasmid is included:
The cells were allowed to package viral particles for three days after transfection. Then they were harvested and lysed via freezing and thawing the scraped cells in a hypotonic lysis solution containing benzonase at 2 U/mL (Novagen). The cell media, which contains an appreciable amount of shed viral particles, was also saved for separate processing with a heparin affinity column.

Figure 2.6 – Plasmid Map of DNA Used in Production of AAV-DAT<sub>wt</sub>

The ~4000 base pair (bp) region between the two inverted terminal repeats (ITR) is the region packaged into the virus, and incorporated into the brain during injection.
The cell lysate fraction was then loaded onto a discontinuous iodixanol gradient containing 15%, 25%, 40% and 60% layers (in PBS). The 15% layer contained 1M NaCl and the alternating layers contained phenol red for visualization. The tubes were spun at 350,000 x g for 1 hour at 18 °C. The material at the 60/40 % interface was collected. This was combined with the isolate from the media fraction and concentrated and dialyzed using a Amicon Ultra 15 centrifugation ultrafilter. The final preparation was in a buffer of PBS with 0.001% pluronic F-68 (Sigma-Aldrich) and was titered by real-time PCR.

*Surgeries and Microinjection of Viral Vectors*

The stereotaxic injection setup consisted of a Hamilton syringe and tubing system, primed with water and connected to a 33 gauge injector cannula (Plastics One, Roanoke VA). The injection was monitored visually by a calibrated reticule in the system with a small air bubble as a marker. The viral vector was backfilled into the injector, downstream of this air bubble before the surgery.

The mice were anesthetized with a mixture of 100 mg/kg ketamine and 15 mg/kg xylazine (Sigma-Aldrich). Using aseptic surgical procedures, the mice were then fixed into a stereotaxic frame (Stoelting Co., IL.) and a small skin incision was made over the skull. The skull was made level, and the location of the bregma landmark was recorded.

Microinjections of viral vectors were carried out for three different brain regions: the rostrolateral striatum (lCPu), the dorsal striatum (dCPu), and the medial accumbens (mNAc). The coordinates targeted for each of the regions were as follows:
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Axis: adjustment relative to Bregma (in mm)</th>
<th>Approach Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior/Posterior</td>
<td>Medial/Lateral</td>
</tr>
<tr>
<td>lCPu</td>
<td>+1.5</td>
<td>±1.2</td>
</tr>
<tr>
<td>dCPu</td>
<td>0.0</td>
<td>±2.2</td>
</tr>
<tr>
<td>mNAc</td>
<td>+1.5</td>
<td>±0.5</td>
</tr>
</tbody>
</table>

**Table 1 – Stereotaxic Coordinates of AAV Microinjection Sites**

The vectors were at a concentration of approximately $1 \times 10^{11}$ vg/mL and were infused at a rate of 0.25 µL/min. Between 1 – 2 µL of virus was injected into each hemisphere, depending on the number of boli administered along the dorsoventral path of the injector through the burr holes at the above coordinates. For the dCPu and mNAc regions, one bolus of 1 µL per hemisphere was injected. For the lCPu region, the coordinates denote the ventral-most bolus (0.5 µL), but two more boli, each being 0.75 µL large and 1 mm more dorsal than the previous bolus, were infused along the angled path in each hemisphere. After infusion, the injector was left in place for 2 minutes, then raised. The mice were then sutured and administered post-operative care for one week. Stable expression of the vector genome was allowed during a four week recovery.
Drugs Administered

Mice were administered all drugs dissolved in a vehicle of 0.9% saline at a concentration such that 10 µL/g body weight would deliver the desired dose. Cocaine HCl was provided by the NIDA drug supply program, and administered at 10 and 20 mg/kg doses intraperitoneally (i.p.). A DAT selective inhibitor, 2β-carbopentoxy-3β-(4-chlorophenyl)tropane (or RTI-113) was provided by the Research Triangle Institute, and administered at 5 mg/kg i.p.

Ketamine and xylazine were administered for anesthesia during the stereotaxic surgeries preceding the behavioral experiments, at doses of 100 mg/kg and 15 mg/kg respectively (i.p.).

Conditioned Place-Preference Test

A conditioned place-preference (CPP) apparatus was used to measure cocaine-induced reward and hyperlocomotion simultaneously. The apparatus was a 12.5 cm x 42.5 cm acrylic box subdivided into three interconnected compartments: two side compartments (12.5 cm x 17.5 cm) and a center compartment (12.5 cm x 7.5 cm). The paradigm was unbiased with respect to unconditioned preferences (via counterbalancing) and consisted of a pre-conditioning test (first day), a cocaine conditioning phase (next 8 days), and a post-conditioning test (last day) during which the expression of a cocaine conditioned place-preference would be measured. Unlike some conventional paradigms (Tilley et al. 2009), locomotion was measured during the conditioning phase, for a total of 10 days of measurement. Each day consisted of one 30-minute session.
Measurements of total time spent in each of the three compartments, as well as of total distance traveled, were automatically carried out by the AnyMaze video tracking system (Stoelting Co.).

Mice were habituated to handling for three days, prior to the pre-conditioning test. On the pre-conditioning test day, the three compartments were made distinct from one another by visual and tactile cues, creating three different “environments.” Mice were placed into the center compartment and allowed to explore all three compartments. Their preference was defined as the difference in time spent in one side compartment versus the other. Their unconditioned preexisting preference was counterbalanced in each group by designating individual mice to receive cocaine in either their initially preferred or initially non-preferred environment – such that the group bias was minimized.

After assignments, the conditioning phase proceeded by configuring the entire apparatus with only one of the two environmental cue sets. The mice were then administered the treatment corresponding with the environment for that day (either saline or cocaine). On the following day, the opposite agent was administered in the appropriate environment, and these alternations proceeded for 8 days (a total of four “pairings”). Analytical groups were counterbalanced for treatment order.

The apparatus was then configured as three-environments for the post-conditioning test, and the mice were tested exactly the same way as on the pre-conditioning test day. No treatments were administered during either the pre or post-conditioning tests.
**Immunohistochemistry**

Immunohistochemical localization of the injection site was performed on floating sections. Mice were sacrificed and perfused with 4% paraformaldehyde in order to harvest their brains, after the last trial. The brain was sectioned coronally at 60 µm through the striatal region on a freezing microtome. The sections were stained for the HA-tagged DAT expressed by the AAV in a series of antibody and reagent incubations. All incubations were carried out at room temperature in PBS. Each incubation was followed by washing 3 times in PBS (5 minutes each wash). The sections were first incubated in a blocking buffer of 1% BSA for 20 minutes. They were then incubated in a 1:6000 dilution of mouse monoclonal anti-HA primary (Sigma). They were then incubated in a 1:1000 dilution of goat-anti mouse secondary (Sigma) followed by an incubation in a 1:1000 dilution of peroxidase-conjugated mouse anti-peroxidase (Jackson ImmunoResearch). The sections were then washed, and the labeled tertiary was reacted with a chromogen via a nickel-intensified, glucose oxidase-catalyzed procedure detailed elsewhere (Tian et al. 2008). The sections were mounted onto slides and visualized at 1.25x magnification on a CarlZeiss axioscope.

**Fast Scanning Cyclic Voltammetry**

In a separate experiment aimed at validating the above system, DAT-CI mice were injected with AAV-DAT_{wt} in the ICPu region unilaterally. Coronal brain slices (400 µm thickness) containing the injected region were prepared, and electrically-evoked dopamine overflow was measured using FSCV as described previously (Chen et al. 2006;
Zhou et al. 2005). On each of the sides, a baseline reuptake curve was measured, followed by a curve in the presence of 2.5 µM cocaine.
Chapter 3: Pharmacology of psychostimulant-induced locomotor behavior

This chapter is adapted from a previous publication (O’Neill B. & Gu H.H.)

3.1 Abstract

We previously generated a knock-in mouse line with a cocaine-insensitive dopamine transporter (DAT-CI mice). These mice lost several behavioral responses to cocaine, but retained their response to amphetamine. DAT-CI mice are hyperdopaminergic due to reduced DAT function, and may thus be a good model for studying attention deficit hyperactivity disorder (ADHD). These mice had been behaviorally characterized while they were on a mixed genetic background. However as the colony was propagated over time, the mixed genetics were shifted toward a pure C57Bl/6J background – via a common breeding scheme known as “backcrossing.” Several phenotypes appeared to have changed during this time frame. In this study, we investigated whether backcrossing altered the hyperlocomotive phenotype and behavioral responses to amphetamine, a drug used to treat ADHD.

C57-congenic DAT-CI mice had high spontaneous locomotor activity that could be suppressed by low doses of amphetamine. Furthermore, their locomotion was not stimulated by very high doses of amphetamine (20 mg/kg). After the reversion to a mixed genetic background by breeding with the 129 strain, the C57:129 hybrid DAT-CI
mice displayed reduced basal locomotor activity compared to the C57-congenic mutant mice, and regained locomotor stimulation by high-dose amphetamine. The calming effect of amphetamine at low doses was retained in both strains.

In summary, reduced DAT function in DAT-CI mice leads to a hyperdopaminergic state, and an ADHD-like phenotype in both strains. The data show that the genetic background of DAT-CI mice affects their locomotor phenotypes and their responses to amphetamine. Since the differences in genetic background between the strains of mice have a significant impact on the ADHD-like phenotype and the response to amphetamine, further study with these strains could identify the genetic underpinnings affecting the severity of ADHD-related symptoms and the treatment response.

3.2 Introduction

In the recent past, knock-out/in mouse generation was limited by the lack of hardiness of stem cells derived from inbred embryos other than those from the 129 strain. Viable stem cells are derived from the 129Sv/J strain of mice relatively easily, and are still almost exclusively used in the generation of knock-out/in mice. In contrast, the large majority of behavioral experiments are performed on inbred C57Bl/6J mice, because of the unique robustness of this strain’s behavioral repertoire (Owen et al., 1997). It is therefore conventional to backcross the chimeric founders with C57 mice. However, this backcrossing process produces >99% C57-congenic mice only after 10 generations of breeding – and it is commonplace for initial behavioral experiments to be conducted
before this time-consuming process has been completed. This may not be ideal for building upon seemingly related data from inbred lines.

Nonetheless, the initial characterization of many genetically engineered knock-out/in mice is performed and published on a mixed C57:129 background, whereas subsequent studies are performed on a C57-congenic background. This results in a gradual shift of the strain genetic background. If such a shift leads to changes in phenotype, future studies may reveal genetic variants responsible for the phenotypic changes.

In the present study, we test whether the strain background is critically involved in the locomotor behavior of our mouse line. Previously, we generated a knock-in mouse line with a cocaine-insensitive dopamine transporter (DAT-CI mice) (Chen et al., 2006). As expected, cocaine responses were altered in these mice while amphetamine responses were not. Specifically, 10 mg/kg amphetamine stimulated locomotion in DAT-CI mice while the line was on a mixed C57:129 background. However, after the mice were backcrossed to a C57-congenic background, we no longer observed locomotor stimulation to 10 mg/kg amphetamine. This prompted us to test the hypothesis that the backcrossing procedure caused a shift in phenotypes related to amphetamine-induced locomotion.
3.3 Results

Two mouse lines were used in this study: 1) DAT-CI mice on a C57-congenic background and 2) DAT-CI mice on a 129B6 (hybrid) background. The corresponding wild-type littermates were used as controls.

A side-by-side characterization of the locomotor behavior of the C57-congenic and the 129B6 mice was performed in two separate testing paradigms. First, spontaneous locomotion and locomotor habituation were observed; then, the amphetamine dose-response curves were determined for locomotion.

129B6 DAT-CI mice are less hyperactive and habituate more efficiently than C57-congenic DAT-CI mice

In addition to hyperactivity, another characteristic feature of ADHD is a deficit in habituation of locomotion over time (ie. within-session) – especially within familiar environments (ie. across sessions). This feature has been recapitulated in mice – as the dopamine transporter knock-down mouse displays a less efficient locomotor habituation over a 1 hour session and sustained hyperactivity throughout the 6 days of testing (Zhuang et al., 2001). A similar paradigm was used in our study in order to measure the relative activity and locomotor habituation characteristics of the strains of mice (Fig. 3.1 & 3.2).

We placed untreated mice in open field apparatuses and their locomotor activities were monitored for 30 min. The same experiments were performed for 6 consecutive days. We found that on day 1 DAT-CI mice in both strains are hyperactive relative to
wild-type mice (Fig. 3.1B), as was reported previously (Chen et al., 2006). Importantly, C57-congenic DAT-CI mice show a deficit in within-session habituation, and are more hyperactive than the 129B6 DAT-CI mice (Fig. 3.1A). In the subsequent five days, we found that the C57-congenic strain of DAT-CI mice grew more hyperactive each day and their locomotor activities were significantly higher on day 6 than those on day 1 (Fig. 3.2).

Specifically, a two-way ANOVA showed that there was a main effect of genetics ($F_{3, 52} = 41.03; p < 0.001$) on spontaneous activity. Figure 3.1B shows that both strains of DAT-CI mice are hyperactive relative to their wild-type counterparts (Day 1: $p < 0.05$; Day 6: $p < 0.001$). The results also show that there is a lack of habituation over days, in both strains of DAT-CI mice relative to their wild-type controls (Fig. 3.2). In a repeated-measures ANOVA for locomotion over days, there was a day x genotype interaction ($F_{3, 24} = 8.080; p < 0.01$). Wild-type mice exhibit habituated locomotion on day 6 relative to day 1 (main effect; $p < 0.01$) whereas DAT-CI mice have either unchanged (hybrids) or higher locomotion (C57-congenic: $p < 0.01$) after repeated exposure (Fig. 3.2). The C57-congenic DAT-CI mice are significantly more hyperactive than their 129B6 counterparts after repeated exposure ($p < 0.001$).

In addition to altered between-session habituation, there are differences in within-session habituation between the strains of DAT-CI mice. In a repeated-measures ANOVA for locomotion over 5-minute bins of the day 1 data (Fig. 3.1A), there was a main effect of time ($F_{5, 70} = 11.754; p < 0.001$), of genotype ($F_{1, 14} = 7.899; p < 0.05$), and a time x genotype interaction ($F_{5,70} = 4.287; p < 0.01$). The time-course results also show
that the C57-congenic DAT-CI mice have less efficient habituation than the 129B6 mice. There is a time x strain interaction ($F_{1,14} = 5.961; p < 0.5$) for locomotion, and a one-way ANOVA identifies differences between specific time points across the two strains of DAT-CI mice ($p < 0.01$ for latter 15 minute time-bin). The daggers in figure 3.1A (subdivided by 5-minute bins) denote repeated measures ANOVA pairwise comparisons ($p < 0.05$) between DAT-CI mice.
Mice were placed in 40 x 40 cm boxes for 30 min, and their horizontal movement was recorded in six, daily sessions. (A) The time-course for the locomotion of two strains of DAT-CI mice in the first session – the C57-congenic DAT-CI mice (n = 8) habituate less efficiently than the 129B6 mice (n = 8). (B) The total locomotion during the first session for both the mutant (filled bars) and wild-type mice (open bars) for each of the two strains (n = 8, in all four groups). DAT-CI mice are more hyperactive than wild-type mice in both cases (*, p < 0.05 within strains; ‡, p < 0.05 between strains of DAT-CI mice). All data are means ± SEM.
Figure 3.2 – C57-Congenic DAT-CI mice grow more hyperactive over days of repeated testing

Mice were placed in 40 x 40 cm boxes for 30 min, and their horizontal movement was recorded in six daily sessions. Presented is the day 1 (open bars) vs. day 6 (filled bars) comparison of locomotor results for both the wild-type and DAT-CI mice in both strains (129B6 n = 8; and C57-congenic n = 8). Wild-type mice (n = 8 in each strain) display habituation by the sixth session, whereas DAT-CI mice do not (main effects). Notably, the C57-congenic DAT-CI mice undergo significant “reverse habituation”, causing differences between the strains on day six (double dagger). (*, p < 0.05 within strains; †, p < 0.05 between strains). All data are means ± SEM.
Amphetamine has a locomotor suppressive phase of the dose-response curve for both strains of DAT-CI mice

We also aimed to determine whether the change in genetic background, due to backcrossing, was responsible for differences in the amphetamine response of DAT-CI mice which we saw over time. Therefore, a thorough dose-response curve for amphetamine-induced locomotion was characterized (0, 2.5, 5, 10, and 20 mg/kg doses) for DAT-CI mice. Wild-type mice from both strains were also tested with the same doses, with the omission of 10 mg/kg.

For wild-type mice (Fig. 3.3A), a two-way ANOVA shows that there was a main effect of the drug dose ($F_{3,41} = 33.619; p < 0.001$) and of the testing phase ($F_{1,41} = 68.207; p < 0.001$), and there is a phase x dose interaction ($F_{3,41} = 47.624; p < 0.001$). The Bonferroni post hoc results show that 129B6 wild-type mice have significant locomotor stimulation at most doses (2.5A: $p = .903$; 5A: $p < 0.001$; 20A: $p < 0.001$) when comparing post-injection locomotion to their respective pre-injection scores. The C57-congenic wild-type mice have significant locomotor stimulation only at 5 mg/kg amphetamine ($p < 0.001$). The number of mice used, for each strain and at each dose, is as follows: 129B6 – (0A: n = 3, 2.5A: n = 5, 5A: n = 4, and 20A: n = 9) C57 congeneric – (0A: n = 3, 2.5A: n = 6, 5A: n = 7, 20A: n = 8).

For DAT-CI mice (Fig. 3.3B), a two-way ANOVA shows that there was a main effect of the drug dose ($F_{4,68} = 3.073; p < 0.05$) but not of the testing phase ($F_{1,68} = 3.734; p = 0.057$). There was also a significant phase x dose interaction ($F_{4,68} = 20.203; p < 0.001$). The Bonferroni post hoc results show that the C57-congenic DAT-CI mice do
not have locomotor stimulation even at the very high-dose (20 mg/kg) of amphetamine (p = 0.146). On the other hand, the 129B6 hybrid DAT-CI mice have significant locomotor stimulation at this dose (p < 0.001). Furthermore, both strains have the paradoxical calming effect at lower doses (2.5 mg/kg) of amphetamine (Hybrid: p < 0.01 Congenic: p < 0.001). The C57-congenic DAT-CI mice also have suppression to saline (p < 0.05) and 5 mg/kg amphetamine (p < 0.001), which is in contrast to hybrid DAT-CI mice. The apparently reduced activity after saline injection (relative to before injection) is expected if mice have no significant response to saline, but continue to habituate. The number of mice used, for each strain and at each dose, is as follows: 129B6 – (0A: n = 10, 2.5A: n = 6, 5A: n = 10, 10A: n = 7, and 20A: n = 8) C57 congenic – (0A: n = 8, 2.5A: n = 8, 5A: n = 6, 10A: n = 4, 20A: n = 6).
Figure 3.3 – Amphetamine-induced locomotor dose-response curves for both strains of DAT-CI mice

Mice were placed into a 25 x 25 cm open-field apparatus and allowed to habituate for 45 min. (A) Wild-type mice from both strains were injected with amphetamine at the doses indicated (0, 2.5, 5, and 20; in mg/kg), and returned to the apparatus for another 45 min. The locomotion over the 30 minutes before injection (open bars) and after injection (filled bars) is plotted. Both strains of wild-type mice have locomotor stimulation, but never suppression, from amphetamine (*, p < 0.05; relative to pre-injection locomotion). (B) DAT-CI mice from both strains were also injected with amphetamine at the same doses, plus 10 mg/kg, and measured in the same way. Both strains of DAT-CI mice have suppression of locomotion from 2.5 mg/kg amphetamine, but only the 129B6 DAT-CI mice have locomotor stimulation from 20 mg/kg. All statistics are comparisons of post-injection locomotion relative to the corresponding pre-injection score. All data are means ± SEM.
Figure 3.3
3.4 Discussion

In this study, we generate a second line of knock-in mice with a cocaine-insensitive dopamine transporter (DAT-CI mice) that are a 129B6 hybrid, in addition to the C57-congenic mice already available. We used both strains of DAT-CI mice in a side-by-side comparison of spontaneous and amphetamine-induced locomotion. One aim was to determine whether changes in the genetic background could explain the locomotor responses to amphetamine observed recently that are discrepant to those observed in earlier studies. DAT-CI mice have been shown to exhibit phenotypes considered representative of human ADHD symptoms (Castelli et al., 2011; Napolitano et al., 2010). Thus the second aim was to characterize any ADHD-related phenotypes that are also affected by the genetic background. A recent bioinformatics study shows that both genetic and environmental factors contribute to ADHD in humans (Hudziak et al., 2005). Further studies using these two mouse lines may reveal possible genetic factors contributing to ADHD.

We found that DAT-CI mice in both strain backgrounds had elevated locomotion relative to their respective wild-type littermates (Fig. 3.1B), indicating that reduced DAT function leads to hyperlocomotion. We also found that the 129B6 DAT-CI mice had lower spontaneous locomotion relative to their C57-congenic counterparts. Since wild-type 129B6 hybrids also have lower spontaneous locomotion than wild-type C57 mice, the difference in locomotion between the two strains of DAT-CI mice appears to be due to a main effect of strain.
However, we also found that the C57-congenic DAT-CI mice had a less efficient within-session locomotor habituation compared to the hybrid DAT-CI mice (Fig. 3.1A) and a significant “reverse habituation” over repeated testing (Fig. 3.2). The persistence of hyperactivity within a familiar environment is an important characteristic of ADHD present in purported rodent models (Williams et al., 2009), that is not present in normal mice (Voikar et al., 2004). The lack of within-session habituation observed in the C57-congenic DAT-CI mice, but not in the 129B6 DAT-CI mice (Fig. 3.1A), therefore suggests that certain genetic variants that are different between the two strains play an important role in the hyperactive aspect of ADHD. The further potentiation of this effect across sessions, again present in the C57 congeneric but not 129B6 DAT-CI mice (Fig. 3.2), strengthens this conclusion.

Another finding is the difference in the amphetamine response between the two strains of DAT-CI mice. Both strains exhibit a therapeutic calming effect of amphetamine at low doses. In contrast, the 129B6 DAT-CI mice have a stimulatory phase at high doses while the C57-congenic DAT-CI mice are not stimulated by amphetamine, even at 20 mg/kg (Fig. 3.3B). This may be viewed as a main effect of strain, since C57-congenic wild-type mice also do not have locomotor stimulation in response to 20 mg/kg amphetamine (Fig. 3.3A).

Amphetamine, at low doses, has a calming effect in ADHD patients and is one of the frequently prescribed drugs for the disorder. In contrast, a high dose of the drug is a powerful psychostimulant. The double biphasic dose-response curve for amphetamine in 129B6 DAT-CI mice is similar to the amphetamine effect in human ADHD patients.
(Stein et al., 2011). The behavior of other models – such as the complete lack of stimulation found in the C57-congenic DAT-CI mice – is less similar to ADHD patients. A heavily studied rat model of ADHD notably lacks amphetamine’s calming effect (Langen and Dost, 2011). In this regard, the 129B6 hybrid DAT-CI mice have good face and predictive validity as an ADHD animal model.

Although this study focuses on hyperactivity, ADHD has two main components: attention deficit and hyperactivity. Additional studies on the attention of these mice are needed to further validate the mouse models. It may also be interesting and important to characterize impulse control and reward deficits in DAT-CI mice as these related dysfunctions have been seen in ADHD and comorbid disorders, but are less studied (Sagvolden, 2011; Volkow et al., 2009). In this context, having even more than two background strains characterized with the locomotor phenotype would be beneficial, because each may have varying degrees of attentional deficits. Studies containing both genetic and behavioral analysis of multiple DAT-CI strains – each containing partially overlapping phenotypes and genetic backgrounds – would be best suited for identifying common causes of hyperactivity and inattention.

While the specific genetic underpinnings of the ADHD phenotypes and therapeutic responses are not well understood, there is an increasingly clear involvement of a network of related genetic factors (Poelmans et al., 2011). Many of these genes encode products related to the dopamine system, but the involvement of DAT specifically, is controversial (Contini et al., 2010; Kooij et al., 2008) (for review, see (Thapar et al., 2005)). Some studies have indicated that increases in dopamine function
are associated with ADHD, while others have found the opposite effect, or bidirectionality (Granon et al., 2000; Russell, 2002; Viggiano et al., 2003). Still others have found that NET/norepinephrine function is more critically associated with the symptoms (Viggiano et al., 2004). Importantly, imbalances in single neurotransmitters between interacting regions have been observed (Ventura et al., 2004), and likely make many of these distinctions overly simplified. Our results support the dopamine hypothesis, without excluding a potential role for norepinephrine in these phenotypes. Our results also show a clear involvement of “background” genetic elements that do not co-segregate with the DAT mutations selected during genotyping.

These results show that differences in the genetic background of DAT-CI mice affect several ADHD-related phenotypes: the spontaneous locomotion, the habituation to an environment, and the responses to amphetamine, a drug used to treat ADHD. ADHD is a highly heritable and polygenic disorder in humans and the contributing genetic factors are not known. Additional comparison studies using the congenic C57-congenic and 129B6 DAT-CI mice may reveal genetic polymorphisms and underlying mechanisms that contribute to the variations in the severity of ADHD symptoms and the variations in responses to drug treatment.
3.5 Materials and Methods

Animal Subjects

In these studies, a knock-in mouse line containing three point mutations (L104V/F105C/A109V) in the dopamine transporter gene was used. These mice (DAT-CI mice) were generated as described previously (Chen et al. 2006). In brief, a targeting vector containing the point mutations was incorporated into the genome of ES-cells derived from 129/SvJ mice. Positive ES cell clones were used to generate the chimeric founder mice in a series of embryonic procedures (Yale University Core Facility). These chimeras were then backcrossed to C57Bl/6J as is conventional for behavioral analysis. Heterozygous F₁ offspring indicated successful germline transmission of the ES-cell DNA. This F₁ generation would be an approximately 1:1 mixture of the C57 and 129 strains. Until backcrossing to the C57 line was completed, these mice were varying mixtures of the C57 and 129 parent strains. Backcrossing heterozygous DAT-CI mice to C57Bl/6J mice (Jackson Laboratories) for at least 10 generations would produce greater than 99% genetic homology with the C57 strain. Heterozygous male and female F₁₄ mice were bred to produce wild-type and mutant C57-congenic mice used as a behavioral cohort in this study.

In order to generate the other experimental cohorts, the 129 strain background was reintroduced by crossing male homozygous F₁₄ DAT-CI mice with female 129/SvImJ mice (Jackson Laboratories). The heterozygous siblings were crossed,
generating wild-type, heterozygous, and homozygous DAT-CI mutants on an approximately 1:1 mixture of C57Bl/6J and 129/SvImJ backgrounds (129B6 mice). All mice were kept in standard housing conditions, including ad libitum access to food/water and 12 hours each of dark/light. Only male mice were used, and all mice were between 6-10 weeks of age at the time of behavioral testing. All animal procedures were approved by The Ohio State University Internal Laboratory Animal Care and Use Committee (ILACUC).

**Drugs Administered**

Mice were administered either a single dose of amphetamine sulphate (2.5, 5, 10, and 20 mg/kg) dissolved in 0.9% saline, or the vehicle alone, intraperitoneally (i.p.). The solutions were prepared so that 10µL was injected for every gram of body weight.

**Amphetamine dose-response experiments**

Mice were habituated to handling for three days prior to behavioral testing. On the test day, mice were brought into the experiment room and acclimated for one hour. They were then placed individually into 25 x 25-cm acrylic boxes where their locomotor activity was recorded by the AnyMaze (Stoelting) software system for 45 minutes. The mice were then immediately injected with either saline, or amphetamine, and their locomotor activity was recorded for another 45 minutes. The mice were then removed, and their genotypes confirmed.
It should be noted that nearly 75 mice were needed for the double biphasic DAT-CI locomotor dose-response curves for amphetamine, depicted in figure 3.3B. For wild-type controls, only three of the doses were performed (n = 45) because their dose-response curve is not thought to have a suppressive phase at low doses, and therefore requires less definition. Furthermore, a 25 cm² apparatus was used – rather than a more conventional 40 cm² apparatus – for higher throughput because the total number of mice to be tested was very large.

Locomotor habituation experiments

Mice were habituated to handling for three days prior to behavioral testing and acclimated to the room on test days. They were placed into 40 x 40-cm acrylic and their locomotor activity was measured for 30 minutes, once each day. A total of six such sessions were carried out on the subsequent days. The 40 cm² apparatus was used in this case, because direct comparison with similar experiments (Zhuang et al., 2001) is desirable, and the larger apparatus should be better for detecting subtle differences in spontaneous locomotion and habituation.

Data Analysis

For the spontaneous locomotion results, a repeated measures ANOVA was used to detect within-session habituation for the two strains of DAT-CI mice (Fig. 3.1A). A repeated measures ANOVA was also used to detect habituation/hyperactivity induced by
repetitive, daily testing within the groups (Fig. 3.2). The Bonferroni-Dunn procedure was used to determine pairwise differences between various groups (Fig. 3.1A/B & Fig. 3.2).

For the amphetamine dose-response locomotor results (Fig. 3.3), a two-way ANOVA (testing phase and dose as fixed factors) was performed on each of the four genotypes (wild-type and mutant; at both strains). The Bonferroni-Dunn procedure was then used to detect amphetamine effects at specific doses.

The data were analyzed with SPSS (ver. 19). The Bonferroni correction was used for all main effect models, and the Welch ANOVA was used when there were unequal variances (Fig. 3.3A). In the figures, asterisks represent differences within strains, or between DAT-CI mutants and their wild-type littermates. The double daggers represent differences between the strains of DAT-CI mutants.
Chapter 4: Genetic modulation of cocaine-induced reward behavior

This chapter is adapted from a previous publication (O’Neill, B., Tilley M.R., & Gu H.H.)

4.1 Abstract

Cocaine is an inhibitor of the dopamine, norepinephrine, and serotonin reuptake transporters. Because its administration would therefore elevate signaling of all these three neurotransmitters, many studies have been aimed at attributing individual effects of cocaine to specific transmitter systems. Using mice with a cocaine insensitive dopamine transporter (DAT-CI mice), we previously showed that cocaine-induced dopamine elevations were necessary for its rewarding and stimulating effects. In this study, we observe that DAT-CI mice exhibit cocaine-conditioned place aversion, and that its expression depends on their genetic background. Specifically, DAT-CI mice backcrossed to the C57Bl/6J strain background did not display a preference or an aversion to cocaine, whereas DAT-CI mice that were on a mixed 129S1/SvImJ x C57Bl/6J (129B6) background had a robust conditioned place aversion to cocaine. These results indicate that while inhibition of the dopamine transporter (DAT) is necessary for cocaine reward, other cocaine targets and neurotransmitter systems may mediate the aversive properties of cocaine. Furthermore, the aversive effect of cocaine can be observed in the absence of
a DAT-mediated rewarding effect, and it is affected by genomic differences between these two mouse strains.

### 4.2 Introduction

The primary molecular actions of cocaine are the inhibition of the dopamine, norepinephrine, and serotonin transporters; with additional low-affinity targets that seem less relevant to cocaine’s behavioral effects at moderate doses (Han & Gu 2006; Ritz et al. 1987). The behavioral effects of cocaine include motor stimulation and reinforcement in rodents. In humans cocaine produces an intense euphoria and is considered very addictive.

Cocaine is also known to have negative psychomotor effects after the peak of euphoria, which are thought to indirectly reinforce repeated drug taking (Ahmed & Koob 2005; Leventhal et al. 2011; Newton et al. 2003). However, if the negative effects are more concurrent with reward, they would be expected to compete with reward and discourage drug taking. There is an observable approach-avoidance response conflict in rats for cocaine, due apparently to its anxiogenic properties (Guzman & Ettenberg 2007; Koob 1999). This behavior is evidence of such a competition.

Normally, if drugs of abuse are administered to mice in a conditioned place-preference (CPP) paradigm, they result in a positive preference for the drug-conditioned context – indicating that it was a rewarding experience. However, if the animal is placed into the conditioning apparatus with some delay after cocaine is administered, a negative preference or conditioned place aversion (CPA) for the drug-conditioned context is
expressed. This is true for many drugs, even if they are generally addictive (Fudala & Iwamoto 1990). On the other hand, evidence for an acute aversive effect in the place-preference paradigm is lacking.

Additionally, the balance between rewarding and aversive effects may be influenced by genetics. This seems to be the case for methamphetamine in a model where genetically divergent subpopulations of mice display differences in total consumption. The high-consuming group has been shown to be less sensitive to the aversive effects of methamphetamine (Wheeler et al. 2009), and to have gene expression differences for DARPP-32, GLUR1, and GAB1 (Palmer et al. 2005).

In this study, we sought to investigate whether an acute aversive effect could be observed for cocaine and how strain backgrounds may modulate this effect. Previously, we created knock-in mice with a cocaine-insensitive dopamine transporter (DAT-CI mice). It was shown that inhibition of the dopamine transporter is necessary for cocaine reward – as evidenced by the lack of CPP and self-administration of the drug (Thomsen et al. 2009; Tilley et al. 2009). Initially, while the DAT-CI line was still on a mixed 129B6 background, the mice displayed an aversive response to cocaine (Chen et al. 2006). This aversive response seemed to be lost in various experiments performed after backcrossing to the C57 strain for more than 10 generations – when the mice displayed neither a preference nor aversion to cocaine. It was unclear whether this was truly due to strain genetics, or due to epigenetic or experimental differences that may have also changed over time. We therefore recreated a mixed 129B6 background DAT-CI mouse
line, from contemporaneous C57-congenic breeders in order to directly test the influence of strain backgrounds in side-by-side experiments.

4.3 Results

We observed that DAT-CI mice displayed an aversive response to cocaine inconsistently: with an early experiment finding robust CPA to cocaine and a more recent experiment failing to replicate this effect. In this study, we sought to investigate whether genetic drift was responsible for this inconsistency. We compared the cocaine-induced conditioned place-preference/aversion response of three groups of DAT-CI mice. Group 1 mice are from early generations of breeding – before complete backcrossing – and therefore contain a mixture of C57 and 129 strain backgrounds. Group 2 mice are from later generations – after backcrossing – and therefore on a C57-congenic background. We created group 3 mice in order to test the hypothesis that behavioral differences between groups 1 and 2 are due to their genetic differences, and are not an effect of changing experiment or other environmental factors. Therefore, group 1 mice were analyzed separately in a one way ANOVA, and groups 2 and 3 mice were analyzed in a two way ANOVA in order to detect a strain interaction.

Group 1

Group 1 mice were homozygous DAT-CI mice and their wild type littermates. These mice were studied before the extensive backcrossing procedure and thus had a mixed background of 129 and C57 strains as described in detail in the “Methods” section.
The mice were examined with the CPP/CPA procedure and the results are shown in figure 4.1. Two-way analysis of variance (ANOVA) of CPP/CPA post-test scores (open bars in figures) was performed within this data set (Fig 4.1A, B) – with genotype and drug treatment as between-subject factors. There was no main effect of drug ($F_{1,36} = 1.211; p = 0.278$), but there was a main effect of genotype ($F_{1,36} = 16.964; p < 0.001$) and a genotype x drug interaction ($F_{1,36} = 18.389; p < 0.001$). Bonferroni-Dunn post hoc test show that there was a significant CPP ($p < 0.01$) in the group 1 wild-type controls, and a significant CPA ($p < 0.05$) in the DAT-CI littermates for 20 mg/kg cocaine.
Figure 4.1 – Conditioned Place-Preference of Group 1 Mice

Mean ± SEM of CPP scores. Group 1 mice were of an early backcrossing generation (F2) and have a mixed genetic background consisting of a roughly 1:1 ratio of the 129 and C57 strains. CPP scores are defined as the time spent in the drug-designated environment minus the time spent in the saline-designated environment. Control mice receive saline in both environments. (a) Wild-type mice (cocaine: n = 8, saline: n = 10) show a robust CPP induced by 20 mg/kg cocaine. (b) DAT-CI mice (cocaine: n = 14, saline: n = 8) showed significant CPA induced by 20 mg/kg cocaine. All statistics are two-way ANOVA comparisons of the time bias (CPP score) observed post-conditioning (open bars) relative to saline-conditioned control mice within the figure (*, p < 0.05; **, p < 0.01).
Figure 4.1

A  
F₂ wild-type  
- Before Conditioning  
- After Conditioning  
Time in CS - US (s)  
Saline  
Cocaine  

B  
F₂ DAT-CI  
- Before Conditioning  
- After Conditioning  
Time in CS - US (s)  
Saline  
Cocaine  

Figure 4.1
Groups 2 and 3

Group 2 mice are C57-congenic and derived from group 1, by backcrossing with wild-type C57 mice for 12 generations. Group 3 mice were generated by crossing group 2 mice with wild-type 129S1/SvImJ mice, and thus they are analogous to group 1 in that both groups are on a 129B6 mixed strain background. These groups were examined with the CPP/CPA procedure and the results are shown in figures 4.2 and 4.3. Two-way ANOVA of CPP/CPA post test scores was performed within these data sets – with genetics and drug treatment as between-subject factors; with genetics meaning the true genotype, involving both the mutation and strain. There was no main effect of drug (F$_1$, 63 = 3.231; p = 0.078), but there was a main effect of genetics (F$_3$, 63 = 4.977; p < 0.005) and a genetics x drug interaction (F$_3$, 63 = 7.758; p < 0.001).

Bonferroni-Dunn post hoc tests show that there was no significant effect of 20 mg/kg cocaine (compared to saline) in group 2 DAT-CI mice (Fig 4.2B; p = 0.470), but there was a significant CPP in their wild-type controls (Fig 4.2A; p < 0.001). Importantly, Bonferroni-Dunn post hoc tests show that there was a significant CPA in group 3 DAT-CI mice (Fig 4.3B; p < 0.01). However, the large apparent CPP of their wild-type controls did not reach statistical significance (Fig 3a; p = 0.070). This is apparently due to a larger variance in this group, which is perhaps a result of the genetic heterogeneity of 129B6 mice compared to C57-congenic mice.

A priori hypothesis testing shows that group 2 and group 3 DAT-CI mice had significantly different responses to 20 mg/kg cocaine (F$_1$, 63 = 6.417; p < 0.05) with group 3 having significant CPA and group 2 having no response to cocaine. Since group 3
wild-type mice had a reduced CPP score compared to group 2 wild-types, a similar *a priori* hypothesis test was conducted on the wild-type mice. This test does not indicate that there was a significant difference between the wild-type control mice of the two groups, in response to 20 mg/kg cocaine (F$_{1,63} = 2.00$; p = 0.162).
Figure 4.2 – Conditioned Place-Preference of Group 2 Mice.

Mean ± SEM of CPP scores. Group 2 mice have been backcrossed to the C57 strain for 14 generations (F14) and have a >99.9% C57-congenic background. (a) Wild-type control mice (cocaine: n = 7, saline: n = 8) show a robust CPP induced by 20 mg/kg cocaine. (b) DAT-CI littermates (cocaine: n = 8, saline: n = 8) failed to show CPP or CPA to 20 mg/kg cocaine. All statistics denoted by asterisks are two-way ANOVA comparisons of the time bias (CPP score) observed post-conditioning (open bars) relative to saline-conditioned control mice (***, p < 0.001). An a priori hypothesis test was conducted for the between group (2 and 3) comparison of the DAT-CI mice responses (‡, p < 0.05).
Figure 4.2

A  C57-congenic wild-type

- Before Conditioning
- After Conditioning

Saline  Cocaine

B  C57-congenic DAT-CI

- Before Conditioning
- After Conditioning

Saline  Cocaine

Figure 4.2
Figure 4.3 – Conditioned Place-Preference of Group 3 Mice.

Mean ± SEM of CPP scores. Group 3 mice have a roughly 1:1 ratio of 129 and C57 strain backgrounds, via outcrossing the C57-congenic group 2 mice to 129S1/SvImJ mice. (a) Group 3 wild-type control mice (cocaine: n = 16, saline: n = 8) show an apparently large trend toward CPP induced by 20 mg/kg cocaine (p = 0.070), but this result is not statistically significant due to larger variations than in the inbred mice. (b) 129B6 DAT-CI littermates (cocaine: n = 8, saline: n = 8) show a robust CPA induced by 20 mg/kg cocaine in contrast to group 2 DAT-CI mice (a priori contrast, denoted by ‡; p < 0.05). All other statistics are comparisons of the time bias (CPP score) observed post-conditioning (open bars) relative to saline-conditioned control mice (*, p < 0.05; **, p < 0.01).
Figure 4.3
4.4 Discussion

In the present study, we observe that mice with a cocaine insensitive dopamine transporter (DAT-CI mice) express aversion to cocaine – or indifference to it – depending on their genetic background. Other studies, involving delayed conditioning, had shown that an aversive effect can be observed for addictive drugs (Fudala & Iwamoto 1990). In contrast, we report here that DAT-CI mice on a mixed 129B6 background (groups 1 and 3) have a robust conditioned place aversion to immediate conditioning with cocaine. Since our conditioning was not delayed, we conclude that cocaine can have an aversive action that is to some degree acute and concomitant with the rewarding effect, as opposed to a delayed withdrawal effect.

The hedonic allostasis, or opponent process theory states that if the aversive effects of a drug are delayed relative to the rewarding effects, then they may promote drug taking in order to delay the aversion once again (Ahmed & Koob 2005). This mechanism is then used to explain the escalation of intake over time. Indeed, the sensitivity to methamphetamine’s aversive effect has been correlated with total intake in a mouse model of high versus low consumption (Shabani et al. 2011).

The current experimental results are helpful in updating or extending the theory on the aspect of timing of the opponent processes. Apparently, the rewarding and aversive processes may not be much separated in time, as is commonly thought when equating aversion with the withdrawal syndrome. In wild-type littermates, where the DAT-mediated rewarding effect is present, a robust CPP is induced by immediate conditioning with cocaine. In contrast, a robust CPA is induced by immediate cocaine
conditioning in the 129B6 DAT-CI mice. This is an important detail, because it suggests that cocaine’s aversive effects are to some degree concurrent with its rewarding effect in wild-type mice.

In this regard, experiments involving strains of readily available wild-type mice, especially hybrid mice, may be sufficient to study factors involved in reward and aversion on the genetic level. The DAT-CI mutation affords the unique ability to observe the effects of these genetic factors on the behavioral level. For example, eliminating DAT inhibition promotes an aversive effect of cocaine in group 1 and group 3 DAT-CI mice. This is not the case in group 2 DAT-CI mice, where cocaine induces neither a CPP nor a CPA. A previous study which used multiple doses of cocaine in C57-congenic (group 2-like) DAT-CI mice also did not observe CPA (Tilley et al. 2009). Therefore, it appears that the unique loss of both CPP and CPA in group 2 DAT-CI mice is a fully qualitative difference due to genetics.

We can therefore hypothesize that the aversive actions of cocaine are normally mediated by its non-DAT targets, but that these actions are not readily manifest in the C57 strain. Interestingly, we found in another study that the DAT/NET inhibitor methylphenidate does not induce either reward or aversion (Tilley & Gu 2008). Studies using the cocaine conditioned taste aversion test in transporter knock-out mice support the idea that inhibition of NET and/or SERT – as opposed to DAT – are primarily involved in aversion (Jones et al. 2009). Taken together, these data suggest that both NET and SERT inhibition are involved in cocaine’s aversive effect.
Studies with genetically heterogeneous mice, such as mice in group 1, could also identify biomarkers of addiction risk. Several genes such as cFos, Slc6a4 (SERT), and Htr3a (a serotonin receptor) have been correlated with differences in methamphetamine sensitivity across strains (Wheeler et al. 2009). Differences in genes such as these may influence cocaine aversion or any effects mediated through inhibition of SERT and NET. Therefore, strain differences in modifier genes may explain the observed differences in CPA behavior, even though both strains contain the same mutation in DAT. It is important to realize that to some degree, even inbred mice presumed to be genetically homogenous are not so, due to retrotranspositions; and there are certainly different transpositions in the C57 and 129 strains (Akagi et al. 2008).

Not all cocaine-induced negative states proceed rapidly after inhibition of a cocaine target. Most models accept that addiction involves maladaptive, cumulative, drug-induced neurological changes termed “metaplasticity,” within the reward circuit. The mechanisms involved may include long, slow-acting plasticity such as synaptic and whole-cell remodeling, or neurodegeneration (Achat-Mendes et al. 2005; Lee et al. 2011; Russo et al. 2010). However, an acute aversive effect and a delayed or protracted antireward process are not mutually exclusive, and may play different roles in the drug response. The current data suggest an acute action of cocaine as the cause for its negative effects – when they are defined as negative reinforcement, or aversion.

In summary, DAT-CI mice can express an aversion to cocaine, and this effect is modulated by the genetic background. The aversive effect is to some degree concurrent with the rewarding effect that is expressed in wild-type controls. These findings are
important because the aversive effect observed may be the reason for certain individuals having low propensity to cocaine addiction. The DAT-CI mouse strains can be used further, in molecular studies, to identify the differences in the genetic background that are critical in modifying their behavioral responses. Furthermore, behavioral studies that build upon this characterization, with a manipulation of the norepinephrine system for example, may uncover the mechanism of drug-induced aversion. Studies such as these could identify therapeutic strategies that exploit a more potent, acute aversive response in order to halt an affected individual’s drug taking.

4.5 Materials and Methods

Animals Subjects

In these studies, a knock-in mouse line containing three point mutations (L104V/F105C/A109V) in the dopamine transporter gene was used. These mice (DAT-CI mice) were generated as described previously (Chen et al. 2006). Briefly, a site-specific targeting construct was generated by PCR assembly. The construct was introduced into mouse embryonic stem (ES) cells derived from the 129/SvJ strain (129 mice), and positive ES cell clones were used to generate the chimeric founder mice (Yale University Core Facility). These chimeras were then crossed to wild-type C57Bl/6J mice (C57 mice). Heterozygous F1 offspring would be on a 1:1 mixture of C57 and 129 background at this point, due to successful germline transmission of the 129/SvJ derived ES-cell DNA.
Group 1 mice (DAT-CI n = 14, and 8 controls; Wild-Type n = 8, and 10 controls) were directly derived from sibling pairings of the F₁ heterozygotes described above. These mice were then bred to a C57-congenic CRE recombinase-expressing mouse line (Jackson Laboratories, B6.FVB-Tg(EIIa-cre)C5379Lmgd/J) in order to remove the Neomycin (Neo) selection marker that is flanked by two loxP sites. Heterozygous breeders from the Neo-removed (F₂) progeny were used to generate wild-type and homozygous mutant mice for the experiments depicted in figure 4.1. These mice would contain an approximately 1:1 ratio of 129 and C57 backgrounds.

Group 2 mice (DAT-CI n = 8, and 8 controls; Wild-Type n = 8, and 8 controls) were generated by backcrossing the F₂ generation described above to wild-type C57Bl/6J mice for an additional twelve generations, producing F₁₄ mice. Heterozygous male and female F₁₄ mice were paired to generate wild-type and homozygous mutant mice for the experiments depicted in figure 4.2. These mice share greater than 99.9% of their genetic background with the C57Bl/6J strain.

Group 3 mice (DAT-CI n = 8, and 8 controls; Wild-Type n = 16, and 8 controls) were generated by outcrossing the C57-congenic DAT-CI mice described above with wild-type 129S1/SvImJ for one generation. The heterozygous offspring contain a 1:1 mixture of C57 and 129 backgrounds, and were sibling paired in order to generate DAT-CI mutants. Their wild-type and homozygous mutant progeny were used in experiments depicted in figure 4.3. The full designation of the strain is (129S1/SvImJ x C57BL/6J)F₂ and it is abbreviated as 129B6.
All mice were kept in standard housing conditions, including ad libitum access to food/water and 12 hours each of dark/light. Only male mice were used, and all mice were between 6-12 weeks of age at the time of behavioral testing. All animal procedures were approved by The Ohio State University Internal Laboratory Animal Care and Use Committee (ILACUC).

**Drugs Administered**

Cocaine hydrochloride was provided by the National Institute on Drug Abuse drug supply program. Cocaine was dissolved in 0.9% sterile saline and injected intraperitoneally (i.p.) at a dose of 20 mg/kg and a volume of 0.1 mL/10g body weight.

**Conditioned Place-Preference Test**

Conditioned place-preference/aversion testing was performed in 12.5 cm x 42.5 cm acrylic boxes subdivided into three interconnected compartments: two side compartments (12.5 cm x 17.5 cm) and a center compartment (12.5 cm x 7.5 cm). The three compartments were made visually and tactiley distinct from one another by cues, creating three different “environments”. An unbiased paradigm (balanced with respect to pre-conditioning preferences) consisting of a pre-conditioning preference test, a conditioning phase, and a post-conditioning preference test was performed as described previously (Tilley et al. 2009). For the pre/post conditioning preference tests, mice had access to all three compartments. Their preference was defined as the difference in time spent in one side compartment versus the other. Mice were assigned to receive cocaine in
one environment or the other, based on their natural preference determined during the pre-conditioning test. The assignments were selected such that the average natural preference of each experimental group was minimized, by counterbalancing equal-sized subgroups.

On the first day of conditioning, half the mice received a 20 mg/kg cocaine injection (i.p) and half received the vehicle (0.9 % saline). The mice were then immediately confined for 30 minutes to the environment assigned to them for that day/treatment. Each mouse received the alternate treatment (in alternating environments) on subsequent days, for a total of 8 days.

After conditioning (paradigm day 10) mice were tested for their post-conditioning preference using the same unconfined apparatus configuration as the pre-test. Pre/post-test sessions were performed without an injection. During these sessions, mouse behavior was recorded by overhead cameras and analyzed by the AnyMaze software (Stoelting Co.). The time spent in the drug-designated compartment subtracted from the time in the vehicle-designated compartment is a mouse’s preference, or CPP score. Any change in the preference between the pre and post-conditioning tests is thought to be due to the rewarding action of the drug, which can have either a positive or negative reward valence (punishment). Additional groups, for which vehicle was administered in both environments, were also tested as controls. Any change in the bias between the pre and post-conditioning tests in these groups are indicative of noise in the paradigm.
**Statistical Analysis**

Statistics were performed using SPSS statistical software (ver. 19, IBM). Two-way ANOVA was performed, with drug treatment and genotype as fixed factors and with CPP score as the dependent measure. The CPP score was defined as time spent in the drug-designated compartment minus the time spent in the vehicle-designated compartment during the 30-minute preference tests. One-way ANOVA was then performed within genotypes having significant main effects and the Bonferroni-Dunn procedure was used to determine which groups had a significant conditioning effect of the drug. An *a priori* (simple) contrast was performed in order to compare between the group 2 and 3 DAT-CI responses. A significance level of $p < 0.05$ was used for all tests.
Chapter 5: A discussion of experiments on DAT-independent cocaine effects

5.1 Abstract

Cocaine is an inhibitor of the dopamine, norepinephrine, and serotonin transports. We have been discussing a knock-in mouse line with a cocaine-insensitive dopamine transporter (DAT-CI mice) in this dissertation – mostly in the context of the dopamine system. Considering that DAT-CI mice would have normal cocaine-inhibition of the norepinephrine and serotonin transporters (NET and SERT), they could also be useful in the context of understanding these systems. In fact, the finding of DAT-independent conditioned place aversion (chapter 3) is an example of success in this area. This chapter will briefly report several incomplete, or discontinued projects aimed at investigating the NET and SERT systems more completely or more directly. Although the topics are experimental, the chapter is organized unlike the data in chapters 2 – 4, and is organized more similar to the subdivided prose of the introduction and conclusion chapters.

5.2 Attempted generation of SERT-CI mice

Analogous to DAT-CI mice, we attempted to generate knock-in mice with a cocaine-insensitive serotonin transporter (SERT-CI mice). These mice would allow us to study the effects of cocaine-inhibition of SERT, in the presence of DAT and NET blockade. Studies of transporter knock-out mice had implicated SERT/5-HT mediated
signaling (along with dopamine signaling) in producing cocaine-induced reward and locomotor behaviors (Uhl et al. 2002). It would therefore be desirable to test whether cocaine blockade of SERT is necessary for these behaviors – as was done above, in the DAT-CI system. If SERT inhibition is also a necessary cause of cocaine’s behavioral effects, we would expect a similar ablation of these responses in SERT-CI mice – even though DAT inhibition is fully present. Furthermore, we would be able to detect more subtle (but complex) interactions, if those were the case – by crossing the DAT-NET-SERT CI mice. We therefore aimed to identify a suitable cocaine-insensitive SERT mutant, and to generate knock-in mice for such studies.

Our group, and the group of Dr. Randy Blakely, found a single mutant variant of the serotonin transporter (I172M) which has reduced cocaine sensitivity but which retains its uptake function (Henry et al. 2006). We attempted to improve upon this mutation – in terms of increasing the function:sensitivity ratio – by performing additional rounds of random mutagenesis. This strategy was supported by the fact that the successful DAT-CI mice and the analogous NET-CI mice both were generated from similar triply-mutated transporters (Chen et al. 2005; Wei et al. 2009). While the dopamine and norepinephrine transporters are more closely homologous to each other than the serotonin transporter is to either of them, we estimated that a triple mutant with better properties than the I172M SERT mutant could be found after some screening – however no such mutant was found.

The I172M mutant was therefore incorporated into a targeting construct that was assembled by PCR. A diagrammatic representation of the features and assembly strategy of the DNA construct is depicted in figure 5.1.
The targeting construct was a segment of mouse genomic DNA modified by PCR assemblage of exogenous elements and the I172M mutation. The left hand segment (P11 – I172M) contains the positive and negative selection markers TK and NEO. These confer either toxicity (TK) or resistance (NEO) to selection drugs used to verify that the homologous recombination event produced a site-specific knock-in of the I172M mutation. The right hand segment (I172M – P6) is essentially unmodified genomic DNA from the SERT gene that would serve to increase the rate of recombination.

Therefore, recombination in mouse ES cells – and generation of SERT-CI founder mice from these cells – would result in knocking out wild-type SERT and replacing it with a cocaine-insensitive SERT. The NEO cassette would be present in these mice, but it is located in an intron. Furthermore, because NEO is flanked by loxP sites, breeding the SERT-CI mice with CRE-expressing mice would result in a single loxP site in the intron. Thus, the only coding mutation in SERT-CI mice would be the I172M mutation.

**Figure 5.1 – Targeting Construct for Generation of SERT-CI Mice**

The targeting construct was a segment of mouse genomic DNA modified by PCR assemblage of exogenous elements and the I172M mutation. The left hand segment (P11 – I172M) contains the positive and negative selection markers TK and NEO. These confer either toxicity (TK) or resistance (NEO) to selection drugs used to verify that the homologous recombination event produced a site-specific knock-in of the I172M mutation. The right hand segment (I172M – P6) is essentially unmodified genomic DNA from the SERT gene that would serve to increase the rate of recombination.
At this point, the Blakely Lab published a study which characterized the depressive-like behavior and antidepressant response of I172M SERT knock-in mice they had generated (Thompson et al. 2011). For this reason, we discontinued the project at the mouse ES cell-targeting stage. To date, only the forgoing study regarding selective serotonin reuptake inhibitors has been published using the mice, and the Blakely Lab has indicated that there are anomalies with the line’s vigor – apparently stemming from the pieces of exogenous DNA incorporated into the introns of the mice from the construct (personal communications). It is possible that the positioning of “silent” synonymous mutations used in the PCR assembly, and in the neomycin-resistance cassette used in the ES cell-targeting (NEO, figure 5.1), both disrupt functions of the introns unrelated to protein coding.

Importantly, these features differ between the DNA constructs generated in our lab and our colleagues’ lab. If evidence arises implicating these intronic elements in the loss of vigor observed in the mice, generation of I172M SERT knock-in mice from our alternate construct could be an avenue to continuing experiments, which remain to be expanded from SSRI to cocaine research.

5.3 Amphetamine-cocaine cross-sensitization in DAT-CI mice

As mentioned earlier, DAT-CI mice are, in principle, unaltered with respect to amphetamine’s pharmacology. For this reason, amphetamine was used as a control in the initial studies that demonstrated the involvement of DAT-inhibition in cocaine’s acute effects. However, both amphetamine and cocaine have many complex, chronic, or
indirect effects that likely contribute to addiction – many of which may be “non-DAT” (Narayanan et al. 2011; Ritz & George 1993). Additionally, amphetamine is most potent at NET (Han & Gu 2006). Therefore, it is possible to use amphetamine in DAT-CI mice, in order to actually investigate the role of non-DAT influences contributing to psychostimulant addiction.

We attempted to do this by administering cocaine to DAT-CI mice with a history of amphetamine exposure. Normally cocaine has no noticeable effects in DAT-CI mice, but because amphetamine does affect the mice and is administered first, it is reasonable to expect to see cross-sensitization or adaptation of the cocaine effect (Pierce & Kalivas 1997). Especially because amphetamine is a categorically similar drug, it is likely that recruitment of a growing set of targets shared with cocaine – and potentially non-DAT targets such as SERT and NET – occurs over time.

Specifically, we aimed to 1) establish CPP in DAT-CI mice using amphetamine, 2) to allow it to extinguish through prolonged abstinence, and 3) to induce relapse by a subthreshold “tempting” dose of cocaine (to which the mice are normally “insensitive”). In this design, we are isolating the role of the DAT/DA (or non-DAT) targets in the later stages of drug experience – which cannot be done easily in many other systems.

The priming dose of cocaine has a relapse-inducing effect normally, in wild-type mice, as evidenced by many studies using the reinstatement of extinguished CPP (or self-administration of a drug) as a model of relapse (Schmidt et al. 2005). In these models, three “priming” modalities have been identified as potent triggers of relapse: a drug prime, cue prime, and stress prime.
The circuitry underlying relapse to cocaine abuse is strikingly similar to the limbic loop of the basal ganglia, depicted in figure 1.2. This diagram summarizes preexisting data on the circuitry involved in specific trigger modalities: drug-primes, stress-primes, and cue-primes. The color-coding is meant to depict the possible involvement of the transmitter (released into a given area) in modulating the function of that region in the pathways(s) it forms—and is not meant to imply any critical or necessary involvement of that transmitter.

The drug-prime modality is the least applicable to a real-world situation, however it is thought to be subserved by dopamine and/or glutamate in the “final common pathway.” Interestingly, the stress-primed pathway involves noradrenergic mechanisms in brain regions ‘upstream’ of the VTA.

**Figure 5.2 – Diagram of the Circuitry Underlying Relapse to Cocaine Abuse**
It is beginning to be clear that these modalities each have largely overlapping, but somewhat separate underlying pathways and neurochemistries. It is often assumed that there is a common “final” pathway, most closely correlated with the output or response – and subserved by dopamine (Pierce & Kumaresan 2006). The drug-prime is thought to act most directly through this common dopamine-centered pathway (Schmidt & Pierce 2006). This is a pattern of thinking that is perhaps reinforced by the established commonality of all drugs of abuse in elevating dopamine in the nucleus accumbens during their acute phase, and causing subjective pleasure (Di Chiara & Imperato 1988).

On the other hand, the stress-prime pathway is arguably the most distant, with many discoveries on the mechanisms revolving around the amygdala, CRF, and noradrenaline (Brown et al. 2009).

Our thinking was opposite in both cases, since we hypothesized that a drug (cocaine) priming dose elicits its relapse-inducing effect indirectly through a noradrenergic and stress-related mechanism. Therefore, we expected to see significant relapse to the CPP response in DAT-CI mice after administration of cocaine, even though cocaine would not increase dopamine in these mice. This is because cocaine would still inhibit the norepinephrine transporter (NET) as well as elicit its known “non-specific” effects on the immune system and HPA axis (Friedman & Eisenstein 2004).

Upon attempting to test this hypothesis, we encountered the problem of DAT-CI mice not expressing CPP for amphetamine. This was unanticipated, since it had been published that DAT-CI mice can exhibit CPP for amphetamine (Chen et al. 2006), and since amphetamine is known to be equipotent at the wild-type and mutant transporters (in
vitro and in vivo). Many different conditions and CPP configurations were tested in an attempt to finally establish CPP, and move on to the extinction and cocaine reinstatement phases.

Eventually, we surmised that the lack of CPP by amphetamine - and the discrepancy with historical data - were due to mouse strain differences in the genetic background. These numerous “failed” experiments, in fact, were what led to the project studying DAT-CI mice as an ADHD model – and the responses to amphetamine of various strains (see chapters 3 and 4). In those studies, it was confirmed that the background did in fact play a pivotal role in changing the amphetamine and cocaine response with respect to locomotion and CPP, respectively (O’Neill & Gu 2013; O’Neill et al. 2012).
Chapter 6: Concluding Remarks

The first three studies contribute significantly to our understanding of the anatomy, pharmacology, and genetics underlying cocaine-induced reward and hyperlocomotion – especially with respect to the DAT/dopamine system. The final two studies, in the preceding chapter, were attempts to investigate the involvement of NET and SERT inhibition. These fell short of the goal, but ultimately led to a better understanding of the nature of our DAT-CI mice, and knock-in/out mice generally.

The three topics discussed in the introduction are supported by previous studies, primarily involving classical neuropharmacological techniques. These laid the groundwork for understanding any research result in the dopamine or drug abuse field, but especially the results of our studies – which focus directly on reward and locomotion. Importantly, these studies clearly placed an emphasis on the dopamine system – as opposed to the serotonin and norepinephrine systems – in explaining cocaine’s effects (see introduction). However, the cutting edge on which our studies most directly build was defined only more recently, with the start of extensive research using the monoamine transporter knock-out mice. Discussing this literature, specifically in the context of our findings, is now possible and important.

Arguably, the dopamine, norepinephrine, and serotonin transporter knock-out mice were all created for the express purpose of studying cocaine’s actions. Each mouse
had certain differences in phenotype relative to wild-type mice – such as differences in vigor, spontaneous locomotion, and drug-induced locomotion (Rocha 2003) – however all three of the knock-out mice retained a cocaine-induced reward and locomotor response of some sort. Therefore, the results essentially ran counter to what would tend to be the default hypothesis – that cocaine’s effect, or parts of it, would be lost along with the removal of cocaine’s targets. This was especially expected of the dopamine transporter, given the historical evidence for dopamine’s prominent role in reward.

Only after creating a dopamine and serotonin transporter double knock-out was the anticipated loss of cocaine-induced behaviors observed (Sora et al. 2001). This suggested that cocaine exerts its effects through overlapping neurochemical pathways. It is important to note that this interaction is contrary to the anatomical one described in the introduction – in which dopamine is the main neurotransmitter involved. In fact, the multiplication of entities involved in reward and locomotion, on both the molecular and systems levels, was at a height this time.

We previously reported on the generation of mice with a cocaine insensitive dopamine transporter (DAT-CI mice). These mice have removal of the cocaine active site, without removal of the entire protein. Therefore, there is loss of cocaine inhibition of DAT without the complete loss of DAT function itself (as is the case in the knock-out mice). Unlike DAT-KO mice, DAT-CI mice had a complete loss of cocaine-induced reward and hyperlocomotion, even though targetable NET and SERT were still present (Chen et al. 2006). The contradiction between the two systems is likely a result of confounded conclusions in the knock-out studies, due to artifacts of extreme and
chronically elevated levels of neurotransmitters in the mouse model (Jones et al. 1998; Spielewoy et al. 2000). Based on the results from DAT-CI mice, it was possible to again “simplify” our understanding of these two cocaine-induced behaviors – in the sense of ascribing them to dopamine-centered systems.

We then proceeded to determine any potential anatomical or pharmacological factors giving rise to this multiplicity within the DAT/DA system. As discussed, the most natural bifurcation seemed already to be in the literature – and regarded mesoaccumbens reward versus nigrostriatal locomotor function. Since these two systems project to the ventral and dorsal striatum respectively, we hypothesized that DAT-inhibition in the ventral striatum underlies reward and in the dorsal striatum, DAT-inhibition underlies hyperlocomotion instead.

We found that restoration of wild-type DAT to the rostrolateral striatum restored cocaine-induced hyperlocomotion in DAT-CI mice (chapter 2). This manipulation did not restore reward, as measured by CPP, nor did AAV injections of DATwt in the medial accumbens or dorsal striatum – two injections which in fact did not restore either behavior. These findings allow us to conclude that DAT-inhibition in the rostrolateral striatum is critically involved in cocaine-induced hyperlocomotion. The fact that none of the injections restored cocaine induced CPP leaves the neural mechanism no more clear, but does support the idea that reward and locomotion are dissociated on the anatomical level.

The finding that DAT-CI mice do not only have a loss of conditioned place preference, but rather also exhibit a conditioned aversion to cocaine (chapter 4) is very
important. As discussed in that chapter, the direct importance of the finding is that we
can ascribe an *acute* aversive effect to cocaine, and more specifically to its actions at non-DAT targets. In this scenario, cocaine blockade of DAT is responsible for reward,
whereas cocaine blockade of NET and/or SERT is responsible for aversion. Furthermore,
the fact that the net (rewarding versus aversive) behavioral effect of cocaine is affected
by genetic variations between the two mouse strains opens the possibility of comparing
these mice in the future, as a model of individual susceptibility to addiction.

Similarly, the finding that hyperlocomotion in DAT-CI mice is sensitive to the
genetic background (chapter 3) is also important with respect to further genetic studies.
Attention deficit hyperactive disorder (ADHD) is a highly heritable disorder in humans,
with a surprising relationship to reward processes. The most salient result from this study
is the difference in habituation between DAT-CI and wild-type mice, and between the
strains of DAT-CI mice. This result may seem mundane, in part because the
experimentation is very straightforward. However, the habituation response is highly
complex and is potentially related to anxiety, attention, and reward responses to novelty
(Lubow 1997; Volkow et al. 2009) – as well as the change in these processes over time.

Taken together, these findings serve first to strengthen the original conclusion
from DAT-CI mice, that DAT inhibition is necessary for cocaine reward and locomotion.
Some may have doubted the reliability of this conclusion, and especially the claim of any
increased reliability relative to DAT knock-out studies. The predictable reversibility of
the locomotor phenotype in the AAV experiments may lessen this doubt. Furthermore,
there is lack of positive reward (CPP) to cocaine that is consistent across strain backgrounds – indicating that this finding is robust in the face of shifting genotypes.

The findings also serve to build upon the original conclusions. In DAT-CI mice with restoration of DAT_{wt} in the rostrolateral striatum, we observed hyperlocomotion induced by RTI-113 in addition to cocaine (data not shown). Since RTI-113 is a DAT-specific inhibitor, this allows us to conclude that DAT inhibition in this region is sufficient to induce a cocaine-like hyperlocomotion. With this in mind, it appears that DAT inhibition is both necessary, and in this region sufficient to cause of hyperlocomotion. This sort of bidirectional logic to the conclusion is very important, because it suggests that the so-called “DAT is it” hypothesis is accurate – with NET/SERT effects playing only a modulatory role in locomotion. This is a helpful articulation of our understanding of psychomotor stimulation. It would have been extremely impactful if a similar conclusion could be made for the mechanism of cocaine reward. Given the results, it appears likely that multiple neurotransmitters and/or brain regions interact to produce the effect.

In fact, even for a presumably more simple behavior such as locomotion, our own results suggest an interaction of multiple regions. The rostrolateral striatum injection (ICPu) was performed in an attempt to restore both cocaine-induced locomotion and CPP simultaneously – since this injection targets both the dorsal and ventral subregions of the striatum. To our surprise, this injection restored cocaine-induced hyperlocomotion but not CPP. Importantly, we attempted to dissect whether the dorsal or ventral parts of this injection were primarily responsible for the result, and we observed that the two
components seem to be more than additive (data not shown) – with neither region producing an appreciable effect alone.

There are some more rigorously performed studies that have similarly implicated the interaction of dorsal and ventral subregions of the striatum (Belin & Everitt 2008). Firstly, there are anatomical studies that show that the ventral striatum is connected to the dorsal striatum via reciprocal connections to the midbrain dopamine neurons (see figure below). This connectivity has been found to be necessary for expressing cocaine self-administration habits. It is interesting to note that this behavior is perhaps more related to locomotion than is CPP, and could thus explain our unique ability to dissociate reward and locomotion while using CPP as the measure. For instance, CPP does not heavily involve locomotion, and passively assesses reward value and conditioned associations (Takano et al. 2010). The time spent in the drug-paired compartment of a CPP apparatus could be spent completely immobile once the animal walks into that side of the apparatus. On the other hand, self-administration necessitates motion (by definition), and can become unrelated to reward valuation once it is under habitual control (Zapata et al. 2010). Of course, the converse may also be true, where the self-administration paradigm may have revealed integrative functions of our combined dorso-ventral (ICPu) manipulation.
This diagram draws attention to the microcircuitry within the striatum and midbrain which potentially make the dorso/ventral hypothesis oversimplified. The subregions are depicted above (A), and gain their specific functions most obviously from their cortical connections—which are also topographically organized. Interestingly however, there is an organized set of reciprocal connections between the midbrain and striatum (B). Furthermore, the “feedback” connections from the striatum to the midbrain send collaterals (Dotted Arrows) more laterally, such that there is connectivity between each loop. This gives rise to a set of loops not previously appreciated by depictions (such as figures 1.1 and 1.2) of the midbrain as a 2-nuclei source of dopamine. Furthermore, the loops are integrated by the “crosstalking” collaterals, and this integration is now known to play an important role in the function of the dorsal and ventral striatal regions in habit formation (Belin & Everitt 2008).

These microcircuits, and the convergence of multiple loops in the striatum, suggest a more integrated or at least continuum-like conception of striatal function than the dorso-ventral compartmentalization implied by many theories of addiction.

Source:

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Figure 6.1
In conclusion, we have determined that DAT inhibition is necessary for cocaine-induced reward and hyperlocomotive behaviors (previous data; Chen et al. 2006). Furthermore, DAT inhibition in the rostrolateral striatum is sufficient to produce a cocaine-like hyperlocomotion, but not reward. Importantly, both the dorsal and ventral subregions likely contribute to the full magnitude of the hyperlocomotive effect. Separate restoration of DAT blockade to the dorsal or ventral striatal subregions was insufficient to restore cocaine-induced reward or hyperlocomotion in DAT-CI mice. These results are important in understanding the neural mechanisms of reward and locomotion, as well as the potential substrates of neuroadaptations underlying addiction.
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