GENETIC INFLUENCES ON THE HUMAN MESOLIMBIC DOPAMINE REWARD SYSTEM

By

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To my consistently amazing wife, Ashley, for her sense of adventure

and

To my father, Neil, for Saturday mornings at the lab

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ABSTRACT

Current estimates suggest that as many as 9% of Americans meet the DSM-IV criteria for substance use disorders, and the annual economic burden of substance abuse has been assessed at approximately half a trillion dollars. Thus, addiction is a highly prevalent social problem that rivals almost any other public health issue in terms of social and personal costs. However, despite the fact that addiction is significantly heritable, very few specific genetic susceptibility factors have been reliably identified. Moreover, even for the most promising risk genes, the systems-level neural mechanisms that mediate their impact on risk are largely unknown. In this dissertation, I use a dual-scan dopamine receptor imaging approach with the stimulant drug amphetamine to probe the neurogenetic architecture of addiction. Motivated by the key role played by dopamine in drug addiction, I test the hypothesis that genetic variability in three distinct brain signaling systems with conceptual links to addiction converge to exert a sensitizing effect on striatal dopamine responses to drugs of abuse. First, I examine variation at a locus in the *CSNK1E* gene, which encodes a protein kinase that regulates the function of the dopamine signaling integrator DARPP-32. Second, I study an allelic variant in the gene encoding an hypothalamic-pituitary-adrenal (HPA) stress axis factor (*CRH*) that has been previously associated with stress-induced alcohol consumption in non-human primates. Finally, given the prominent psychopathological and neurobiological parallels

between obesity and addiction, I investigate the novel hypothesis that obesity-linked genetic variability in leptin signaling (*LEPR*) may predispose risk for substance abuse by affecting striatal dopamine responses to stimulants. In all cases, individuals who carried the putative risk allele at each of these loci demonstrated marked sensitization of striatal dopamine responses to amphetamine. In turn, the magnitude of striatal dopamine release was positively associated with subjective responses to amphetamine and with individual differences in impulsivity. Taken together, these findings support the involvement of two genes (*CSNK1E* and *CRH*) in risk for addiction, nominate a third (*LEPR*) for enhanced phenotypic investigation, and offer a common neurobiological mechanism – sensitization of striatal dopaminergic function – that may be involved in the conferral of susceptibility by diverse genetic risk factors.

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INTRODUCTION

Current estimates suggest that as many as 9% of Americans meet the DSM-IV criteria for substance use disorders[1, 2], and the annual economic burden of substance abuse including costs relating to crime, lost productivity, treatment, incarceration and law enforcement) has been assessed at approximately half a trillion dollars[3]. Thus, addiction is a highly prevalent social problem that rivals almost any other public health issue in terms of the magnitude of fiscal damage that it wreaks on society. Therefore, understanding the etiology and the pathophysiology of drug dependence represents a major target for scientific investigation and intervention. It is noteworthy that despite the fact that all drugs of abuse are highly reinforcing, only a relatively small percentage of individuals exposed to these drugs go on to develop the destructive pattern of compulsive drug seeking and drug taking that is the hallmark of addiction[4]. Characterizing sources of individual differences in risk and elucidating their mechanisms of action will aid in the identification of novel therapeutic targets for addiction; as such, these research aims represent crucial next steps in advancing treatment options for individuals afflicted with substance use disorders.

Individual Differences in Addiction Liability: Genetic Mechanisms

In considering the etiology of drug addiction, clues can be gleaned from heritability studies in twin and family samples that include addicted individuals. On the whole, such studies converge to suggest that genetic factors account for approximately 50% of the population variance in risk for addiction, with heritability (h^2) estimates ranging from .4-.6, depending on the specific addictive agent [5, 6]. The known heritability of addiction raises two questions: 1) which specific genes are involved in predisposing risk, and 2) what are the specific neurobiological pathomechanisms through which genetic risk factors exert their effects. To address the first question, investigators have mounted a nearly 20-year search for genetic associations to addiction. However, it must be said that the endeavor has, to date, yielded precious little fruit: though some potentially promising risk genes have been identified, non-replications are more the rule than the exception in this literature. These inconsistent genetic associations across studies likely result from the use of taxonomic, clinical diagnosis as a phenotypic end-point. However, it has been recognized that the likelihood that any given individual with a certain genotype will express a putatively associated phenotype (genetic "penetrance") varies depending on the type of phenotype under investigation. This suggests that we may be able to accelerate the process of gene finding and pathomechanism characterization by identifying and employing more penetrant phenotypes.

Crucially, compared to diagnostic or behavioral phenotypes, penetrance is generally greater for so-called "endophenotypes" – phenotypes that are closer to

the level of a variant's direct physiological effect[7-9]. Practically, this means that significantly larger effect sizes are observed for genetic effects on endophenotypes – particularly systems-level biological endophenotypes – relative to those seen for clinical diagnosis or self-reported traits[9]. These neurobiological endophenotypes – especially those derived from *in-vivo* human neuroimaging measures (Figure 1) – allow an investigator to probe the brain at the systems level to give a quantitative functional readout of the impact of a genetic variant on brain structure, function, neurochemistry or metabolism. This strategy both enhances the ability to detect genetic effects and affords a means of discovering plausible neurobiological risk mechanisms.

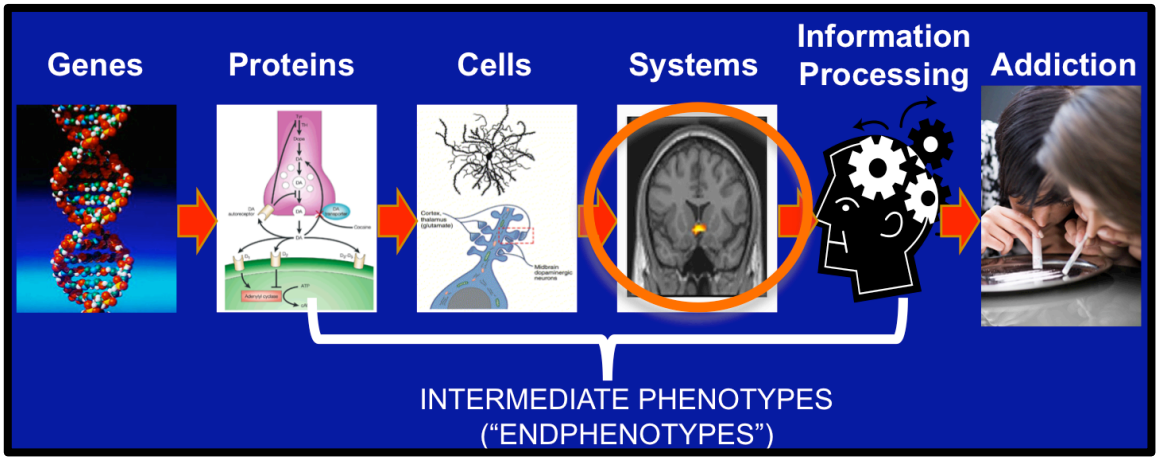

Figure 1. Endophenotypes can aid in the characterization of genetic pathomechanims for psychiatric illness

Addiction as a Disease of Reward and Motivation

In general, when attempting to identify etiopathophysiological pathways through which heritable factors might exert their effects on susceptibility for a given disorder, it is instructive to consider the core cognitive and behavioral domains that are disrupted in that disorder[8]. Addiction is fundamentally a disease

of reward and motivation, and it is commonly accepted that addiction develops through the arrogation of evolutionarily conserved neural systems for processing survival-critical natural rewards (e.g. palatable food, sex) by drugs of abuse [10-14]. This singular fact raises the intriguing possibility that genetic risk factors may shape susceptibility by altering the functional properties of brain reward circuitry. Therefore, endophenotypes related to reward and motivation make especially appealing targets for identifying potential genetic risk factors and for elucidating the neurobiological mechanisms through which they convey liability for addiction. In particular, the strong link between reward, motivation, and the mesolimbic dopamine system[15-18] implies that brain imaging endophenotypes related to mesolimbic dopamine function can be used to further our understanding of the neurobiological mechanisms of genetic risk.

Addiction, Reward, and Nucleus Accumbens Dopamine

Initial interest in DA as a neurochemical substrate for addiction developed from work which demonstrated that all drugs of abuse increase synaptic DA in the nucleus accumbens (NAcc)[19]; showed that animals will work for the opportunity to self-administer DA potentiating drugs[20-22]; and appeared to suggest that such drugs reinforce instrumental behavior only to the extent that they elevate DA[17]. These and related findings led Wise to develop the hedonia hypothesis of DA, which held that "dopamine junctions represent a synaptic way station … where sensory inputs are translated into the hedonic messages we experience as pleasure[23]." This hypothesis is the conceptual foundation for many

of the dominant neurobiological theories of drug addiction (e.g. the reward allostasis model of Koob and LeMoal [12]), which share the view that addiction is a disorder of meso-accumbens DA "pleasure" systems. Wise's formulation of reward neurochemistry was premised on the assumption that the hedonic and motivational values of a stimulus are so inextricably linked as to be indistinguishable. However, more recent work suggests a neurobiological dissociation between these two facets of reward, and this separation has significant implications for how best to study addiction neurobiology in humans.

Specificity of NAcc DA for Reward "Wanting"

Berridge and Robinson were among the first to argue for a clear differentiation of hedonics and motivation in reward processing, which they term 'liking' and 'wanting,' respectively[24]. 'Liking' refers to the hedonic impact of a stimulus – the positively valenced sensory experience that immediately follows reward receipt. By contrast, 'wanting' or 'incentive salience' refers to the motivational value of that reward – that is, its ability to drive goal-directed behavior. Neurobiological discrimination of "liking" and "wanting" processes arose from the finding that experimentally manipulating striatal DA levels appears to have a dissociable impact on behavioral measures of each. Namely, using experimental measures that permit an empirical parsing of hedonic and motivational responses to rewards, Berridge and Robinsons have shown that altering mesolimbic DA signaling has a specific and profound effect on reward 'wanting,' while reward 'liking' is unaltered by such changes [25]. For example, 6-

hydroxy-dopamine (6-OHDA) lesions of ascending DA-ergic projections have no effect on hedonic responses to sucrose, despite almost completely depleting DA levels in NAcc and dorsal striatum[26, 27]. In addition, D2R blockade does not alter 'liking' responses (to sucrose) or 'disliking' responses (to quinine)[28]. Similarly, neither systemic administration of amphetamine[29], amphetamine microinjections into NAcc[30], or electrical stimulation of the medial forebrain bundle[31] affect liking reactions to sucrose reward, although all three of these manipulations significantly potentiate manifestations of reward 'wanting,' such as food seeking and ingestive behaviors. Notably, genetically hyperdopaminergic and hypodopaminergic mice (DAT and TH knockouts, respectively) show striking and directionally consistent alterations in reward 'wanting' behavior (DAT knockouts increased, TH knockouts decreased) in the absence of corresponding changes in hedonic response[32-36]. On the whole, these data strongly and selectively implicate NAcc DA in energizing motivated, instrumental behavior to obtain rewards.

Relevance of NAcc DA-mediated Reward Wanting for Addiction

Based on the findings outlined above, Berridge and Robinson have argued that mesolimbic DA mediates the dynamic attribution of "incentive salience." This value, when ascribed to a reinforcing stimulus, "transforms mere sensory information about rewards and their cues … into attractive, desired, riveting incentives … to make [them] a 'wanted' target of motivation.[25]" Incentive salience "tags" a stimulus as a target for goal-directed behavior and ensures that

an organism will prioritize resources towards obtaining that stimulus over others. Noting that that the key neurobiological nexus for the actions of drugs of abuse – meso-accumbens DA circuitry – is critically involved in ascribing incentive salience to environmental stimuli, Berridge and Robinson have hypothesized that drug addiction involves a dysregulation of incentive salience processing. Their "Incentive Sensitization" hypothesis is based on the observation that drugs of abuse induce a profound and long-term hypersensitivity of this system to rewards and to reward-predicting cues. Repeated administration of a wide range of addictive drugs causes animals to become sensitized to their psychomotor effects (e.g. elevated locomotor, exploratory and approach behavior). Strikingly, repeated exposure to psychoactive drugs also induces sensitization to their incentive motivational effects, even as tolerance develops to their hedonic effects. For example, pre-exposure to amphetamine decreases the dose and the time required for an animal to subsequently learn to self-administer the drug, and increases the amount of work they will expend to gain access to it [22, 37, 38]. The expression of sensitization is strongly influenced by associative learning mechanisms, with drug-associated cues promoting excessive 'wanting' behavior long after the last drug exposure[16]. The development of sensitization is paralleled by structural adaptations in NAcc dendritic spines, and by cellular alterations within the VTA and at NAcc/PFC synapses[39-41]. In sum, the Incentive Sensitization hypothesis posits that repeated exposure to an addictive drug sensitizes meso-accumbens circuitry for incentive motivation, leading to an excessive attribution of incentive salience to the drug.

Taken together, these preclinical findings strongly suggest that DA selectively mediates reward 'wanting,' and that excessive (i.e. sensitized) NAcc DA function following exposure to drugs of abuse promotes an inflexible and long-lasting attribution of salience to drugs and drug cues, which in turn leads to compulsive drug taking behavior. While suggestive, the relevance of these findings for human mesolimbic function, reward experience, and addiction risk is not immediately clear. However, recent neuroimaging work in humans suggests strong cross-species parallels.

Human Mesolimbic DA Function and Addiction Risk

Human NAcc Activity and Reward Wanting

Human functional neuroimaging studies recapitulate the distinction between wanting and liking by elucidating distinct neuroanatomical substrates for each, and suggest that reward-related NAcc DA function in humans is specific to anticipatory reward "wanting." Several early fMRI studies demonstrated that monetary reward and drugs of abuse robustly activate mesolimbic and mesocortical DA terminal fields in humans[42-46]. In addition, primate electrophysiological work by Schultz revealed differences in the response patterns of NAcc and orbitofrontal neurons to the expectation and delivery of rewards, suggesting a neuroanatomical basis for the distinction between appetitive and consummatory phases of reward recognized by ethologists[47].

Drawing on this body of work, as well as its conceptual links to Berridge and Robinson's incentive salience model of reward, Knutson and colleagues have shown that anticipating and receiving monetary reward activates distinct neural circuits. NAcc is active following the presentation of cues that signal the opportunity to emit an instrumental response to obtain reward, but not during the receipt of that reward; by contrast, medial prefrontal cortex is active following the attainment of monetary reward, but not during the anticipatory period preceding reward receipt[48-51]. Similar results have been observed during the anticipation and receipt of taste reward[52]. Further support for the notion that human NAcc is sensitive to the motivational aspects of reward, rather than reward hedonics, is offered by data showing that NAcc response to monetary reward is contingent on stimulus saliency[53] and dependent on the production of an instrumental response[54, 55].

Motivation-related NAcc Activity in Addiction

There is considerable support for the notion that dysregulated – specifically, sensitized – motivation-related activity in the ventral striatum (VS), a region that encompasses the NAcc, is linked to risk for addiction in humans. For example, VS activation following acute cocaine administration is positively correlated with subjective ratings of drug craving, but negatively correlated with subjective ratings of drug "high" (liking)[56]. In addition, enhanced VS engagement by drug cues has been found in substance dependent individuals, and is positively correlated with measures of drug craving [57-61]. Notably, a recent study

found increased motivation-related VS activity in response to non-drug (monetary) reward cues in substance dependent individuals, suggesting a general hyper-reactivity of NAcc to motivationally salient stimuli in addiction. Finally, several human fMRI studies have found heightened VS activation during reward tasks in individuals without of history of substance abuse, but who possessed high levels of addiction-linked traits (e.g. sensation-seeking, impulsivity, impulsive-antisociality)[62-64] indicating that VS hyper-reactivity may constitute a trait-like factor that precedes, and contributes to risk for, the development of drug addiction.

These findings imply a specific and circumscribed role for NAcc in human reward motivation, and suggest that VS hyper-reactivity may comprise an important component of addiction pathophysiology. Further, given the clear links between DA, VS function, reward wanting and addiction, these fMRI studies suggest that sensitized dopaminergic function may also be associated with risk for addiction. However, while some human [63, 65] and preclinical [66] research support the notion that the VS fMRI reward signal is driven by DA signaling, fMRI cannot provide compelling information about the neurochemical alterations that may lead to changes in measured MR signal. Radioligand PET studies – particularly in combination with DAergic drugs – provide an essential confirmation of a role for human VS DA in reward "wanting" and its putative dysregulation in addiction.

Dopaminergic Mechanisms for Addiction-related NAcc Dysregulation

A series of radioligand PET studies provide a critical complement to the fMRI work outlined above by demonstrating that hyper-reactive DA signaling within the NAcc is associated with excessive reward wanting and risk for drug abuse. First, Leyton and colleagues found that the magnitude of amphetamine-induced increases in VS DA release is strongly positively correlated with changes in self-reported 'drug wanting' and with individual differences in "novelty seeking" trait scores [67], which have been found to be elevated in substance abusers[68]. Boileau and colleagues extended this work by demonstrating long lasting stimulant-induced sensitization of VS DA release in humans. Remarkably, the magnitude of sensitized response was strongly correlated with individual differences in novelty seeking trait scores and other self-report impulsivity measures that are also related to addiction risk [69]. Of note, it has been shown that amphetamine-associated conditioned cues increase VS DA release to an extent that is comparable to the drug itself[70], providing a parallel to fMRI data that implicate the VS in cue-induced craving.

Importantly, using 18F-fallypride, we have recently found evidence that impulsive traits – which are heritable, stable, and strongly linked to addiction liability[68] – are associated with exaggerated VS DA release following amphetamine administration. This enhancement in DA release in impulsive individuals appears to be driven, in part, by diminished somatodendritic autoreceptor control over midbrain DA neuron firing. Critically, we also showed that the magnitude of amphetamine-induced VS DA tracks subjective ratings of drug wanting, with increased DA release in highly impulsive subjects predicting

stronger drug wanting responses in these subjects[71]. Given that heightened subjective "wanting" responses are associated with risk for drug dependence[72], and that trait impulsivity has itself been found to predict drug wanting in substance dependent individuals[73], these data raise the possibility that excessive DA release during early stage drug use in impulsive individuals promotes and excessive, inflexible, and long-lasting attribution of salience to drugs and drug cues. On the whole, this series of PET studies are highly consistent with the hypothesis that risk for addiction is associated with VS dopaminergic hyper-reactivity.

Taken together, the body of work described above indicates that human VS DA is associated with reward "wanting", and demonstrates that individual differences in the sensitivity of the mesolimbic DA system are related to risk for addiction. Furthermore, given that genetic factors play a key role in determining addiction liability, these findings raise the suggestion that heritable individual differences in the responsiveness of mesolimbic DA circuitry to reinforcers – including drugs of abuse – may be a crucial factor mediating individual susceptibility to addiction. Accordingly, genetic variants that enhance the responsiveness of mesolimbic DA circuitry to reinforcers may increase addiction liability by causing an exaggerated attribution of incentive salience to reward stimuli. In this manner, genetically mediated individual differences in VS DA sensitivity may predispose the development of addiction following exposure to drugs of abuse. While intriguing, this hypothesis remains untested: to date, no

studies have examined the impact of addiction-linked genetic variation on individual differences in drug-induced VS DA release.

Specific Aims

The purpose of the experiments described in this dissertation is to examine the impact of genetic variability on individual differences in ventral striatal DA reactivity. To that end, I employ molecular imaging with PET to assess the effects of polymorphic variants in three addiction-linked brain-signaling pathway genes on VS DA reactivity to a psychostimulant drug of abuse. A central goal of these experiments is to show that genetic risk factors in diverse brain signaling systems may converge to increase susceptibility through a final common neurobiological mechanism: sensitization of VS DA responses to drugs of abuse.

Aim 1: Characterize the impact of risk-linked allelic variation in DA signaling on psychostimulant-induced DA release.

Individuals with heightened subjective responses to stimulant drugs are at increased risk for the development of future drug dependence. Therefore, genetic variants that predispose exaggerated stimulant responsiveness may be good candidate addiction risk factors. Chapter II of this dissertation tests the hypothesis that the effect of one such variant, located in the DA signaling pathway gene *CSNK1E*, is driven by its sensitizing influence on stimulant-induced VS DA release.

Aim 2: Test for functional interactions between genetically-mediated differences in stress responsiveness and human DA function.

Stress is a prominent risk factor for both the development of compulsive drug use and for relapse behavior following a period of abstinence. This strong association implicates individual variability in the function of stress-linked neurobiological factors – particularly those comprising the hypothalamic-pituitary-adrenal (HPA) stress axis – in addiction risk. However, despite an extensive preclinical literature demonstrating regulation of striatal DA signaling by HPA-axis factors, compelling demonstration of a relationship between variation in stress responsiveness and striatal DA function in humans is lacking. In chapter III, I investigate the possibility that genetic variability in one HPA-axis gene – *CRH*, encoding the corticotropin-releasing hormone – potentiates striatal DA release, which may provide a translational mechanism accounting for the known sensitizing effects of stress and stress susceptibility on DA function and drug-taking behavior in animals.

Aim 3: Demonstrate regulation of human striatal DA function by energy-regulating hormones.

Obesity is burgeoning public health problem with prominent psychological and neurobiological parallels to addiction. Notably, the most well characterized genetic risk factor for obesity – the energy-regulating hormone leptin – is known to modulate mesolimbic DA system function in rodents. However, it is unknown whether individual differences in energy-regulating hormone signaling might also play a role in regulating human mesolimbic DA system reactivity. Chapter IV of

this dissertation provides genetic evidence for leptin-DA interactions in the human striatum; such interactions may provide a plausible mechanism explaining the psychobiological parallels between obesity and addiction.

At the conclusion of this dissertation, I will detail the contributions of these experiments to our understanding of the neurogenetic architecture of addiction. Further, I will outline a proposal for bootstrapping this new understanding to devise novel approaches to gene finding in addiction. For example, while a candidate gene approach based on the known pathobiology of addiction has been critical in identifying risk-linked genetic markers, there are likely many as-yet-undiscovered risk factors lurking in the genome. Exploratory, data-driven approaches such as genome-wide association studies provide one route for novel gene discovery, but the issues inherent to diagnosis-based genetic association identified above make this approach problematic. I will offer that genome-wide association studies using validated quantitative biological endophenotypes – such as stimulant-induced DA release – may provide a more robust method for gene finding in substance abuse.

CHAPTER II

ALLELIC VARIATION IN THE DOPAMINE REGULATING GENE CSNK1E SENSITIZES STIMULANT-INDUCED STRIATAL DOPAMINE RELEASE

Drug abuse is a serious public health problem with significant costs to affected individuals and to society as a whole. While approximately 9% of Americans are estimated to meet the criteria for DSM-IV substance use disorders[1, 2], many more people are thought to use drugs in a recreational (non-compulsive or dependent) fashion. Indeed, it is noteworthy that despite the fact that all drugs of abuse strongly affect neural systems for reward and motivation and are highly reinforcing, only a relatively small percentage of individuals who are exposed to drugs of abuse go on to develop a destructive pattern of compulsive drug seeking and taking that persists even in the face of significant adverse consequences – the hallmark of addiction[4]. Thus, there appear to be significant individual differences in susceptibility to drug addiction following drug exposure[14]. However, we do not yet have a full accounting of the specific risk factors that predict conversion from recreational drug use to drug addiction, and neurobiological correlates for even the most well characterized risk factors are still largely unknown[5, 74]. Therefore, the identification of specific predictors of addiction liability and the elucidation of their underlying pathophysiological mechanisms represents a critical scientific goal.

While the risk architecture for addiction conversion (i.e. the transition from early-stage, recreational drug use to compulsive drug-taking) is complex and multiply determined, one factor that has emerged as potentially important is an individual's subjective responses during their initial or early exposure to a drug of abuse. For example, subjective responses to alcohol are a strong predictor of alcohol use problems, such that high-risk individuals can be distinguished by the presence of enhanced "stimulant" effects and diminished sedative effects of the drug[75]. Similarly, Lambert and colleagues found that individuals who report higher subjective "wanting" responses during their initial exposure to cocaine are at markedly increased risk for meeting criteria for cocaine dependence[72]. These findings accord well with preclinical data indicating that individual differences in behavioral responses during initial exposure to a drug of abuse significantly predict whether or not an animal goes on to develop addiction-like behavior (e.g. drug self-administration)[21, 22]. Of note, subjective responses to drugs of abuse are significantly correlated with other established trait risk factors, such as sensation seeking[76], providing convergent evidence for the relevance of subjective responses in determining individual liability to addiction conversion.

The high heritability of addiction[5, 6] implies that genetic mechanisms may predispose risk, and further, raises the possibility that heritable mechanisms may play a role in determining individual differences in subjective drug responses. Available evidence suggests that this is in fact the case. For example, subjective responses tend to track other established genetic risk factors for substance abuse, such as the presence of a family history of alcoholism[75, 77]. Furthermore,

several groups have reported that genetic factors account for a significant degree of variance in subjective responses to alcohol, tobacco, and marijuana [78-80]. Interestingly, multivariate genetic modeling in those studies delineated a role for both common and drug-specific genetic factors in accounting for individual variance in subjective responses[78]. On the whole, these findings strongly suggest that alterations in subjective responses to drugs of abuse may be one psychological mechanism through which genetic susceptibility factors may exert their effects[81-83]. Such alterations – especially, the sensitization of positive effects – may in turn promote the transition to an addicted state.

Consistent with the data of Haberstick and colleagues illustrating the importance of genetic factors *generally* in predisposing individual differences in drug responses, a number of investigators have found associations between *specific* genetic variants and aspects of subjective experience following drug exposure. For example, polymorphisms in the *GABRA2, CNR1, OPMR1, and ALDH2* genes have been linked to subjective responses to alcohol consumption[84-89], and alleles in *CSNK1E, SLC6A4, SLC6A2, SLC6A3* and *FAAH* have been shown to predict subjective responses to stimulant administration[90-96]. Of these stimulant-associated polymorphic variants *CSNK1E* is a particularly intriguing candidate, with especially strong translational evidence supporting a causal role for this gene in determining responses to drugs of abuse.

CSNK1E encodes the epsilon isoform of casein kinase 1 (CK1E), a serine/threonine kinase that is an important regulator of the striatally-enriched DA signaling protein DARPP-32 (dopamine and cAMP-regulated phosphoprotein of

molecular weight 32 kDa)[97]. By way of review, DARPP-32 is highly expressed in midbrain dopamine neuron terminal field regions, with peak expression in the medium spiny neurons (MSNs) of the caudate, putamen and nucleus accumbens, and is known to play an important role in orchestrating second and third messenger signaling cascades within MSNs following exposure to all known classes of abusable drugs[98]. In the context of the present discussion, it is notable that DARPP-32 has also been found to mediate both the locomotor activating and reinforcing effects of amphetamine[99, 100]. The involvement of DARPP-32 in determining psychostimulant responsiveness is likely a function of the role that this molecule plays in amplifying the effects of DA D1 receptor stimulation on protein kinase A (PKA) signaling within MSNs[101].

Amphetamine administration leads to higher levels of synaptic DA, which results in enhanced stimulation of post-synaptic D1 receptors on MSNs. D1 stimulation, in turn, activates PKA, which phosphorylates DARPP-32 at Thr-34, dramatically potentiating its affinity as an inhibitor of protein phosphatase I (PP-1)[102]. PP-1 inhibition causes enhanced phosphorylation at multiple target proteins, including receptors for excitatory amino acids in the neuronal plasma membrane and nuclear-localized cAMP response element binding protein (CREB), a crucial regulator of gene expression (Figure 1). CK1e positively regulates the DARPP-32 mediated inhibition of PP-1 by phorphorylating DARPP-32 at Ser-137; this, in turn, decreases the rate of Thr-34 dephosphorylation by protein phosphatase-2B (PP2B or calcineurin) and, by so doing, increases the overall level of phosphorylation of DARPP-32 by PKA at Thr-34. Thus, the net

effect of CK1E is to increase the phosphorylation state of the Thr-34 residue of DARPP-32, thereby magnifying the impact of drug-induced stimulation of post-synaptic D1 receptors in striatal MSNs[103-105]. Overall, these data hint that CK1E, by acting to modify the phosophorylation state of DARPP-32, may impact stimulant responsiveness.

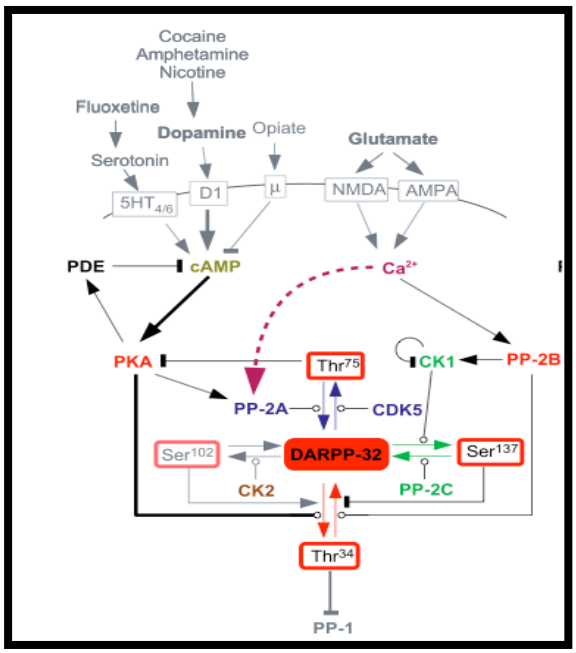

Figure 1. CK1e regulates phosphorylation of DARPP-32 at Ser[137], potentiating the PKA-mediated phosphorylation at Thr[34] required for inhibition of PP-1

Specific evidence for the involvement of CK1E in psychostimulant responsiveness can be gleaned from a series of translational studies. First, using a model of cocaine sensitization in *Drosophila*, Andretic and colleagues found blunted responses to acute cocaine and abolished sensitization in flies carrying a mutated form of the *Drosophila* protein *doubletime*, which has significant homology to the epsilon isoform of human CK1E[106]. Next, using a behavioral quantitative trait locus (bQTL) approach, Palmer and colleagues investigated the

linkage between genomic variability and individual differences in psychostimulant responsiveness – specifically, locomotor activation following methamphetamine administration – in mice that were bred selectively to highlight such differences. This identified a responsiveness-linked region on chromosome 15 that encompassed the murine *CSNK1E* gene. Subsquent microarray-based QTL analysis using nucleus accumbens tissue revealed an *expression* QTL for stimulant responsiveness that also mapped to the *CSNK1E* gene region, suggesting that variability in the behavioral phenotype might be caused by genetic factors that drive changes in accumbens CK1E expression[107]. Further supporting the hypothesis that accumbens CK1E activity is a critical regulator of responsiveness through its facilitatory actions on DARPP-32 Thr-34 phosphorylation, a recent study showed that selective pharmacological inhibition of accumbens CK1E activity dramatically attenuated locomotor responses to methamphetamine, while at the same time abolishing stimulant-induced phosphorylation of DARPP-32[108].

Extending these findings to humans, Veenstra-VanderWeele and coworkers examined associations between SNPs in the human *CSNK1E* gene and subjective responses to an oral dose of D-amphetamine (placebo, 10mg and 20mg; administered in a double-blind, randomized, three-session crossover design). They found one SNP downstream from the 3' untranslated region (UTR) of the *CSNK1E* gene (rs135745) that was associated with two measures of subjective drug response: the Drug Effects Questionnaire (DEQ) "Feel Drug" scale, and the ARCI "euphoria" scale. Individuals carrying one or more C allele at

this locus had significantly greater subjective responses as measured by both scales relative to G allele homozygotes [95]. Taken together, these translational findings indicate that CK1E is a critical determinant of stimulant responsiveness – likely through its modulatory effects on DARPP-32 phosphorylation – and that polymorphic variation at the human gene encoding the CK1E epsilon isoform (*CSNK1E)* predicts individual differences in human subjective responses to stimulant administration. Thus, *CSNK1E* represents a compelling candidate gene for addiction conversion. However, despite the suggestive linkages described above, the specific neurobiological mechanisms through which variation in *CSNK1E* exerts its effect on subjective responses and (putatively) addiction risk remain unknown.

To gain purchase on the systems-level biological effects that may underpin the linkage between *CSNK1E* genetic variation and stimulant responsiveness in humans, we employed positron emission tomography (PET) imaging with the high-affinity D2/D3 ligand [18F]fallypride, in concert with an amphetamine challenge. This approach provides an in-vivo measure of stimulant-induced DA release, which we compared across *CSNK1E* rs135745 genotypes in our sample of healthy community volunteers. Given the robust linkage – preclinically and in humans – between ventral striatal (VS) DA levels and drug wanting, the selective associations between VS CK1E and drug responsiveness, and the impact of *CSNK1E* rs135745 genotype on positive subjective responses to amphetamine, we hypothesized that *CSNK1E* rs135745

C allele carriers would release significantly more DA within VS in response to amphetamine administration.

Methods

Participants

We studied 59 individuals (29 males; age range = 18-33, mean = 23; 48 Caucasian, 5 African-American, 3 Asian, and 1 individual of mixed Caucasian descent; Table 1) using a dual-scan placebo-controlled paradigm with [18F]fallypride PET and d-Amphetamine (AMPH). All participants were medically and psychiatrically healthy adults, age 18 to 35, with estimated IQ greater than 80. Subjects were excluded if they had any history of substance abuse, current tobacco use, alcohol intake greater than 8 ounces of whiskey or equivalent per week, use of psychostimulants (excluding caffeine) more than twice in the subject's lifetime or at all in past 6 months, any psychotropic medication for the past 6 months other than occasional use of benzodiazepines for sleep, history of psychiatric illness, significant medical condition, any condition which would interfere with MRI or PET studies (e.g., extreme obesity, claustrophobia, cochlear implant, metal fragments in eyes, cardiac pacemaker, neural stimulator, and metallic body inclusions or other metal implanted in the body which may interfere with MRI scanning, pregnancy, or anemia). Female participants were studied during the early follicular phase of their menstrual cycle.

Following initial screening, subjects were given an interview of their medical history and a structured psychiatric interview (SCID-NP;[109]). In addition to the regular questions in the non-alcohol substance dependence section of the SCID-NP, subjects were asked to indicate the number of times that they have taken any drug that they reported having tried, and asked to indicate any usage within the last 2 months. Any illicit drug use in the last 2 months was grounds for exclusion, even in subjects who did not otherwise meet criteria for substance abuse. Urine drug screens were performed to test for the presence of amphetamines, cocaine, marijuana, PCP, and opiates, benzodiazepines, and barbiturates.

Table 1.Demographic Information for CSNK1E PET Sample

	rs135745 Genotype		
	C/C	**C/G**	**G/G**
Sex (m/f)	4/9	13/14	12/5
Age (mean/SD)	23.33/3.9	22.67/3.17	25.59/4.03
PET Scanner (1/2)	7/6	7/20	10/7
Blind (Non-Blind/Blind)	3/10	5/22	6/11
% Change Left Ventral Striatal BP_{ND} (mean/SD)	8.43/6.38	5.09/4.81	3.40/9.04
% Change Right Ventral Striatal BP_{ND} (mean/SD)	7.37/6.13	4.32/4.99	2.95/8.64
Ethnicity (Cauc/Asian/AA/Hisp)	11/2/0/0	25/1/0/1	12/2/3/0

Table 1. Demographic information for *CSNK1E* PET Sample

Genotyping

Saliva was collected from each subject using DNA Genotek Oragene-250 collection kits. Genomic DNA was extracted per manufacturer's protocols and banked at the Vanderbilt University Center for Human Genetics Research DNA Resources Core. Genotyping for rs135745 was conducted by Vanderbilt DNA Resources Core through the use of the Sequenom massARRAY genotyping platform, based on a single-base primer extension reaction coupled with mass

spectrometry. Genotyping for the participants reported in this study was performed in two separate genotyping runs, as part of a larger batch of genotyping that included DNA collected as part of an unrelated study. The genotyping success rate for this marker was high (96.6%). Allele frequencies were as follows: C/C = 13, C/G = 27, G/G = 17, and did not deviate from Hardy-Weinberg Equilibrium ($p = 0.72$). Genotype was not significantly associated with age ($F = 0.36$, $p = 0.71$), but we did find trends for imbalances in genotype distribution across sex ($X^2 = 4.83$, $p = 0.09$) and self-reported ethnicity ($X^2 = 10.38$, $p = 0.11$) (see Table 1). Therefore, we controlled for the potentially confounding effects of this imbalance in subsequent between-groups genetic analyses.

PET

Image Acquisition and Analysis

All PET images were acquired using [^{18}F]fallypride. ((*S*)-*N*-[(1-allyl-2-pyrrolidinyl)methyl]-5- (3[^{18}F]fluoropropyl)-2,3-dimethoxybenzamide), a substituted benzamide with very high affinity for D2/D3 receptors[110]. Unlike other D2/D3 ligands, [^{18}F]fallypride allows stable estimates of D2-like binding in both striatal and extrastriatal regions[111]. Our current resolution (see below) allows visualization of [18F]fallypride binding potential in the substantia nigra (SN)/ventral tegmental area (VTA), [for a discussion of the spatial resolution requirements for detecting activity in the SN see [112]]. However, this resolution does not permit us to cleanly distinguish between different DA cell populations,

preventing a clear parcellation of the VTA from the neighboring SN, which possesses higher levels of D2-like receptors. Previous studies have demonstrated good intersubject and intratest-retest reliability for measurement of [^{18}F]fallypride binding potential for the DA midbrain at the current resolution[113-115]. [^{18}F]fallypride binds with high affinity to both presynaptic ("D2-short") and postsynaptic ("D2-long") D2-like receptors[116]. However, because DA receptor expression in the midbrain is dominated by the D2-short receptor isoform [117] variance in [^{18}F]fallypride BP_{ND} within the midbrain is presumed to be driven by individual differences in these D2-short autoreceptors.

In addition, [^{18}F]fallypride has been found to be sensitive to endogenous DA release [114, 118], particularly in the striatum, making it an ideal ligand for use in conjunction with a dual scan strategy that allows assessment of both baseline receptor availability and individual differences in induced DA release. Baseline binding of [^{18}F]fallypride is also influenced by endogenous DA levels, and thus provides a metric of receptor availability, rather than absolute receptor density. However, receptor availability has proven a highly useful measure in quantifying individual differences in DA functioning, and indeed in some ways may be a more relevant variable than receptor density examined in isolation (as only available receptors can be engaged at a given point in time).

Protocols for PET image acquisition and analysis were derived from a larger ongoing study and have been previously published [114, 115]. Subjects received two PET scans using [^{18}F]fallypride. The first scan was a baseline placebo scan; the second scan was performed while the subject received an

amphetamine (d-AMPH) challenge. We used a single-blind drug administration regimen for the majority of participants (n = 43); however, we also included in our analyses 14 subjects who participated in the study during a pilot phase, and who were not blind to drug. We do not observe an imbalance of genotypes between blind and non-blind subjects (Pearson X^2 = 1.6, p = 0.45); nevertheless, blinded status was included as a nuisance covariate in all analyses where it was appropriate to do so (i.e. in analyses that included non-blind participants). PET imaging was performed on a GE Discovery LS scanner located at Vanderbilt University Medical Center that was upgraded to a Discovery STE system during the course of the study. All subjects received their baseline and d-AMPH scans on the same scanner. To ensure the validity of combining data across scanners, we performed a voxel-wise analysis comparing DA release between the two scanners. No clusters survived whole brain correction at t = 2.5 (lowest cluster-level p-value >.90). Moreover, no differences were observed in our anatomical region of interest, the ventral striatum. (left and right VS; both p-values > 0.45). 24 participants were scanned on scanner 1, and 33 participants were scanned on scanner 2. We did observe a trend for an imbalance of genotypes between the two scanners (pearson X^2 = 5.58, p = 0.06; Table 1). We therefore included scanner (i.e. scanner 1 vs. scanner 2) as a nuisance covariate in all of our regression analyses. Following reconstruction both scanners had similar in plane and throughplane resolution. [^{18}F]fallypride was produced in the radiochemistry laboratory attached to the PET unit, following synthesis and quality control procedures described in US Food and Drug Administration IND 47,245. Scans

were timed to start 3 hours after 0.43mg/kg oral d-AMPH administration, which was timed to coincide with the period of peak plasma d-AMPH. 3-D emission acquisitions scans were performed following a 5.0 mCi slow bolus injection of [^{18}F]fallypride (specific activity greater than 3000 Ci/mmol). Serial scans were started simultaneously with the bolus injection of [^{18}F]fallypride and were obtained for approximately 3.5 hours, with two 15-minute breaks for subject comfort. CT transmission scans were collected for attenuation correction prior to each of the three emission scans.

Binding Potential Maps

Each subject's serial PET scans were first corrected for motion across scanning periods and then co-registered to the subject's structural T1-weighted MRI image. To determine the success of the coregistration in the midbrain, in a prior study of 34 subjects[115] we manually labeled several landmarks around the midbrain, including the posterior edge of the right and left inferior colliculus, the anterior-most point of the right and left cerebral peduncle and the interpeduncular fossa at z = 10, and the inferiormost point of the supramammillary commissure. Of these 34 subjects, all but one showed excellent midbrain coregistration, with no tag varying by 2mm in any direction from the mean coordinate of the tag (across these 33 subjects, the mean distance in any direction from the average tag was 1mm for every tag examined). Given the spatial resolution of the PET images, this degree of misregistration is at the subvoxel level, and would have negligible impact on the results.

Regional D2/D3 binding potential (nondisplaceable; BP_{ND}) was calculated on a voxelwise basis using the full reference region method[119], with cerebellum chosen as the reference region because of its relative lack of D2/D3 receptors[120]. Voxelwise kinetic modeling was executed using Interactive Data Language. Prior studies in our lab indicate that the reference region method produces binding potential estimates that are in close agreement with estimates derived from Logan plots [121] using a metabolite corrected plasma input function. Because [^{18}F]fallypride binding values exhibit significant variability across different regions (e.g., striatum vs. prefrontal cortex; PFC), we used variance estimates at the voxelwise level rather than the pooled variance used in typical parametric analyses [122]. Individual images of percent-change in [^{18}F]fallypride binding from placebo to amphetamine (representing percent-change in DA release) were created by subtracting each subject's amphetamine scan from their placebo scan and dividing the resulting imaging by the placebo scan, using the "imcalc" image math routine in SPM5.

Region of Interest Analyses

Based on the large body of preclinical and human research linking psychostimulant responsiveness to ventral striatal dopamine [24, 67, 71, 123, 124], we limited our analysis of the effects of *CSNK1E* genetic variation on stimulant-induced DA release to the ventral striatum. To that end, we constructed an anatomical VS region of interest (ROI) by manually editing the striatum ROI derived from the LONI Probabilistic Brain Atlas 40 (LPBA40;[125]) according to the

criteria outlined in Mawlawi et al. (2001)[126, 127] (Figure 1). Percent-change values were averaged across all voxels within left and right VS ROIs to create a single percent-change value for each individual's left and right VS mask.

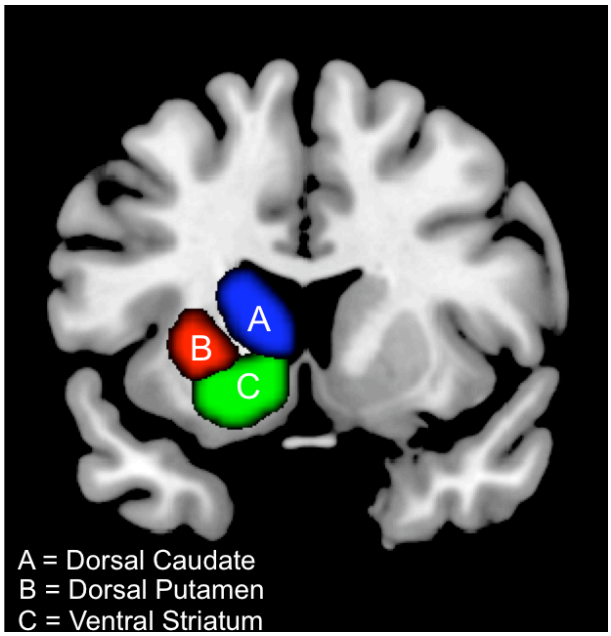

Figure 2. Striatal Regions of Interest (ROIs). ROIs rendered on a T1-weighted reference image, coronal slice at Y = 11 (MNI space). ROIs defined by anatomical criteria of Mawlawi et al (2001). Only pre-commisural ROIs shown.

Measurement of Subjective Response to AMPH

To measure subjective responses to amphetamine, we administered the Drug Effects Questionnaire (DEQ) at 60-minute intervals following the administration of drug and placebo. The DEQ consists of four questions: whether the subject feels the drug, whether the subject likes the drug, whether the subject feels high, and whether the subject wants more of the drug. Subjects indicated their response on a labeled magnitude scale [128] from 0-100, with 0 indicating "Not

At All" and 100 indicating "Most Imaginable." Participants made these ratings 5 times over the course of a session, approximately every 60 minutes after the administration of drug or placebo. We used the peak rating within the AMPH day timecourse for our genotype effect analyses and our DA release correlation analyses.

Statistical analysis

Statistical analyses using were performed using SPSS 17.0 for the Macintosh. To test for genotype effects on VS DA release, we used a linear regression model with rs135745 genotype (dummy coded as 1 = C/C, 2 = C/G, 3 = G/G) as a predictor of left and right % change in DA release (separate models for left and right VS). For each ROI, we used the mean percent-change in DA release value averaged across all voxels within that ROI (described above). PET scanner, ethnicity, and sex were included in the regression model as nuisance covariates. In follow-up control analyses, we added blinding status and VS BP_{ND} as additional nuisance covariates. Two-tailed tests were used, with alpha = 0.05.

Whole-brain genotype effect analyses were performed using SPM5 running on Matlab R2007b for the Macintosh. We used a linear regression model with rs135745 genotype (dummy coded as 1 = C/C, 2 = C/G, 3 = G/G) as a predictor of voxelwise DA release (i.e. we used voxelwise images of percent-change in DA release following AMPH administration, as described above). Scanner, ethnicity, blinding status and sex were included as nuisance covariates.

To test for genotype effects, we used an uncorrected exploratory threshold of $p < 0.005$, coupled with a cluster extent threshold of 20 voxels.

To test for an association between CSNK1E genotype and subjective responses to AMPH, we performed a multivariate general linear model (GLM) analysis of covariance (MANCOVA) with each of the four DEQ scales as dependent measures, CSNK1E rs135745 genotype as a fixed factor, and sex and ethnicity as covariates. Two-tailed tests were performed, with alpha = 0.05. We used the peak responses on the amphetamine day for our genotype comparisons and for the correlations with amphetamine-induced VS DA release.

Results

Descriptive and inferential statistics for VS BP_{ND} and AMPH-induced DA release

Across the entire sample, mean (standard deviation) baseline BP_{ND} values in the left and right VS ROIs were 22.07 (3.77) and 21.05 (3.83). Mean post-AMPH BP_{ND} values in the left and right VS ROIs were 20.83 (3.83) and 19.99 (3.73). Repeated-Measures Analysis of Variance (ANOVA) confirmed a significant reduction in BP_{ND} in both left ($F_{1,56} = 40.52$, $p = 0.000000038$) and

right VS ($F_{1,56}$ = 33.46, p = 0.000000340) following AMPH administration (Fig 2).

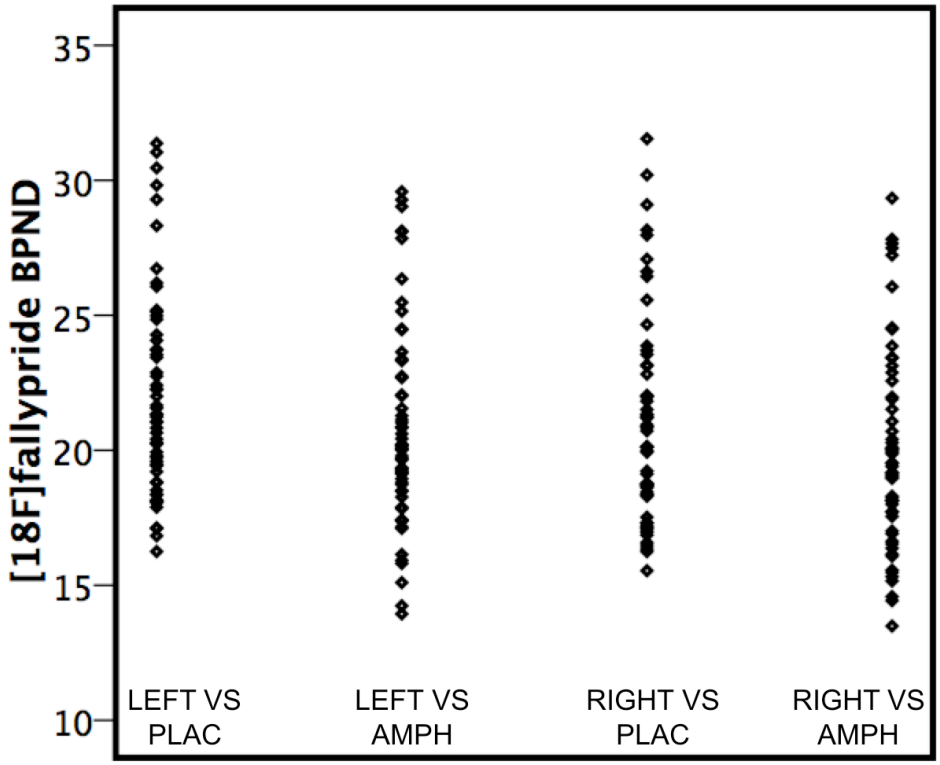

Figure 3. Amphetamine significantly reduced [18F]fallypride binding potential in ventral striatum. Binding potential (BP_{ND}) values on placebo (PLAC) and amphetamine (AMPH) depicted for left and right ventral striatum for 59 participants with dual-scan PET data.

CSNK1E genotype effect on VS DA release

To test for an association between CSNK1E rs135745 genotype and AMPH-induced DA release, we regressed rs135745 genotype against mean percent-change AMPH-induced DA release values calculated from left and right VS ROIs. This analysis revealed that rs135745 genotype was a significant predictor of AMPH-induced DA release in both left (β = -0.33, p = 0.02) and right (β = -0.29, p = 0.04) VS (Figure 3). These effects remained significant ($p \leq 0.05$) when blinding status was included in the model. *CSNK1E* genotype was not

significantly associated with baseline D2/D3 binding within VS (left or right; both p-values > 0.1), and *CSNK1E* remained a significant predictor of VS DA release even after controlling for individual variation in VS D2/D3 BP_{ND} (left VS, p = 0.03; right VS, p = 0.07).

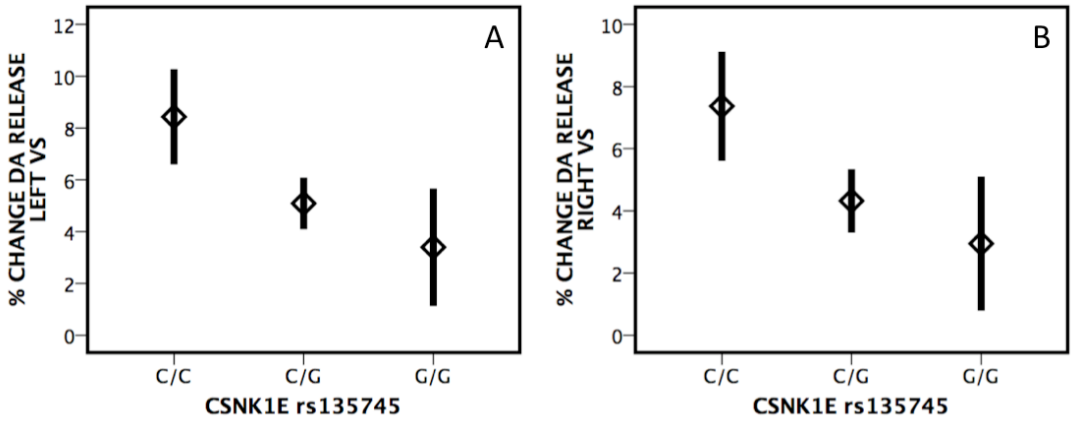

Figure 4. Effects of *CSNK1E* rs135745 genotype on AMPH-induced DA Release in left (A) and right (B) ventral striatum. Error bars indicate ± 1 standard error of the mean,

Whole brain exploratory analysis

To examine unhypothesized effects of CSNK1E rs135745 genotype outside of the striatum, we performed a whole-brain regression analysis in SPM5. At a liberal (uncorrected) exploratory statistical threshold, we did not observe any extrastriatal voxels in which AMPH-induced DA release was significantly predicted by genotype. Similarly, we did not observe any effects of *CSNK1E* genotype on baseline D2/D3 levels in any brain region at our exploratory threshold.

CSNK1E Genotype Effect on Subjective Responses to AMPH

Given the findings of Veentra-Vanderweele and colleagues, we sought to examine the relationship between CSNK1E rs135745 genotype and subjective responses to AMPH. DEQ data were available for 43 participants (CC = 10, CG = 21, GG = 11). Repeated-Measures ANOVA confirmed that AMPH increased scores on each of the four DEQ scales ("Want", "Like", "Feel" and "High"; all p's < 0.01). However, we did not find a significant association between genotype and subjective response in this small sample any of the 4 scales (p's >0.1).

VS DA Release Predicts Subjective Responses to AMPH

Although we did not find a significant relationship between CSNK1E rs135745 genotype and subjective responses to AMPH, CSNK1 may exert an indirect effect on subjective responsiveness by influencing AMPH-induced DA release. That is, while no direct relationship was observed in the current sample, finding a correlation between AMPH-induced DA release and subjective responses to AMPH would support the idea that the effect of genetic variation in CSNK1E on AMPH responsiveness found previously by Veenstra-VanderWeele and colleagues [95] is mediated by an effect of CSNK1E rs135745 on stimulant-induced DA release. To test this notion, we performed a partial correlation (controlling for sex and PET scanner; subjective response data was not obtained for non-blinded participants) between VS AMPH-induced DA release and each of the four DEQ scales. We found a significant correlation between bilateral VS DA release and DEQ Wanting (Left VS: r = 0.37, p = 0.018; Right NAcc: r = 0.41, p =

0.007), consistent with the idea that the magnitude of DA release in VS in response to AMPH is directly related to the magnitude of an individual's subjective craving response to AMPH. Correlations between the three other scales (Like, Feel, and High) were not significant (p's > 0.1).

Discussion

Here, we present data indicating that a polymorphic variant in the *CSNK1E* gene – a compelling candidate gene for variation in stimulant responsiveness – is associated with the magnitude of stimulant-induced DA release in the human ventral striatum. Each rs135745 C allele was associated with a step-wise increase in ventral striatal DA release, which was in turn associated with subjective craving responses to AMPH. The present data suggest a neurobiological mechanism – striatal DA hyperreactivity to AMPH – that may account for a prior association with the same allele to positive subjective responses to AMPH[95]. Thus, our findings support the view that *CSNK1E* genetic variability contributes to the heritability of stimulant responsiveness. Furthermore, insofar as individual differences in stimulant response predict risk for stimulant abuse, these data suggest that *CSNK1E* warrants more extensive investigation as a susceptibility gene for addiction.

The molecular biology of CK1E alone makes it an intriguing candidate for investigation as an addiction liability factor. As detailed previously, CK1E is richly expressed in striatum where it acts as an important regulator of DARPP-32 activity[105]. In particular, CK1E stabilizes the D1 activation-mediated

phosphorylation of DARPP-32 at Thr-34, thereby potentiating its inhibition of PP1. Stimulant-induced phosphorylation at Thr-34 appears to be an essential mechanism underpinning DARPP-32's role in mediating locomotor responses to stimulant administration. Therefore, CK1E is well situated to modify the impact of stimulant drugs on post-synaptic DA signaling by potentiating DARPP-32's inhibition of PP-1, which in turn impacts a range of molecular targets, including fast-acting neurotransmitter receptors and voltage-gated ion channels[103-105]. The importance of CK1E for modulating stimulant regulation of this pathway is highlighted by recent evidence that CK1E is necessary for stimulant responsiveness: pharmacological inhibition of CK1E significantly attenuates stimulant-induced locomotion, and does so by downregulating the phosphorylation state of the DARPP-32 Thr-34 residue following stimulant administration[108].

In addition to its effects on DA signaling through DARPP-32, CK1E is a potent regulator of circadian gene pathways. Given that circadian clock proteins such as Clock, Period2 (Per1) and Period2 (Per2) are increasingly recognized to play an important role in reward and motivation[129-133], interactions between CK1E and these molecules represent another potential mechanism through which allelic variability in *CSNK1E* might impact striatal dopaminergic function and drug responses. In considering how individual differences in CK1E signaling might affect this pathway to sensitize stimulant responsiveness, a wealth of data implicate Per2 as a likely target. Per2 is known to play an important role in the locomotor stimulatory and reinforcing effects of stimulant drugs: in particular,

Per2 mutants demonstrate dramatically enhanced cocaine sensitization and conditioned place preference compared to wild-type animals[134], and *Per2* gene expression is significantly upregulated in the striatum following cocaine self-administration[135]. Moreover, Hampp and colleagues have reported that Per2 mutant mice show significantly higher levels of DA release compared with wild-type animals, an effect that is likely due to downregulated *MAOA* expression within VTA and ventral striatum in *Per2* mutants[136]. This finding is all the more intriguing given evidence that *Per2* mutants show enhanced alcohol consumption, and in light of the fact that genetic variability in the human *PER2* gene is linked to alcohol intake[137]. Critically, CK1E plays an essential role in the stabilization and localization of Per2 protein. CK1E downregulates Per2 activity by targeting it via phosphorylation for proteosomal degradation, and this residue-specific phosphorylation is a critical determinant of Per2 half-life[138, 139]. Thus, CK1E is in a position to have a significant and broad impact on Per2 function, providing one potential route for explaining how genetic variation in CK1E could affect mesolimbic DA reactivity.

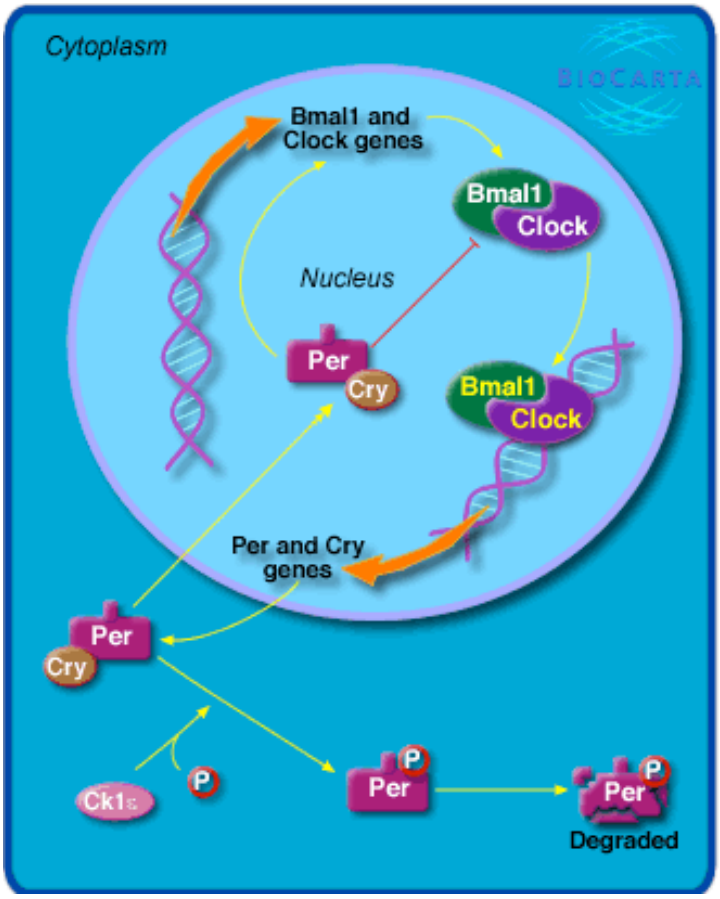

Figure 5. CK1e phosphorylation is necessary for the proteasomal degradation of PER2. Image obtained from biocarta.com.

Though the precise functional effect of the rs135745 SNP on gene expression or post-translational modification is not known, it is nevertheless tempting to hypothesize a potential mechanism for CK1E's involvement in VS DA responses based on several known factors. First, rs135745 is located in the 3' UTR of the CSNK1E gene (Figure 4), raising the possibility that variation at this locus disrupts an miRNA target site; such a disruption would interfere with miRNA-associated translational repression of CK1E protein, leading to higher CK1E levels.

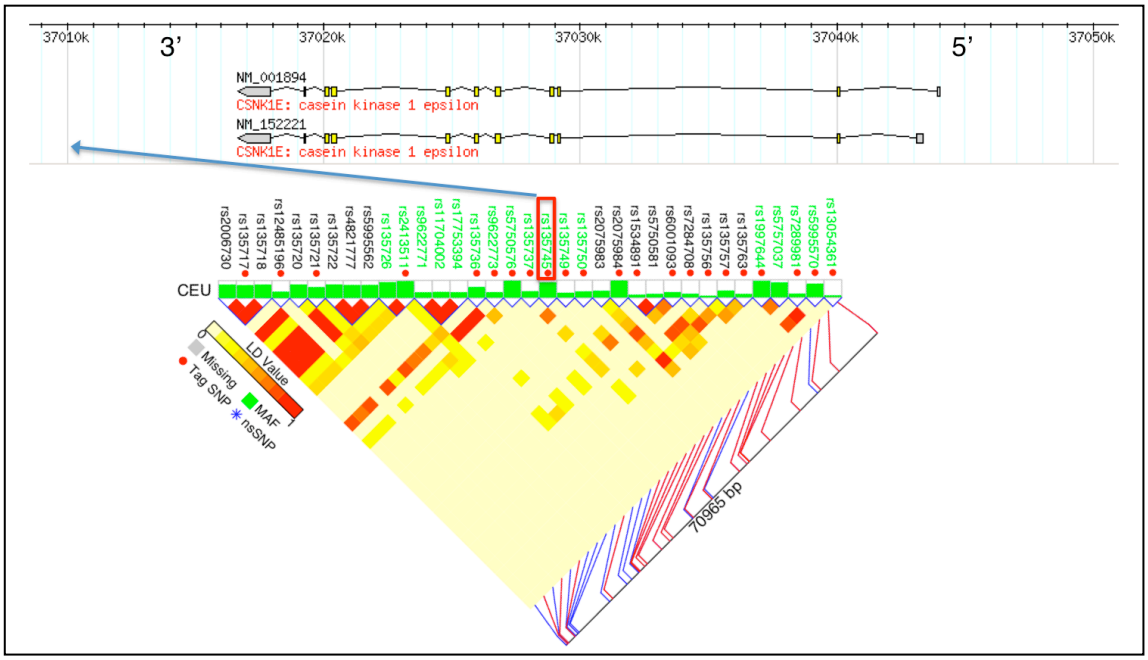

Figure 6. Exonic structure and genomic context of *CSNK1E* gene on chr.22. Depicts linkage disequilibrium structure of the region surrounding *CSNK1E* gene, as well as the position of HapMap SNPs (Release 3, Version 2). rs135745 highlighted with red box and genomic position indicated with blue arrow.

This is particularly of interest in light of a prior QTL study in mice showing that heightened ventral striatal CK1E mRNA expression was associated with exaggerated behavioral responses to amphetamine[107]. Higher CSNK1E expression could, in turn, lead to hyperphosphorylation and enhanced proteasomal degradation of PER2. As PER2 mutants show reduced MAOA expression and hyperdopaminergia in mesolimbic circuitry, CSNK1E-mediated acceleration of PER2 degradation could indirectly affect the amount of presynaptic DA available for release. Additionally, PER2 is under direct regulatory control by PP1: reducing PP1-mediated dephosphorylation – as would occur if higher levels of CSNK1E potentiated DARPP-32 inhibition of PP1 — accelerates PER2 degradation. Though speculative, allelic variation in the 3'

region of CSNK1E could enhance CSNK1E expression; this enhanced CSNK1E expression could, in turn, downregulate PER2, leading to reduced MAOA expression and mesolimbic DA hypereactivity.

This study has several important limitations to consider. First, as mentioned, the functional effect of variation at the rs135745 locus is not currently known. More study of this variant is required to understand if it is functional in its own right or is, rather, in linkage disequilibrium with the true causative variant. Even if the effects that we observe on stimulant-induced DA release, and which others have observed on subjective stimulant responses, are in fact due to the rs135745 SNP, this single variant in isolation is only going to account for a small proportion of the variance in a complex and multiply determined trait such as drug responsiveness. To fully characterize the genetic architecture of this trait – that is, to more completely account for the known heritability of individual differences in subjective drug responses – much more work is necessary. Future investigations would do well to focus on a genome-wide survey of potential candidate SNPs using stimulant responsiveness as a quantitative trait. In addition, exome sequencing of gene networks – such as the CSNK1E interactome – that might be linked to this trait may also bear significant fruit. Furthermore, although one might predict on the basis of available data that CSNK1E might be involved in risk for substance abuse (particularly, in risk for stimulant abuse), it remains unclear from research to date whether this is in fact the case. There is only one reported examination of association between CSNK1E and stimulant (methamphetamine) dependence, and this was negative

for association with rs135745. However, that study utilized a Japanese sample, and allele frequencies for rs135745 are dramatically different between European and Japanese (HapMap Release 28, August 2010; dbSNP 126)[140]. Interestingly, one study did find a nominally significant *CSNK1E* haplotype association to heroin addiction, though the SNP reported here (rs135745) was not tested for association in that study. Finally, our sample was ethnically heterogenous, raising the possibility that our observed associations are due to population admixture. The decision to include non-caucasian participants was motivated by a desire to obtain as large a sample size as possible, and our study recruitment was blind with respect to ethnicity. To address potential concerns related to stratification, we included self-reported ethnicity as a nuisance covariate in all of our general linear model analyses. Further, the impact of rs135745 on VS DA release remains significant even after we reanalyzed our data excluding non-Caucasian participants (all p's < 0.05). However, we acknowledge that we cannot completely rule out the possibility of spurious association due to cryptic ethnic stratification. The use of ancestry informative markers in future studies will enable us to detect population structure in our data and correct for its effects.

In this paper, we report that a polymorphic variant in the *CSNK1E* gene is associated the magnitude of striatal DA release in response to AMPH. As this same variant has been shown previously to predict euphoric subjective responses to AMPH, we believe that dopaminergic hyper-reactivity may account for this effect. Insofar as stronger subjective and striatal DA responses to drugs of abuse predict risk for drug dependence, these data support a role for

genetically-mediated individual differences in *CSNK1E* signaling in addiction liability.

CHAPTER III

GENETIC VARIABILITY IN HPA-AXIS FUNCTION ALTERS HUMAN STRIATAL DOPAMINERGIC REACTIVITY

Considering both direct (e.g. treatment and incarceration) and indirect costs (e.g. lost productivity, opportunity costs), it has been estimated that the annual economic liability associated with substance abuse in the United States approaches 500 million dollars[3]. Thus, In terms of sheer economic costs alone, substance abuse is one of the most expensive public health problems that we face today and represents a critical target for scientific investigation and intervention. Epidemiological investigations have significantly advanced our understanding of the specific social and environmental factors that predict the development of addiction. In particular, these studies converge to highlight stress as a profound risk factor for substance abuse and dependence. Stressors such as adverse childhood events – particularly forms of childhood maltreatment – increase risk for adult substance abuse in a dose-dependent manner; the presence of multiple and/or more severe ACEs (e.g. repeated childhood sexual abuse) predicts lower age of initiation for substance use, increased likelihood for developing substance dependence, and increased susceptibility to relapse after a period of abstinence [141-154]. For example, in a well controlled, large-scale study of twins reared together, Kendler and colleagues found that female twins exposed to childhood sexual abuse were at significantly greater risk (Odds Ratio

>3) for developing substance use disorders compared to unexposed co-twins, suggesting a causal link between maltreatment and addiction [151]. However, while such associations are consistent and robust, the specific neural adaptations underlying the link between stress and addiction are still unclear. Therefore, elucidating the biological mechanisms through which stress acts to affect risk for addiction is an essential step in characterizing its neurobiological risk architecture

Though human data are sparse, a wealth of preclinical findings argue that stress-induced dysregulation of mesocorticolimbic DA circuitry may play a key role in mediating the stress-substance abuse linkage. Experimental manipulations that induce stress (e.g. perinatal social isolation and maternal separation procedures) profoundly alter behavioral responses to drugs of abuse. In particular, stress during development is associated with sensitized locomotor responses to psychostimulants [155-157] and reward-associated conditioned stimuli [156], increased behavioral sensitization to psychostimulants [155, 158, 159], enhanced acquisition and maintenance of psychostimulant self-administration [160-163] and greater ethanol consumption [164]. Critically, stress-associated sensitization of behavioral responses to drugs of abuse occurs in parallel with a sensitization of mesolimbic DA neurochemistry. Stress increases basal extracellular nucleus accumbens DA concentrations [157, 165, 166], enhances psychostimulant-induced increases in ventral striatal DA levels [157, 165-171], and alters electrophysiological responses in prefrontal pyramidal neurons following VTA stimulation [172]. Further, developmental stress is associated with increased D1 and decreased DAT levels in the nucleus accumbens [155, 173, 174], decreased D2 levels in the VTA [164] and

mPFC [175], and functional downregulation of D2 receptors in nucleus accumbens [165]. Altogether, these findings clearly demonstrate that stress profoundly sensitizes mesocorticolimbic DA system function and behavioral responses to drugs of abuse. In considering the proximal biological mechanisms that may underpin these effects, it seems reasonable to suggest that stress-linked alterations in the mesolimbic reward pathway may be a downstream consequence of changes in neural systems that are more proximally involved in brain stress response. Indeed, this explanation has empirical support: the effects of stress on mesolimbic function appear to be due, in part, to stress-induced disruptions in the hypothalamic-pituitary-adrenal (HPA) stress axis.

There is considerable evidence that the sensitizing effects of stress on mesolimbic DA are mediated by the effect of stress on circulating HPA stress hormones, which in turn directly regulate mesolimbic DA function and drug seeking behavior[176]. Importantly, HPA stress hormones strongly regulate behavioral responses to drugs of abuse in animal models. For example, corticosterone treatment sensitizes locomotor responses to amphetamine[177], enhances the acquisition of cocaine and amphetamine self-administration in rodents [178, 179] and increases reinstatement of extinguished cocaine seeking[180]. Adrenalectomized rats show attenuated locomotor responses to cocaine [181] and fail to self-administer cocaine [182]; both of these effects are dose-dependently reversed by exogenous corticosterone[180, 181]. Additionally, pharmacological blockade of CRH1 (corticotropin releasing hormone receptor 1; also called corticotropin releasing factor receptor 1 or CRF1) and glucocorticoid (GC)

receptors reduces cocaine self-administration[183-185] and prevents stress- and conditioned cue-induced reinstatement of extinguished cocaine seeking[186-188]. Moreover, GCs are necessary for the facilitatory effects of acute stress on drug self-administration[189], and glucocorticoid receptor knockout mice show drastically reduced cocaine self-administration and behavioral sensitization[186]. Together, these data suggest that genetic factors influencing HPA-axis function may impact mesolimbic DA sensitivity in humans, and raise the intriguing possibility that genetic variability in HPA-axis function may affect risk for addiction, in part, by sensitizing mesolimbic DA circuitry.

Considered in this context, it is enlightening that variability in several genes encoding HPA-axis components – including *CRH* (encoding the corticotropin-releasing hormone), *CRH1* (encoding the corticotropin-releasing hormone receptor 1), and *CRH-BP* (encoding the corticotroping releasing hormone binding protein) – have been linked to stress responsiveness, to stress-induced drug consumption, and to substance abuse risk following exposure to childhood maltreatment[190-202]. *CRH* in particular is an intriguing candidate in light of several recent findings in non-human primates. Barr and colleagues reported that a functional single nucleotide polymorphism within the promoter region of the rhesus *CRH* gene (rh*CRH* -2232C→G) predicted alterations in CRH and ACTH levels (low baseline CSF CRH and high baseline CSF ACTH), greater behavioral disinhibition, and greater alcohol consumption[191]. This same group subsequently demonstrated that a separate functional *rhCHR* SNP (also located within the promoter region: -248C→T) predicts enhanced phasic HPA-axis stress reactivity,

behavioral inhibition following an acute stressor, and increased stress-induced alcohol consumption [190]. These findings are particularly intriguing in light of data indicating that polymorphic variation in the human *CRH* gene region is associated with trait-like behavioral inhibition[203, 204]. As both behavioral inhibition (anxious temperament) and behavioral disinhibition (impulsive temperament) are associated with enhanced risk for drug dependence[6], these data strongly imply a role for *CRH* genetic variability in stress-linked liability for addiction. However, there is little human data linking polymorphic variation at the *CRH* locus to addiction-related clinical diagnoses, and virtually no data relating *CRH* genetic variability to addiction-related neurobiological endophenotypes. Given the robust association between HPA-axis function and mesolimbic DA system reactivity, biological endophenotypes indexing human mesolimbic DA responsiveness would be appear to naturally lend themselves to such a line of inquiry.

To test the hypothesis that human polymorphic variation in *CRH* affects mesolimbic DA system function, we employed positron emission tomography (PET) imaging with the high-affinity D2/D3 ligand [18F]fallypride, in concert with an amphetamine challenge. This approach provides an in-vivo measure of stimulant-induced DA release, which we compared across *CRH* genotypes in our sample of healthy community volunteers. On the basis of the prior finding by Smoller and colleagues (2005) of an association between *CRH* SNPs and behavioral inhibition, we selected the single marker from that study with the strongest evidence of association (rs6999100)[203]. Given our prior work indicating that disinhibited temperament is linked to striatal dopaminergic hyper-reactivity,

we predicted that the allele that was found to be understransmitted to behaviorally inhibited individuals in that family-based association study (the T allele) would be associated with enhanced stimulant-induced DA release.

Methods

Participants

We studied 59 individuals (29 males; age range = 18-33, mean = 23; 50 Caucasian, 5 African-American, 3 Asian, and 1 individual of mixed Caucasian descent; Table 1) using a dual-scan placebo-controlled paradigm with [18F]fallypride PET and d-Amphetamine (AMPH). All participants were medically and psychiatrically healthy adults, age 18 to 35, with estimated IQ greater than 80. Subjects were excluded if they had any history of substance abuse, current tobacco use, alcohol intake greater than 8 ounces of whiskey or equivalent per week, use of psychostimulants (excluding caffeine) more than twice in the subject's lifetime or at all in past 6 months, any psychotropic medication for the past 6 months other than occasional use of benzodiazepines for sleep, history of psychiatric illness, significant medical condition, any condition which would interfere with MRI or PET studies (e.g., extreme obesity, claustrophobia, cochlear implant, metal fragments in eyes, cardiac pacemaker, neural stimulator, and metallic body inclusions or other metal implanted in the body which may interfere with MRI scanning, pregnancy, or anemia). Female participants were studied during the early follicular phase of their menstrual cycle.

Following initial screening, subjects were given an interview of their medical history and a structured psychiatric interview (SCID-NP;[109]). In addition to the regular questions in the non-alcohol substance dependence section of the SCID-NP, subjects were asked to indicate the number of times that they have taken any drug that they reported having tried, and asked to indicate any usage within the last 2 months. Any illicit drug use in the last 2 months was grounds for exclusion, even in subjects who did not otherwise meet criteria for substance abuse. Urine drug screens were performed to test for the presence of amphetamines, cocaine, marijuana, PCP, and opiates, benzodiazepines, and barbiturates.

Table 1.Demographic Information for CRH PET Sample

	rs1137100 A/A	Genotype G-Carriers
Sex (m/f)	9/9	20/21
Age (mean/SD)	23.07/3.39	22.96/3.64
PET Scanner (1/2)	7/11	19/22
Blind (Non-Blind/Blind)	5/13	10/31
% Change Left Ventral Striatal BP_{ND} (mean/SD)	3.28/5.97	7.53/7.12
% Change Right Ventral Striatal BP_{ND} (mean/SD)	2.01/5.24	7.39/6.88
Ethnicity (Cauc/Asian/AA/Hisp)	13/3/2/0	37/2/1/1

Table 1. Demographic information for *CRH* PET Sample

Genotyping

Saliva was collected from each subject using DNA Genotek Oragene-250 collection kits. Genomic DNA was extracted per manufacturer's protocols and banked at the Vanderbilt University Center for Human Genetics Research DNA Resources Core. Genotyping for rs699100 was conducted by the Vanderbilt DNA Resources Core through the use of the TaqMan 5' exonuclease assay (Assay

ID: C____366649_10). Genotyping for the participants reported in this study was performed in two separate genotyping runs, as part of a larger batch of genotyping that included DNA collected as part of an unrelated study. The genotyping success rate for this marker was high (100%). Allele frequencies were as follows: C/C = 2, C/T = 16, T/T = 41, and did not deviate from Hardy-Weinberg Equilibrium ($p = 0.78$). We found no evidence for an imbalance in genotype distribution across sex ($X^2 = 2.26$, $p = 0.32$), nor was genotype significantly associated with age ($F = 2.16$, $p = 0.13$). However, genotype distribution was significantly imbalanced across self-reported ethnicity ($X^2 = 25.12$, $p < 0.001$). Given the low number of C allele homozygotes observed in our sample, these individuals were combined with heterozygotes to create a "C-carriers" group that was used in subsequent analyses. Demographic information is reported in Table 1.

PET

Image Acquisition and Analysis

All PET images were acquired using [^{18}F]fallypride. ((*S*)-*N*-[(1-allyl-2-pyrrolidinyl)methyl]-5- (3[^{18}F]fluoropropyl)-2,3-dimethoxybenzamide), a substituted benzamide with very high affinity for D2/D3 receptors[110]. Unlike other D2/D3 ligands, [^{18}F]fallypride allows stable estimates of D2-like binding in both striatal and extrastriatal regions[111]. Our current resolution (see below) allows visualization of [18F]fallypride binding potential in the substantia nigra (SN)/ventral tegmental area (VTA), [for a discussion of the spatial resolution requirements for detecting activity in the SN see [112]]. However, this resolution

does not permit us to cleanly distinguish between different DA cell populations, preventing a clear parcellation of the VTA from the neighboring SN, which possesses higher levels of D2-like receptors. Previous studies have demonstrated good intersubject and intratest-retest reliability for measurement of [^{18}F]fallypride binding potential for the DA midbrain at the current resolution[113-115]. [^{18}F]fallypride binds with high affinity to both presynaptic ("D2-short") and postsynaptic ("D2-long") D2-like receptors[116]. However, because DA receptor expression in the midbrain is dominated by the D2-short receptor isoform [117] variance in [^{18}F]fallypride BP_{ND} within the midbrain is presumed to be driven by individual differences in these D2-short autoreceptors.

In addition, [^{18}F]fallypride has been found to be sensitive to endogenous DA release [114, 118], particularly in the striatum, making it an ideal ligand for use in conjunction with a dual scan strategy that allows assessment of both baseline receptor availability and individual differences in induced DA release. Baseline binding of [^{18}F]fallypride is also influenced by endogenous DA levels, and thus provides a metric of receptor availability, rather than absolute receptor density. However, receptor availability has proven a highly useful measure in quantifying individual differences in DA functioning, and indeed in some ways may be a more relevant variable than receptor density examined in isolation (as only available receptors can be engaged at a given point in time).

Protocols for PET image acquisition and analysis were derived from a larger ongoing study and have been previously published [115, 205]. Subjects received two PET scans using [^{18}F]fallypride. The first scan was a baseline

placebo scan; the second scan was performed while the subject received an amphetamine (d-AMPH) challenge. We used a single-blind drug administration regimen for the majority of participants (n = 44); however, we also included in our analyses 15 subjects who participated in the study during a pilot phase, and who were not blind to drug. We do not observe an imbalance of genotypes between blind and non-blind subjects (Pearson $X^2 = 0.99$, $p = 0.61$); nevertheless, blinded status was included as a nuisance covariate in all analyses where it was appropriate to do so (i.e. that included the 15 non-blind participants). PET imaging was performed on a GE Discovery LS scanner located at Vanderbilt University Medical Center that was upgraded to a Discovery STE system during the course of the study. All subjects received their baseline and d-AMPH scans on the same scanner. To ensure the validity of combining data across scanners, we performed a voxel-wise analysis comparing DA release between the two scanners. No clusters survived whole brain correction at $t > 2.5$ (lowest cluster-level p-value >.90). Moreover, no differences were observed in our anatomical region of interest, the ventral striatum (left and right VS; both p-values > 0.5). 26 participants were scanned on scanner 1, and 33 participants were scanned on scanner 2. We did not observe a significant imbalance of genotypes between the two scanners (Pearson $X^2 = 0.36$, $p = 0.82$; Table 1). Nevertheless, we included scanner (i.e. scanner 1 vs. scanner 2) as a nuisance covariate in our regression analyses. Following reconstruction both scanners had similar in plane and throughplane resolution. [^{18}F]fallypride was produced in the radiochemistry laboratory attached to the PET unit, following

synthesis and quality control procedures described in US Food and Drug Administration IND 47,245. Scans were timed to start 3 hours after 0.43mg/kg oral d-AMPH administration, which was timed to coincide with the period of peak plasma d-AMPH. 3-D emission acquisitions scans were performed following a 5.0 mCi slow bolus injection of [^{18}F]fallypride (specific activity greater than 3000 Ci/mmol). Serial scans were started simultaneously with the bolus injection of [^{18}F]fallypride and were obtained for approximately 3.5 hours, with two 15-minute breaks for subject comfort. CT transmission scans were collected for attenuation correction prior to each of the three emission scans.

Binding Potential Maps

Each subject's serial PET scans were first corrected for motion across scanning periods and then co-registered to the subject's structural T1-weighted MRI image. To determine the success of the coregistration in the midbrain, in a prior study of 34 subjects[115] we manually labeled several landmarks around the midbrain, including the posterior edge of the right and left inferior colliculus, the anterior-most point of the right and left cerebral peduncle and the interpeduncular fossa at z = 10, and the inferiormost point of the supramammillary commissure. Of these 34 subjects, all but one showed excellent midbrain coregistration, with no tag varying by 2mm in any direction from the mean coordinate of the tag (across these 33 subjects, the mean distance in any direction from the average tag was 1mm for every tag examined). Given the spatial resolution of the PET

images, this degree of misregistration is at the subvoxel level, and would have negligible impact on the results.

Regional D2/D3 binding potential (nondisplaceable; BP_{ND}) was calculated on a voxelwise basis using the full reference region method[119], with cerebellum chosen as the reference region because of its relative lack of D2/D3 receptors[120]. Voxelwise kinetic modeling was executed using Interactive Data Language. Prior studies in our lab indicate that the reference region method produces binding potential estimates that are in close agreement with estimates derived from Logan plots [121] using a metabolite corrected plasma input function. Because [^{18}F]fallypride binding values exhibit significant variability across different regions (e.g., striatum vs. prefrontal cortex; PFC), we used variance estimates at the voxelwise level rather than the pooled variance used in typical parametric analyses [122]. Individual images of percent-change in [^{18}F]fallypride binding from placebo to amphetamine (representing percent-change in DA release) were created by subtracting each subject's amphetamine scan from their placebo scan and dividing the resulting imaging by the placebo scan, using the "imcalc" image math routine in SPM5.

Region of Interest Analyses

Based largely on the preclinical research described above showing that stress-induced sensitization of behavioral and ventral striatal dopamine responses to stimulants may be mediated by glucocorticoid signaling, we limited

our analysis of the effects of *CRH* genetic variation on stimulant-induced DA release to the ventral striatum. To that end, we constructed an anatomical

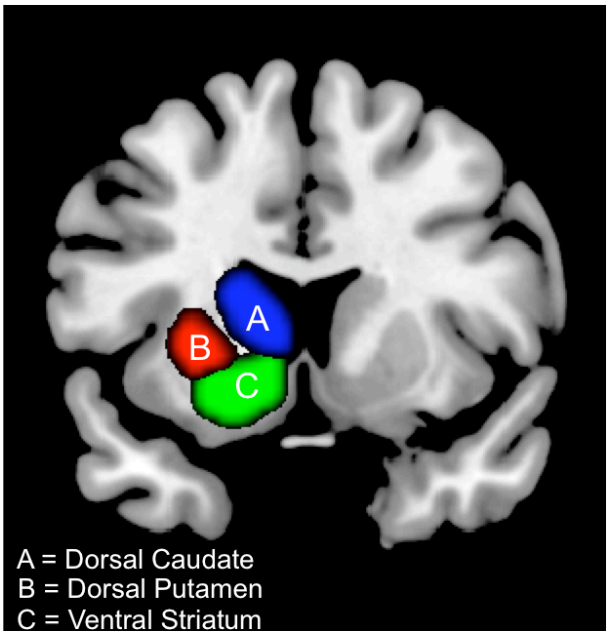

Figure 1. Striatal Regions of Interest (ROIs). ROIs rendered on a T1-weighted reference image, with a coronal slice at Y = 11 (MNI space). ROIs defined by anatomical criteria of Mawlawi et al (2001). Only pre-commisural ROIs are shown.

VS region of interest (ROI) by manually editing the striatum ROI derived from the LONI Probabilistic Brain Atlas 40 (LPBA40)[125] according to the criteria outlined in Mawlawi et al. (2001)[127]. To permit subregion-selective assessment of *CRH* genotype effects within the striatum, we parcellated the LPBA40 striatum ROI into 4 additional striatal subregion ROIs: dorsal caudate rostral to the anterior commissure (AC), dorsal putamen rostral to the AC, post-commissural caudate, and post-commissural putamen, also using previously described criteria[126, 127] (Figure 1). Percent-change values were averaged across all voxels within left

and right ROIs to create a single percent-change value for each individual's ROI mask.

Personality Measures

Impulsivity was assessed with the 30-item Barratt Impulsiveness Scale, version 11 (BIS-11)[206], which is one of the most widely used self-report measures of impulsive personality traits[207-216]. The BIS-11 yields scores for 6 subscales (Attention, Motor, Self-Control, Cognitive Complexity, Perseverance, and Cognitive Instability) and 3 factors (Attentional, Motor, Non-Planning), as well as a full-scale score. We used full-scale scores only for correlation analyses. The PPI comprises 187 multiple-choice items, and yields a total score, as well as scores for eight subscales: Impulsive Nonconformity, Blame Externalization, Machiavellian Egocentricity, Carefree Nonplanfulness, Stress Immunity, Social Potency, Fearlessness, and Coldheartedness. Based on the work of Benning and colleagues, and as reported previously in our work[63, 217], Impulsive-Antisocial (PPI-IA) factor scores were obtained by summing z-scores for the Machiavellian Egocentricity, Blame Externalization, Carefree Nonplanfulness, and Impulsive Nonconformity subscales. Within our PET sample, 44 participants had both personality and genetic data (13 C-carriers and 31 T/T individuals). BIS-11 scores in these 44 participants ranged from 43 to 84, with a mean (standard deviation) of 58.37 (9.37). For the PPI, mean, standard deviation, and range for each of the scales comprising the PPI-IA score were: 62.61, 12.33, 40-89 (Machiavellian Egocentricity); 28.59, 6.7, 19-41 (Blame Externalization); 35.02,

6.66, 23-51 (Carefree Nonplanfullness), 35,3, 8.38, 22-57 (Impulsive Nonconformity).

Statistical analysis

Statistical analyses using were performed using SPSS 17.0 for the Macintosh. To test for genotype effects on VS DA release, we used a linear regression model with rs6999100 genotype (dummy coded as 1 = C-carriers, 2 = T/T) as a predictor of % change in DA release (separate models for left and right VS). For each ROI, we used the mean percent-change in DA release value averaged across all voxels within that ROI (described above). PET scanner, ethnicity, and sex were included in the regression model as nuisance covariates. In follow-up control analyses, we added blinding status and VS BP_{ND} as additional nuisance covariates. Two-tailed tests were used, with alpha = 0.05.

To test for genotype effects outside of our primary VS region of interest, we performed a multivariate analysis of covariance (MANCOVA) using *CRH* genotype as a predictor of AMPH-induced DA release within left and right VS, dorsal caudate, dorsal putamen, post-commisural caudate, and post-commisural putamen (ROI creation described above). Sex, ethnicity, PET scanner and blinding status were included as nuisance covariates.

Whole-brain genotype-effect analyses were performed using SPM5 running on Matlab R2007b for the Macintosh. We used a linear regression model with rs6999100 genotype (dummy coded as 1 = C-carriers, 2 = T/T) as a predictor of voxelwise DA release (i.e. we used voxelwise images of percent-

change in DA release following AMPH administration, as described above). Scanner, ethnicity, blinding status and sex were included as nuisance covariates. To test for genotype effects, we used an uncorrected exploratory threshold of $p < 0.005$, coupled with a cluster-extend threshold of 20 voxels.

We used two MANCOVA analyses to investigate the impact of *CRH* genotype on impulsive temperament. For the BIS-11 analysis, *CRH* rs6999100 genotype was included as a predictor of BIS-11 total, subscale (Attention, Motor, Self-Control, Cognitive Complexity, Perseverance, and Cognitive Instability) and factor scores (Attentional, Motor, Non-Planning), with sex and ethnicity included in the model as nuisance covariates. For the PPI analysis, *CRH* rs6999100 genotype was included as a predictor of PPI total, subscale (Machiavellian Egocentricity, Social Potency, Fearlessness, Coldheartedness, Impulsive Nonconformity, Blame Externalization, Carefree Nonplanfulness, and Stress Immunity) and the PPI-IA factor scores, with sex and ethnicity included in the model as nuisance covariates. For both analyses, two-tailed tests were used, with alpha = 0.05. Reported p-values obtained from the test of Between-Subjects effects.

To test for a relationship between impulsive temperament and VS DA release, we performed two separate partial correlation analyses between bilateral VS DA release and BIS-11 total scores and between bilateral VS DA release and PPI-IA factor scores. In both cases, sex and PET scanner were used as covariates (personality data was only available for participants who were blind to drug administration). Two-tailed tests were used, with alpha = 0.05.

Descriptive and inferential statistics for VS BP_{ND} and AMPH-induced DA release

Across the entire sample, mean (standard deviation) baseline BP_{ND} values in the left and right VS ROIs were 22.04 (3.71) and 21.01 (3.77). Mean post-AMPH BP_{ND} values in the left and right VS ROIs were 20.79 (3.77) and 19.94 (3.68). Repeated-Measures Analysis of Variance (ANOVA) confirmed a significant reduction in BP_{ND} in both left ($F_{1,58}$ = 43.52, p = 0.0000000139) and right VS ($F_{1,58}$ = 36.36, p = 0.00000012) following AMPH administration (Figure 2).

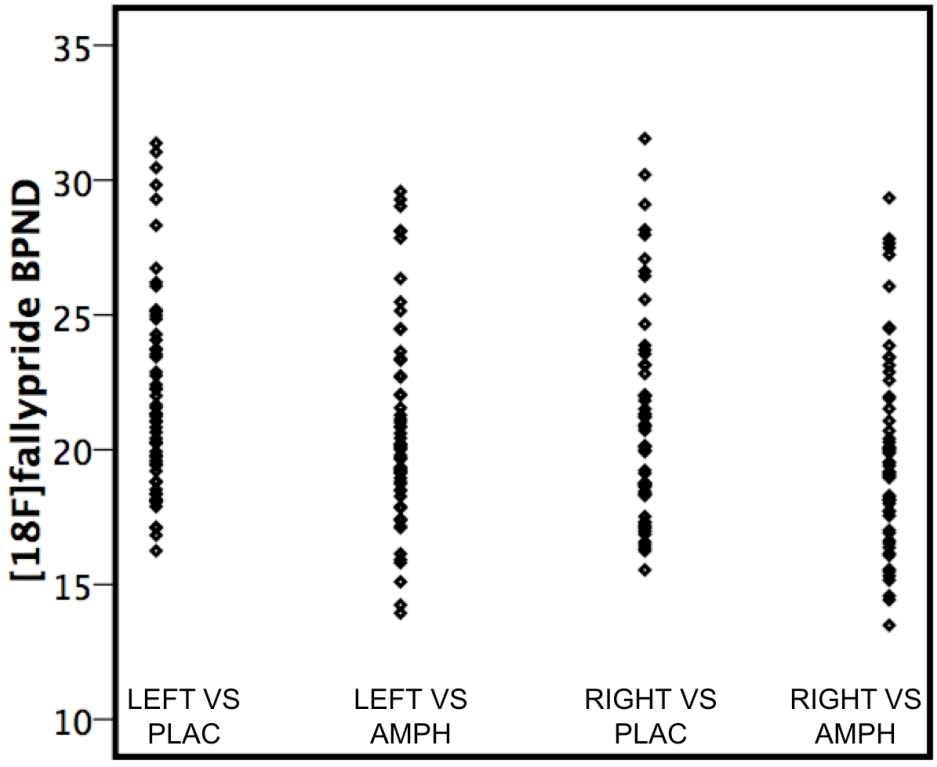

Figure 2. Amphetamine significantly reduces [18F]fallypride binding potential in ventral striatum. Binding potential (BP_{ND}) values on placebo (PLAC) and amphetamine (AMPH) depicted for left and right ventral striatum in 59 participants.

CRH Genotype effect on VS DA release

To test for an association between CRH rs6999100 genotype and AMPH-induced DA release, we regressed our participants' genotypes against their mean percent-change in DA release within left and right VS ROIs. This analysis revealed that rs699100 genotype was a significant predictor of AMPH-induced DA release in both left ($\beta = 0.38$, $p = 0.003$) and right ($\beta = 0.41$, $p = 0.002$) VS, such that T/T individuals showed significantly greater DA release in response to AMPH compared to C-carriers (Figure 3). These effects remained significant ($p < 0.01$) when blinding status was included in the regression model. We did not find a significant effect of *CRH* genotype on baseline D2/D3 binding in either left or right VS (both p-values > 0.5), and *CRH* genotype remained a significant predictor of AMPH-induced DA release even when controlling for baseline VS D2/D3 levels (both p-values < 0.005)

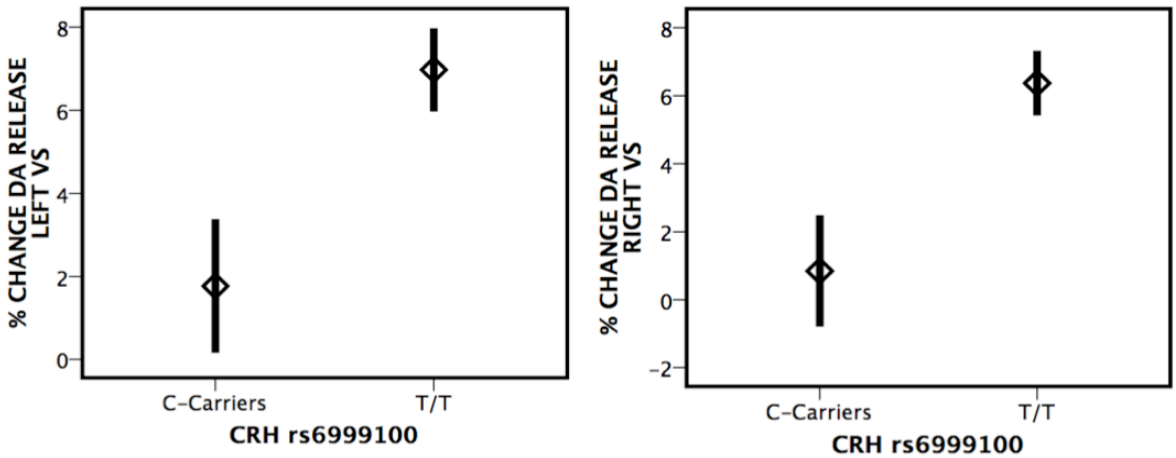

Figure 3. Effects of *CRH* rs6999100 genotype on AMPH-induced DA Release in left and right ventral striatum. Error bars indicate ± 1 standard error of the mean.

Striatal subregional specificity of *CRH* effects

While our a-priori hypothesis centered on a potential impact of *CRH* genetic variability in VS, we endeavored to test empirically the subregional selectivity for *CRH* rs6999100 effects within the striatum. To that end, we examined genotype effects within the following striatal subregions: (left and right) VS, dorsal caudate, dorsal putamen, post-commisural caudate, and post-commisural putamen. This analysis confirmed an effect for *CRH* genotype on DA release in left and right VS ($p = 0.005$, left VS; $p = 0.003$, right VS). In addition, we found a nominally significant effect in dorsal putamen (left and right, both p-values = 0.04); however this effect did not survive a Benjamini-Hochberg false-discovery rate (FDR) correction for multiple comparisons [218], which yielded adjusted p-values of 0.1 for the effect of genotype on left and right dorsal putamen. We did not find even nominally significant effects of *CRH* genotype on AMPH-induced DA release in any of the other ROIs (p-value range: 0.08-0.91).

Whole brain exploratory analysis

To examine unhypothesized effects of *CRH* rs6999100 genotype outside of the striatum, we performed a whole-brain regression analysis in SPM5. At a liberal, uncorrected exploratory statistical threshold, we did not observe any extrastriatal voxels in which AMPH-induced DA release was significantly predicted by genotype. In addition, we investigated the impact of *CRH* genetic variation on baseline D2/D3 binding. At the same exploratory threshold noted

above, we found no significant effect of variability at the *CRH* rs6999100 locus on D2/D3 levels anywhere in the brain.

Relationships between *CRH* genotype, Impulsive Traits, and DA Release

Taken together, our PET findings indicate that *CRH* genetic variation selectively affects AMPH-induced DA release within the VS. Specifically, T-allele homozygotes demonstrated sensitized VS DA responses to AMPH, compared to C-allele carriers. Given that the T-allele was found to be under-transmitted to individuals with behavioral inhibition in a prior study (i.e. the T-allele – and the common haplotype it monitors – was less likely to be found in chromosomes of behaviorally inhibited individuals), we sought to test for an association between *CRH* genetic variation and disinhibited temperament. To that end, we regressed rs699100 T-carrying status against scores on two trait measures of impulsivity (BIS-11 and PPI) that have been shown previously to index risk for addiction. We did not find compelling evidence for an association between *CRH* genotype and these measures in our small sample. For PPI total, subscale, and PPI-IA factor scores, all p-values were > 0.25, with the exception of Carefree Nonplanfullness, where the nominal p-value = 0.055 (T/T mean = 36.23, C-carrier mean = 32.08). For BIS-11 total and subscale scores, all p-values were > 0.3.

However, as we and others have shown previously that striatal DA responses to AMPH is positively correlated with these traits, we endeavored to confirm the relevance of increased DA release in T/T individuals to risk for behavioral disinhibition by demonstrating an association between DA release and

impulsive traits in the current sample. Consistent with our prior findings in a smaller sample, we found a positive relationship between left and right VS DA release and BIS-11 total scores ($r = 0.34$, $p = 0.03$, left VS; $r = 0.44$, $p = 0.004$, right VS) and between left and right VS DA release and PPI Impulsive-Antisocial factor scores ($r = 0.4$, $p = 0.009$, left VS; $r = 0.54$, $p = 0.0003$, right VS). This suggests that, even though we may have been underpowered to detect an association between rs6999100 and impulsive temperament, T-allele carriers showed a pattern of neurobiological response to AMPH that is, in turn, associated with high levels of impulsivity.

Discussion

Here, we report novel evidence that genetic variation in glucocorticoid signaling can impact striatal DA responses to a stimulant drug of abuse. In particular, we found that homozygous carriers of the *CRH* rs6999100 T-allele – previously demonstrated to be *under*transmitted to individuals with behavioral inhibition – show sensitization of striatal DA responses to AMPH, a response pattern that is itself linked to behavioral disinhibition. Of note, these data accord well with prior research on genetic variability at the *CRH1*, which encodes the cognate receptor for CRH (CRH1). CRH1 expression is regulated by stress [219-221] and CRH1 activity is known to be important for drug-induced sensitization[189, 222, 223]. Genetically mediated variation in CRH1 function is associated with HPA-axis hypersensitivity [199, 224], risk for stress-induced drug relapse in animal models[194], and with enhanced penetrance of environmental risk in predisposing

the development of alcohol abuse[200, 201, 225]. The current data add to a growing literature which, as a whole, endorses the notion that genetic variation in HPA-axis factors linked to stress responsivity can contribute to risk for addiction by affecting mesolimbic DA system function.

In considering a biologically tractable mechanism for the observed association between *CRH* genotype and DA function, we note that there is ample evidence that the impact of glucocorticoids on addiction-related behaviors is subserved by their direct effects on mesolimbic DA neurotransmission. Corticosterone elevates DA release in the NAcc and stimulates locomotor activity in a DA dependent manner[226], and stress-induced sensitization of DA release and behavioral responses to drugs of abuse is GC dependent[227, 228]. By contrast, adrenalectomy and glucocorticoid receptor antagonism decrease basal NAcc DA levels and suppress NAcc DA release and locomotor activity induced by drugs of abuse and acute stress[226 229, 230]. Crucially, CRH has been shown to stimulate NAcc DA release by acting on CRH1 receptors [231, 232] and the ability of cocaine to increase DA efflux in NAcc is blocked by pharmacological antagonism of CRH1 receptors[233] in a manner that is correlated with changes in VTA firing[234], suggesting that GCs modify DA signaling by acting directly on midbrain DA neurons. Supporting this notion, GC and CRH1 receptors are expressed on DAergic neurons of the VTA [235, 236], and both corticosterone and CRH potentiate NMDA-mediated VTA synaptic transmission[237, 238]. Further, CRH has been shown to act on VTA CRH1 receptors to increase VTA firing[239] and to elicit increased calcium release from intracellular stores[240], implicating GCs in stress-

induced neuroplastic processes within the DAergic midbrain. It is thus noteworthy that acute stress results in GR-dependent VTA synaptic adaptations that are also common to drugs of abuse[237, 238]. On the whole, these findings emphasize the fact that stress-associated sensitization within the mesolimbic DA system is mediated by stress-induced dysregulation of the HPA-axis, and lend credence to the idea that genetically-mediated differences in stress hormone signaling could affect risk for addiction by dysregulating mesolimbic DA function.

This study has several important limitations. First, the functional effects of the rs6999100 variant are unclear at this time. While this SNP has been identified as a *CRH* variant, it is in fact located within an intronic region in a gene (TRIM55) that is located immediately adjacent to the 3' end of CRH on the forward strand of chromosome 8q13.1 (Figure 4). As TRIM55 (also known as MURF-2 or RNF29) encodes a muscle-specific zinc finger protein[241], it is unlikely that the functional effects of rs6999100 allelic variation on mesolimbic DA are due to alterations in TRIM55 function or expression. However, it does seems reasonable to suggest that rs699100 may not itself be the causative variant driving the observed genotype-linked changes in DA signaling, but rather may in linkage disequilibrium (LD) with the true causative SNP, perhaps located within the *CRH* gene region proper. Indeed, rs6999100 is found within a substantial block of LD that encompasses the entire CRH gene, and extends nearly 31kb into its upstream region (Haploview 4.2, Version 3, Release 2, CEU population data; Figure 4).

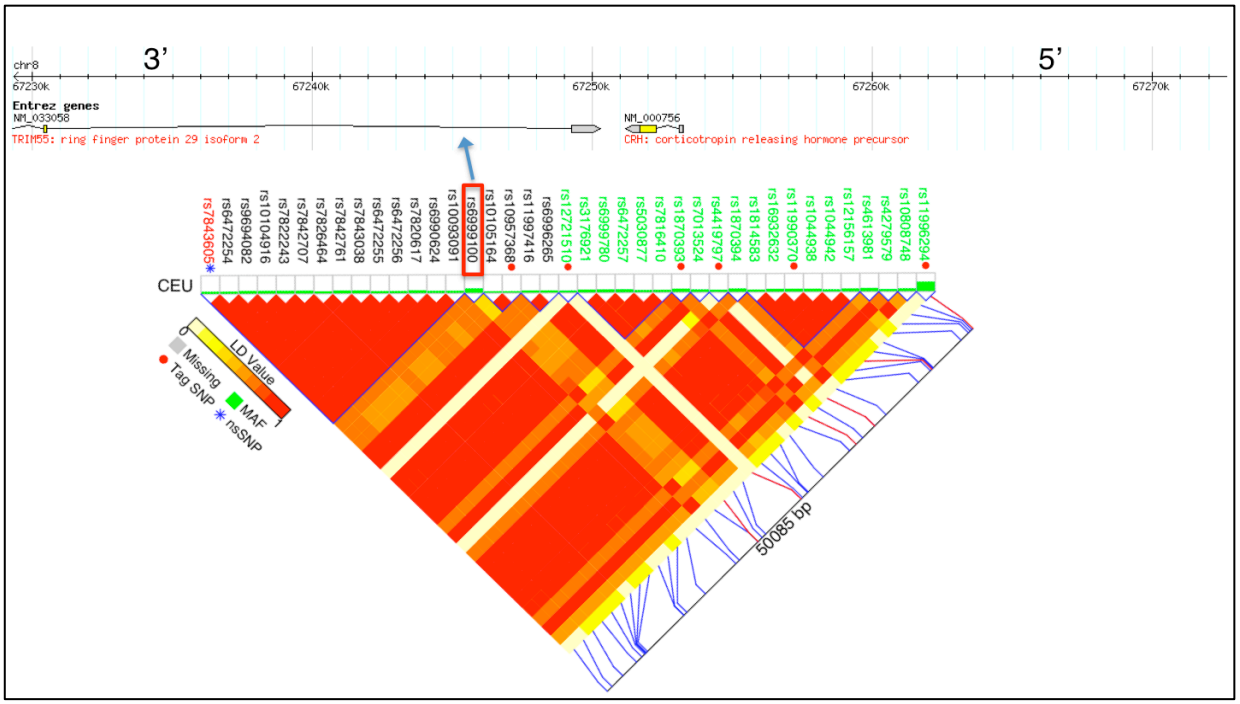

Figure 4. Exonic structure and genomic context of *CRH* gene on chr.22. Depicts linkage disequilibrium structure of the region surrounding C*RH* gene, as well as the position of HapMap SNPs (Release 3, Version 2). rs6999100 highlighted with red box and genomic position indicated with blue arrow.

Second, we did not find a significant association to trait phenotypes linked to addiction. This negative association may be due to the small sample size in this study (44 individuals had both personality and genetic data) compared to many other candidate gene association studies of traits, where sample size commonly exceeds 100 participants. However, we did observe that *CRH* variation was linked to enhanced DA release, which was in turn linked to impulsivity. While this does support our contention that the higher level of striatal DA released in response to AMPH in rs6999100 T/T participants (approximately 6% more compared to C-carriers) is relevant to individual differences in risk for disinhibitory psychopathology, a direct association would provide a more

compelling form of evidence. To that end, we suggest that these findings nominate *CRH* for focused association analysis targeting externalizing phenotypes such as addiction, antisocial personality disorder, and attention-deficit/hyperactivity disorder. By the same token, given that other groups have found a significant interaction between allelic variation in the *CRH1* receptor and environmental stress in predicting risk for alcohol abuse, and in light of empirical support for a similar interaction with *CRH* in non-human primates, the present data strongly highlight the need to more thoroughly explore the possibility of interplay between *CRH* genetic variability and environmental stressors in promoting substance abuse.

Third, though this is one of the largest studies of stimulant-induced DA release ever reported, the sample is extremely small by the standards of genetic association. While the expense of the dual-scan PET approach ($8-10k/subject) tends to prohibit large datasets, multi-site replication and data pooling for meta-analytic approaches would be useful in confirming the effects reported here, and may help to circumvent some of the traditional limits to PET sample size placed by cost. In addition, our sample was ethnically heterogeneous, raising the possibility that our observed associations are due to population admixture. The decision to include non-Caucasian participants was motivated by a desire to obtain as large a sample size as possible, and our study recruitment was blind with respect to ethnicity. We note that we included self-reported ethnicity as a nuisance covariate in all of our general linear model analyses, and also that our reported effects of *CRH* variability on VS DA release remain significant even

when non-Caucasians are excluded from analysis (all p's < 0.01). However, we acknowledge that we cannot completely rule out the possibility of spurious association due to cryptic ethnic stratification. The use of ancestry informative markers in future studies will enable us to detect population structure in our data and correct for its effects.

In conclusion, we report first-ever evidence that genetically-mediated variation in human HPA stress axis signaling affects ventral striatal DAergic responses to the administration of a stimulant drug of abuse. Homozygosity for the rs6999100 T allele – previously found to be undertransmitted in individuals with behavioral inhibition – predicted relatively sensitized ventral striatal DA release in response to amphetamine, the magnitude of which was in turn associated with higher levels of impulsive traits. These findings buttress prior preclinical associations of *Crh* variants to addiction-linked behaviors, and nominate the human *CRH* gene for more thorough phenotypic analysis.

CHAPTER IV

OBESITY-LINKED GENETIC VARIABILITY IN LEPTIN SIGNALING PREDICTS ALTERATIONS IN MESOLIMBIC DOPAMINE NEUROCHEMISTRY

Obesity – defined as a body mass index of ≥ 30 kg/m^2 – predicts increased morbidity from a range of somatic illnesses, including hypertension, type II diabetes, coronary heart disease, stroke, and a number of distinct cancers[242]. Current estimates suggest that fully 1/3 of the U.S. population meets the criteria for obesity[243]. Given its high prevalence and associated health risks, it is perhaps unsurprising that obesity is estimated to cost nearly $150 billion per year[244]. Further, these costs are likely to increase: obesity rates have increase dramatically over the past 30 years and continue to rise in some especially high-risk demographics[245]. Taken together, the cost in dollars and quality of life marks obesity as a significant public health problem that is worthy of scientific investigation and intervention.

While classic accounts tended to construe obesity strictly in terms of nutrition and metabolism, current conceptualizations stress the psychological pathomechanisms that characterize the pattern of compulsive, non-homeostatic consumption of palatable (high-fat/high-calorie) foods that is associated with the development and maintenance of obesity. Of note, it is on the basis of this compulsive behavioral component that some have called for obesity to be included in the next revision of the Diagnostic and Statistical Manual (DSM-V) as a form of

addiction[246]. Indeed, to a large extent, focus on the psychopathological dynamics that underlie obesity – in particular, obesity that is caused by an excessive motivational drive for high-fat/high-calorie food – centers on the striking parallels between obesity and drug addiction[247, 248]. Both addiction and obesity involve a transition from the volitional or recreational pursuit and intake of a substance (a drug or palatable food) that is valued because of the subjective pleasure produced by its consumption, to the compulsive pursuit of that substance that persists despite adverse consequences and the loss of control over its intake[246]. Indeed, Volkow and O'Brien have argued that each of the DSM-IV criteria for substance dependence – tolerance; withdrawal; overconsumption; desire for and failure to achieve a reduction in usage; significant time spent obtaining, consuming and recovering from the substance; abdication of social, occupation, and recreational activities due to substance use; and persistent use despite knowledge chronic physical and/or psychological problems caused by use – have parallels in obesity[246]. For example, considering tolerance and withdrawal, an obese individual may be forced to increase the amount of food they consume to reach satiety and may experience distress during periods of abstinence from their desired palatable food (i.e. during dieting). Intriguingly, obese individuals demonstrate a personality and neuropsychological profile that is markedly similar to individuals with substance use problems, including impulsive decision-making on the Iowa Gambling Task (IGT), greater delay-discounting, more impulsivity, and higher novelty seeking scores on the Temperament and Character Inventory (TCI)[249-252]. Moreover, relationships between obesity and impulsivity can be observed in early

childhood and adolescence[253, 254], suggesting that they co-develop. On the whole, the conspicuous psychopathological parallels between obesity and substance abuse tend to validate the construal of obesity as form of addiction or dependence (i.e. to palatable food), and raise the possibility of common biological origins.

Indeed, such parallels strongly imply the presence of shared pathophysiology between the two disorders, and preclinical research highlights dysregulation within mesolimbic dopamine reward circuitry as a potential common neurobiological factor that may underpin both substance abuse and obesity. All drugs of abuse act to promote the release of dopamine from nerve terminals in the ventral striatum, both directly via stimulatory effects on dopaminergic neurons of the substantia nigra and ventral tegmental area or by facilitating transmitter efflux from presynaptic boutons (or, alternatively, through direct inhibitory effects on synaptic dopamine clearance), and indirectly by inhibiting the influence of GABAergic interneurons on VTA activity[255-257]. Palatable food consumption appears to impinge on mesolimbic DA reward circuitry as well: several studies have shown that exposure to palatable food – and to food-conditioned cues – enhances ventral striatal DA release[258-260]. This regulation of striatal DA by food is mediated through the impact of palatable food on opioid signaling in GABAergic striatal medium spiny neurons (MSNs) and in GABAergic interneurons that synapse on DAergic VTA neurons[11, 261-263], and by downstream effects of palatable food on circulating levels of peptides that modulate dopaminergic function (e.g. leptin, insulin)[264, 265]. Importantly, instrumental responding for both food and drug rewards is abolished following pharmacological blockade of striatal DA receptors or

lesioning of striatal DA nerve terminals[266], demonstrating the shared requirement of striatal DA function for feeding-related and drug-related motivational drive.

Human neuroimaging data also emphasize common patterns of mesolimbic DA dysfunction in obesity and addiction. For example, food cravings (induced by dietary manipulation, in combination with a cue-induction technique) produce a pattern of brain activation in mesolimbic DA reward circuitry that is strikingly similar to that produced during drug craving[267]. This circuitry is engaged following the presentation of high calorie (but not low calorie) food in obese individuals[268], and in non-obese subjects with temperamental risk factors for obesity[269]. Importantly, a recent prospective study showed that mesolimbic response to high calorie food predicts future increases in BMI[270], suggesting that alterations in mesolimbic sensitivity to natural rewards are involved in the etiology of obesity. Remarkably, obesity is also associated with reduced NAcc D2 receptor density[271] – mirroring findings in drug addicted individuals[272-275] – and in animals that have developed compulsive patterns of palatable food intake[276]. Further, an addiction-linked variant in the D2 receptor gene that is associated with lower striatal D2 expression also modulates mesolimbic reward response to high calorie foods in obese subjects[270]. Taken together, these findings show that a natural reward (palatable food) activates the mesolimbic DA reward system in a manner that parallels drugs of abuse, and that individuals who demonstrate behavioral dyscontrol over natural rewards present with changes in mesolimbic DA neurochemistry and neurophysiology that echo changes seen in individuals who abuse drugs.

The striking degree of psychopathological and neurobiological synchrony between obesity and addiction suggests the possibility of shared etiopathophysiological mechanisms. Though the risk architectures of substance abuse and obesity are complex, heritability estimates range from 40%-60% for addiction[5, 6] and 45%-85% for BMI[277, 278], implying an important role for genetic susceptibility factors in both disorders. While empirical data on shared genetic variance between addiction and obesity are currently unavailable, it seems reasonable to propose – on the basis of the parallels highlighted above – that a shared genetic risk factor (or set of genetic risk factors) may act in a pleiotropic fashion to increase susceptibility to both addiction and obesity. This notion is also supported, in part, by evidence of comorbidity between the two disorders: individuals seeking treatment for obesity are significantly more likely to present with substance use problems compared to a general population sample[279].

If it is true that the psychopathological and neurobiological overlap reflects shared genetic risk between obesity and addiction, it may be the case that this common risk factor exerts a relatively deleterious influence over a neurobiological pathway that is jointly impacted in both disorders, with environmental factors pushing the expression of psychopathology in one direction or the other (i.e. towards obesity versus substance abuse). This would accord with the known importance of disorder-general genetic factors and disorder-specific environmental factors in predisposing disease outcomes in externalizing psychopathology[280]. For example, social and cultural dynamics clearly play a role in the development of both obesity and addiction, but these factors do not necessarily overlap. A factor

that influences an individual's level of physical activity and/or access to high-calorie/high-fat foods (especially processed and junk food) would, on a background of this common genetic risk, likely predispose the development of obesity but not (necessarily) addiction.

A putative common susceptibility factor should affect neurophysiological domains that are relevant to both disorders (e.g. motivation) rather than those that would be specific to one or the other (e.g. taste-related sensory processing). One avenue for exploring this possibility is to examine the impact of specific genetic risk factors for one disorder or the other on neurobiological phenotypes that are linked to both disorders. For example, one could probe the impact of an obesity-linked genetic variant on a measure of brain function that has been shown previously to be affected in both obesity and addiction. Such an effect, if found, would nominate the obesity-linked variant for enhanced phenotypic investigations in addiction. In this chapter we adopt this strategy, focused on characterizing the impact of an obesity-linked genetic variant in the leptin receptor gene (LEPR) on mesolimbic DA system function.

Leptin, perhaps the best-known and most well-characterized hormonal regulator of food intake, is a 16 kDa protein comprised of 146 amino acids[281, 282]. Secreted from adipose tissue, leptin crosses the blood brain barrier to exert central effects by acting on it cognate receptor. The leptin receptor (Lepr) is a one transmembrane domain spanning protein that is coupled to the Janus kinase-signal transducer and activation of transcription (JAK-STAT) pathway, and which belongs to the cytokine receptor superfamily[283-286]. The leptin signaling pathway

was the first genetic factor linked to obesity: a series of landmark studies examining two strains of spontaneously obese mice (so called *ob* and *db* mice) demonstrated that mutations in the leptin and leptin receptor genes were responsible for producing the obesity and hyperphagia phenotypes[281, 283, 287-289]. In both cases, the mutations resulted in impaired leptin signaling. Subsequent investigations suggested that peripheral leptin levels – which rise after feeding – act on hypothalamic leptin receptors as a satiety signal to inhibit feeding activity, explaining the association between leptin deficiency and obesity[281].However, more recent work suggests that leptin's modulatory impact on mesolimbic DA function may critically influence its effects on feeding behavior. Functional receptors for leptin are prominently expressed in the VTA, particularly in neurons that co-express tyrosine hydroxylase[290, 291], and local infusion of leptin into the VTA suppresses food consumption in a dose-dependent manner[291]. Further, leptin reduces the firing rate of VTA DA neurons *in vivo* and *in vitro* VTA slice preparations, and leptin infusion decreases synaptic DA concentrations in the NAcc[291, 292]. Critically, VTA-specific leptin receptor knockdown increases food intake, locomotor activity and sensitizes behavioral responses to food reward[291].

Human studies confirm that genetically mediated variability in leptin signaling alters human feeding behavior. Rare mutations in the leptin (LEP) and leptin receptor (LEPR) genes have been identified in several families, resulting in congenital leptin deficiency and marked obesity[293-295]. Of note, leptin replacement therapy in LEP mutation carriers partially rescues the obese phenotype by reducing food intake in a manner that is correlated with changes in ratings of food

"wanting[296]." Crucially, functional neuroimaging studies suggest that these changes are mediated by LEP-associated changes in the responsiveness of striatal reward circuitry to appetitive food stimuli[297, 298]. While congenital leptin deficiency is extremely rare, common single nucleotide polymorphisms (SNPs) in LEP and LEPR have been linked to increased risk for obesity, elevated body mass index, risky eating behaviors, and type-II diabetes[299-318]. Thus, common individual genetic differences in leptin signaling affect motivation to obtain food reward, and may do so by altering the reactivity of striatal reward circuitry. Given that DA is a powerful regulator of feeding motivation (as detailed above), and taking into account the known role for leptin in modulating mesolimbic DA circuit activity, these findings strongly suggest that obesity-associated variants in leptin signaling genes may affect risk by altering mesolimbic DA reactivity. Further, as proposed above, if true – that is, if an obesity-linked variant affects mesolimbic DA system function – this would nominate that variant as a novel putative risk variant for addiction. To test this notion, we examined the effect of two nonsynonymous coding variants in the LEPR gene with prior positive associations to BMI and obesity: rs1137101 (Q233R) and rs137100 (K109R)[319] on a measure of striatal DA function (amphetamine-induced DA release) that is sensitive to individual differences in risk for addiction.

Methods

Participants

We studied 59 individuals (29 males; age range = 18-33, mean = 23; 50 Caucasian, 5 African-American, 3 Asian, and 1 individual of mixed Caucasian descent; Table 1) using a dual-scan placebo-controlled paradigm with [18F]fallypride PET and d-Amphetamine (AMPH). Body Mass Index (BMI) data were available for 43 participants with rs137100 genotype data and 42 participants with rs137101 genotype data. All participants were medically and psychiatrically healthy adults, age 18 to 35, with estimated IQ greater than 80. Subjects were excluded if they had any history of substance abuse, current tobacco use, alcohol intake greater than 8 ounces of whiskey or equivalent per week, use of psychostimulants (excluding caffeine) more than twice in the subject's lifetime or at all in past 6 months, any psychotropic medication for the past 6 months other than occasional use of benzodiazepines for sleep, history of psychiatric illness, significant medical condition, any condition which would interfere with MRI or PET studies (e.g., extreme obesity, claustrophobia, cochlear implant, metal fragments in eyes, cardiac pacemaker, neural stimulator, and metallic body inclusions or other metal implanted in the body which may interfere with MRI scanning, pregnancy, or anemia). Female participants were studied during the early follicular phase of their menstrual cycle. See Table 1 for demographic information.

Table 1.Demographic Information for LEPR PET Samples

	rs1137100 A/A	Genotype G-Carriers	rs1137101 A/A	Genotype G-Carriers
Sex (m/f)	12/17	16/12	11/18	18/11
Age (mean/SD)	23.07/3.39	22.96/3.64	24.06/3.76	22.66/3.44
PET Scanner (1/2)	11/18	14/14	9/20	16/13
Blind (Non-Blind/Blind)	6/23	9/19	3/26	12/17
% Change Left Ventral Striatal BP_{ND} (mean/SD)	3.28/5.97	7.53/7.12	3.95/6.28	6.40/7/10
% Change Right Ventral Striatal BP_{ND} (mean/SD)	2.01/5.24	7.39/6.88	5.57/7.32	6.37/5.73
Ethnicity (Cauc/Asian/AA/Hisp)	25/3/0/1	25/2/3/0	25/3/0/1	24/2/3/0

Table 1. Demographic information for *LEPR* PET Sample

Following initial screening, subjects were given an interview of their medical history and a structured psychiatric interview (SCID-NP;[109]). In addition to the regular questions in the non-alcohol substance dependence section of the SCID-NP, subjects were asked to indicate the number of times that they have taken any drug that they reported having tried, and asked to indicate any usage within the last 2 months. Any illicit drug use in the last 2 months was grounds for exclusion, even in subjects who did not otherwise meet criteria for substance abuse. Urine drug screens were performed to test for the presence of amphetamines, cocaine, marijuana, PCP, and opiates, benzodiazepines, and barbiturates.

Genotyping

Saliva was collected from each subject using DNA Genotek Oragene-250 collection kits. Genomic DNA was extracted per manufacturer's protocols and banked at the Vanderbilt University Center for Human Genetics Research DNA Resources Core. Genotyping for rs1137101 and rs1137100 was conducted by the Vanderbilt DNA Resources Core through the use of the Sequenom massARRAY genotyping platform, based on a single-base primer extension reaction coupled

with mass spectrometry. Genotyping for the participants reported in this study was performed in two separate genotyping runs, as part of a larger batch of genotyping that included DNA collected as part of an unrelated study. The genotyping success rate for rs1137101 and rs1137100 for these 59 participants across the two genotyping runs was high (98.3% and 96.6%, respectively), leaving 58 participants with both PET data and rs1137101 genotype and 57 participants with both PET data and rs1137100 genotype. Allele frequencies were as follows: A/A = 29, A/G = 20, G/G = 9 (rs1137101) and A/A = 29, A/G = 19, G/G = 9 (rs1137100), and did not deviate from Hardy-Weinberg Equilibrium (p's > 0.05). The two markers were in low-moderate linkage disequilbrium (D' = 0.51) in our sample. We found no evidence for an imbalance in genotype distribution across sex (rs1137101: $X^2 = 3.6$, p = 0.17; rs1137100: $X^2 = 3.68$, p = 0.2) or PET scanner (rs1137101: $X^2 = 4.15$, p = 0.13; rs1137100: $X^2 = 1.01$, p = 0.6), nor was genotype significantly associated with age (rs1137101: F = 0.57, p = 0.57; rs1137100: F = 0.007, p = 0.93). Genotype distribution was significantly imbalanced across self-reported ethnicity and blinding status for rs1137101 ($X^2 = 18.67$, p = 0.005; $X^2 = 11.64$, p = 0.003) but not rs1137100 ($X^2 = 8.22$, p = 0.22; $X^2 = 0.97$, p = 0.62). Given the low number of G/G individuals for both markers, these participants were grouped with heterozygotes into a G-carrier group for subsequent analyses.

PET

Image Acquisition and Analysis

All PET images were acquired using [^{18}F]fallypride. ((*S*)-*N*-[(1-allyl-2-pyrrolidinyl)methyl]-5- (3[^{18}F]fluoropropyl)-2,3-dimethoxybenzamide), a substituted benzamide with very high affinity for D2/D3 receptors[110]. Unlike other D2/D3 ligands, [^{18}F]fallypride allows stable estimates of D2-like binding in both striatal and extrastriatal regions[111]. Our current resolution (see below) allows visualization of [18F]fallypride binding potential in the substantia nigra (SN)/ventral tegmental area (VTA), [for a discussion of the spatial resolution requirements for detecting activity in the SN see [112]]. However, this resolution does not permit us to cleanly distinguish between different DA cell populations, preventing a clear parcellation of the VTA from the neighboring SN, which possesses higher levels of D2-like receptors. Previous studies have demonstrated good intersubject and intratest-retest reliability for measurement of [^{18}F]fallypride binding potential for the DA midbrain at the current resolution[113-115]. [^{18}F]fallypride binds with high affinity to both presynaptic ("D2-short") and postsynaptic ("D2-long") D2-like receptors[116]. However, because DA receptor expression in the midbrain is dominated by the D2-short receptor isoform [117] variance in [^{18}F]fallypride BP_{ND} within the midbrain is presumed to be driven by individual differences in these D2-short autoreceptors.

In addition, [^{18}F]fallypride has been found to be sensitive to endogenous DA release [114, 118], particularly in the striatum, making it an ideal ligand for use in conjunction with a dual scan strategy that allows assessment of both baseline receptor availability and individual differences in induced DA release. Baseline

binding of [^{18}F]fallypride is also influenced by endogenous DA levels, and thus provides a metric of receptor availability, rather than absolute receptor density. However, receptor availability has proven a highly useful measure in quantifying individual differences in DA functioning, and indeed in some ways may be a more relevant variable than receptor density examined in isolation (as only available receptors can be engaged at a given point in time).

Protocols for PET image acquisition and analysis were derived from a larger ongoing study and have been previously published [114, 115]. Subjects received two PET scans using [^{18}F]fallypride. The first scan was a baseline placebo scan; the second scan was performed while the subject received an amphetamine (d-AMPH) challenge. We used a single-blind drug administration regimen for the majority of participants; however, we also included in our analyses 15 subjects who participated in the study during a pilot phase, and who were not blind to drug. To control for any confounding effect of between-group differences in blinding, blinded status was included as a nuisance covariate in all analyses. PET imaging was performed on a GE Discovery LS scanner located at Vanderbilt University Medical Center that was upgraded to a Discovery STE system during the course of the study. All subjects received their baseline and d-AMPH scans on the same scanner. To ensure the validity of combining data across scanners, we performed a voxel-wise analysis comparing DA release between the two scanners. No clusters survived whole brain correction at t = 2.5 (lowest cluster-level p-value >.90). Moreover, no differences were observed in our anatomical region of interest, the ventral striatum (left and right VS; both p-values > 0.45). 25

participants were scanned on scanner 1, and 33/32 (rs1137101/rs1137100) participants were scanned on scanner 2. To account for any potentially confounding effect of between-group differences in PET scanner, scanner (i.e. scanner 1 vs. scanner 2) was included as a nuisance covariate in our regression analyses. Following reconstruction both scanners had similar in plane and throughplane resolution. [^{18}F]fallypride was produced in the radiochemistry laboratory attached to the PET unit, following synthesis and quality control procedures described in US Food and Drug Administration IND 47,245. Scans were timed to start 3 hours after 0.43mg/kg oral d-AMPH administration, which was timed to coincide with the period of peak plasma d-AMPH. 3-D emission acquisitions scans were performed following a 5.0 mCi slow bolus injection of [^{18}F]fallypride (specific activity greater than 3000 Ci/mmol). Serial scans were started simultaneously with the bolus injection of [^{18}F]fallypride and were obtained for approximately 3.5 hours, with two 15-minute breaks for subject comfort. CT transmission scans were collected for attenuation correction prior to each of the three emission scans.

Binding Potential Maps

Each subject's serial PET scans were first corrected for motion across scanning periods and then co-registered to the subject's structural T1-weighted MRI image. To determine the success of the coregistration in the midbrain, in a prior study of 34 subjects[115] we manually labeled several landmarks around the midbrain, including the posterior edge of the right and left inferior colliculus, the

anterior-most point of the right and left cerebral peduncle and the interpeduncular fossa at *z* = 10, and the inferiormost point of the supramammillary commissure. Of these 34 subjects, all but one showed excellent midbrain coregistration, with no tag varying by 2mm in any direction from the mean coordinate of the tag (across these 33 subjects, the mean distance in any direction from the average tag was 1mm for every tag examined). Given the spatial resolution of the PET images, this degree of misregistration is at the subvoxel level, and would have negligible impact on the results.

Regional D2/D3 binding potential (nondisplaceable; BP_{ND}) was calculated on a voxelwise basis using the full reference region method[119], with cerebellum chosen as the reference region because of its relative lack of D2/D3 receptors[120]. Voxelwise kinetic modeling was executed using Interactive Data Language. Prior studies in our lab indicate that the reference region method produces binding potential estimates that are in close agreement with estimates derived from Logan plots [121] using a metabolite corrected plasma input function. Because [^{18}F]fallypride binding values exhibit significant variability across different regions (e.g., striatum vs. prefrontal cortex; PFC), we used variance estimates at the voxelwise level rather than the pooled variance used in typical parametric analyses [122]. Individual images of percent-change in [^{18}F]fallypride binding from placebo to amphetamine (representing percent-change in DA release) were created by subtracting each subject's amphetamine scan from their placebo scan and dividing the resulting imaging by the placebo scan, using the "imcalc" image math routine in SPM5.

Region of Interest Analyses

As our own prior work – as well as that of others – indicates that stimulant-induced DA release in the ventral striatum is a robust predictor of individual differences in trait risk factors for substance abuse[63, 67, 69, 71, 320, 321], we limited our analysis of the effects of *LEPR* genetic variation on stimulant-induced DA release to the ventral striatum. To that end, we constructed an anatomical VS region of interest (ROI) by manually editing the striatum ROI derived from the LONI Probabilistic Brain Atlas 40 (LPBA40)[125] according to the criteria outlined in Mawlawi et al. (2001)[126, 127]. To permit subregion-selective assessment of *LEPR* genotype effects within the striatum, we parcellated the LPBA40 striatum ROI into 4 additional striatal subregion ROIs: dorsal caudate rostral to the anterior commissure (AC), dorsal putamen rostral to the AC, post-commissural caudate, and post-commissural putamen, also using previously described criteria[126, 127]. Percent-change values were averaged across all voxels within left and right VS ROIs to create a single percent-change value for each individual's left and right VS ROI.

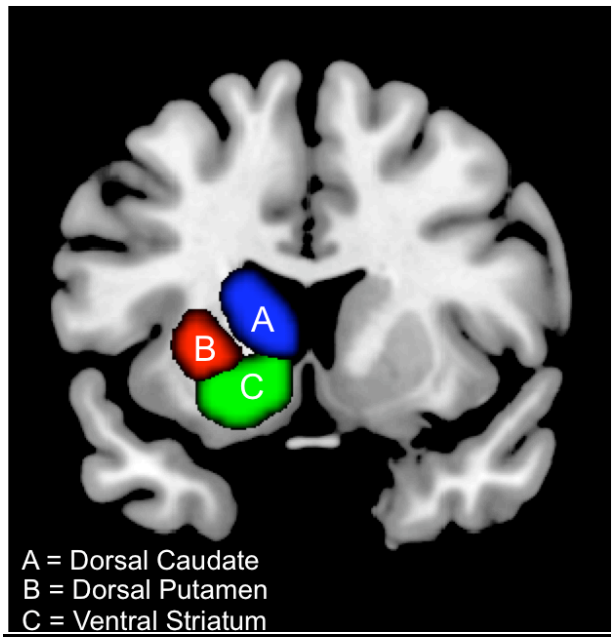

Figure 1. Striatal Regions of Interest (ROIs). ROIs rendered on a T1-weighted reference image, with a coronal slice at Y = 11 (MNI space). ROIs defined by anatomical criteria of Mawlawi et al (2001). Only pre-commisural ROIs are shown.

Personality Measures

Personality data were available for 43 participants with rs1137101 genotype data and 42 participants with rs1137100 genotype data. Impulsivity was assessed with the 30-item Barratt Impulsiveness Scale, version 11 (BIS-11)[206], which is one of the most widely used self-report measures of impulsive personality traits[207-216]. The BIS-11 yields scores for 6 subscales (Attention, Motor, Self-Control, Cognitive Complexity, Perseverance, and Cognitive Instability) and 3 factor scores (Attentional, Motor, Non-Planning), as well as a full-scale score. We used full-scale scores only for correlation analyses. Mean (standard deviation) and range of BIS-11 scores for the rs1137101 sample: 58.35 (9.47), 43-84. Mean (standard deviation) and range for the rs1137100 sample: 58.74 (9.33), 43-84. The PPI comprises 187 multiple-choice items, and yields a total score, as well as

scores for eight subscales: Impulsive Nonconformity, Blame Externalization, Machiavellian Egocentricity, Carefree Nonplanfulness, Stress Immunity, Social Potency, Fearlessness, and Coldheartedness. Based on the work of Benning and colleagues, and as reported previously in our work [63, 217], Impulsive-Antisocial (PPI-IA) factor scores were obtained by summing z-scores for the Machiavellian Egocentricity, Blame Externalization, Carefree Nonplanfulness, and Impulsive Nonconformity subscales. For the rs1137101 sample, mean (standard deviation), and range for each of the scales comprising the PPI-IA score were: 62 (11.79), 40-87 (Machiavellian Egocentricity); 28.35 (6.58), 19-41 (Blame Externalization); 34.95 (6.72), 23-51 (Carefree Nonplanfullness); 34.91 (8.1), 22-57 (Impulsive Nonconformity). For the rs1137100 sample, mean (standard deviation), and range for each of the scales comprising the PPI-IA score were: 62.4 (12.27), 40-89 (Machiavellian Egocentricity); 28.67 (6.76), 19-41 (Blame Externalization); 35.12 (6.8), 23-51 (Carefree Nonplanfullness); 34.93 (7.82), 22-56 (Impulsive Nonconformity).

Statistical Analysis

Statistical analyses using were performed using SPSS 17.0 for the Macintosh. To test for genotype effects on VS DA release, we used two linear regression models with rs1137100 and rs1137101 genotypes (dummy coded as 1 = A/A, 2 = G-carriers) as separate predictors of % change in DA release (separate models for left and right VS). For each ROI, we used the mean percent-change in DA release value averaged across all voxels within that ROI (described above).

PET scanner, ethnicity, and sex were included in the regression model as nuisance covariates. In follow-up control analyses, we added blinding status and VS BP_{ND} as additional nuisance covariates. Two-tailed tests were used, with alpha = 0.05.

To test for genotype effects outside of our primary VS region of interest, we performed two multivariate analyses of covariance (MANCOVA) using *LEPR* genotypes as predictors of AMPH-induced DA release within left and right VS, dorsal caudate, dorsal putamen, post-commisural caudate, and post-commisural putamen (ROI creation described above). Sex, ethnicity, PET scanner and blinding status were included as nuisance covariates.

Whole-brain genotype-effect analyses were performed using SPM5 running on Matlab R2007b for the Macintosh. We used two linear regression models with *LEPR* rs1137100 and rs1137101 genotypes (dummy coded as 1 = A/A, 2 = G-carriers) as predictors of voxelwise DA release (i.e. we used voxelwise images of percent-change in DA release following AMPH administration, as described above). Scanner, ethnicity, blinding status and sex were included as nuisance covariates. To test for genotype effects, we used an uncorrected exploratory threshold of $p < 0.005$, coupled with a cluster-extend threshold of 20 voxels.

We used four MANCOVA analyses to investigate the impact of *LEPR* genotype on impulsive temperament. For the BIS-11 analyses, *LEPR* rs1137100 and rs1137101 genotypes were included as separate predictors of BIS-11 total, subscale (Attention, Motor, Self-Control, Cognitive Complexity, Perseverance, and Cognitive Instability) and factor scores (Attentional, Motor, Non-Planning), with sex

and ethnicity included in the model as nuisance covariates. For the PPI analysis, *LEPR* rs1137100 and rs1137101 genotypes were included as separate predictors of PPI total, subscale (Machiavellian Egocentricity, Social Potency, Fearlessness, Coldheartedness, Impulsive Nonconformity, Blame Externalization, Carefree Nonplanfulness, and Stress Immunity) and the PPI-IA factor scores, with sex and ethnicity included in the model as nuisance covariates. For both analyses, two-tailed tests were used, with alpha = 0.05. Reported p-values obtained from the test of Between-Subjects effects.

To test our hypothesis that sensitized VS DA release mediated the relationship between *LEPR* rs1137100 genotype and impulsive traits, we estimated the indirect of genotype on PPI-IA and BIS-11 scores through left and right VS DA release using a nonparametric approach as outlined in Preacher et al (2008)[322], and implemented in the SPSS macro indirect.sbs. We tested four mediation models: for each model *LEPR* rs1137100 was used as the predictor (X) of BIS-11 (Y1) or PPI-IA scores (Y2), using left VS DA (M1) or right VS DA (M2) as a mediator. Thus, the four models were: 1) X→M1→Y1, 2)X→M2→Y1, 3)X→M1→Y2, and 4)X→M2→Y2. For each model, sex, ethnicity and PET scanner were included as nuisance covariates, and a bootstrap resampling procedure (5000 resamples) was used to generate point estimates of and 95% confidence intervals for each indirect effect (bias corrected and accelerated).

Results

Descriptive and inferential statistics for VS BP_{ND} and AMPH-induced DA release

For the rs1137101 sample, mean baseline BP_{ND} in the left and right VS ROIs were 21.89 (3.57) and 20.89 (3.69). Mean post-AMPH BP_{ND} values in the left and right VS ROIs were 20.7 (3.73) and 19.86 (3.66). For the rs1137100 sample, mean baseline BP_{ND} in the left and right VS ROIs were 22.11 (3.75) and 21.09 (3.81). Mean post-AMPH BP_{ND} values in the left and right VS ROIs were 20.86 (3.82) and 20.02 (3.72). Repeated-Measures Analysis of Variance (ANOVA) confirmed a significant reduction in BP_{ND} in both left and right VS following AMPH administration (rs1137100 sample: p = .00000003, left VS and p = 0.0000003, right VS; rs1137101 sample: p = .000000026, left VS and p = 0..00000024, right VS) (Figure 2).

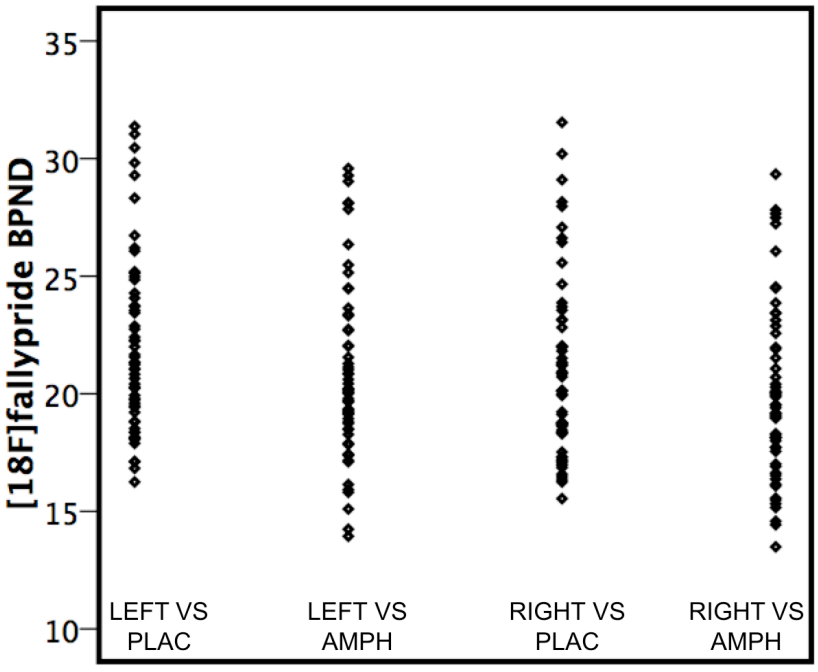

Figure 2. Amphetamine significantly reduced [18F]fallypride binding potential in ventral striatum. Binding potential (BP_{ND}) values on placebo (PLAC) and amphetamine (AMPH) depicted for left and right ventral striatum for 59 participants with dual-scan PET data.

LEPR Genotype effect on VS DA release

To test for an association between *LEPR* genotype and AMPH-induced DA release, we regressed *LEPR* genotypes against mean percent-change AMPH-induced DA release values calculated from left and right VS ROIs. These analyses revealed a significant effect of rs1137100 genotype on AMPH-induced DA release in both left (β = 0.34, p = 0.015) and right (β = 0.43, p = 0.002) VS, such that G-carriers showed significantly greater DA release in response to AMPH compared to A/A individuals (Figure 3). However, AMPH-induced DA release did not vary as a function of rs1137101 genotype in either left or right VS (both p's > 0.1). We did not find a significant effect of *LEPR* genotype on baseline D2/D3 binding in either left or right VS for either rs1137100 or rs1137101 (p-values for rs1137100 > 0.8; p-values for rs1137100 > 0.1). Importantly, rs1137100 remained a significant predictor of AMPH-induced DA release even when controlling for baseline VS D2/D3 levels (i.e. baseline left and right VS BP_{ND} included as a nuisance covariate; left VS, p = 0.017; right VS, p = 0.002).

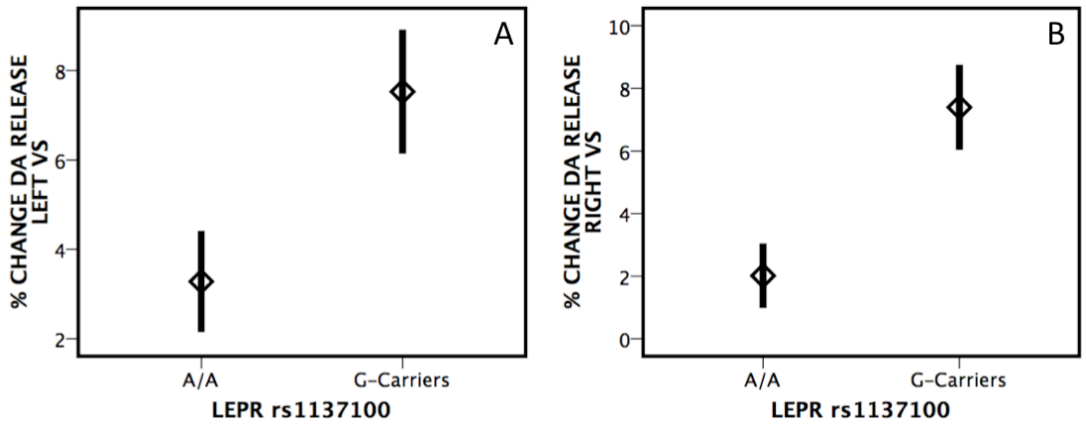

Figure 3. Effects of *LEPR* rs1137100 genotype on AMPH-induced DA Release in left (A) and right (B) ventral striatum. Error bars indicate ± 1 standard error of the mean.

Striatal subregional specificity of *LEPR* genotype effects

While our a-priori hypothesis centered on a potential impact of *LEPR* genetic variability in VS, we endeavored to test empirically the subregional selectivity for *LEPR* rs1137100 effects within the striatum (left and right VS, dorsal caudate, dorsal putamen, post-commisural caudate, and post-commisural putamen). This analysis revealed nominally significant effects in post-commisural putamen (left VS, $p = 0.012$; right VS, $p = 0.041$) and left dorsal putamen ($p = 0.047$); however these did not survive a false-discovery rate correction for multiple comparisons[218]. We did not find even nominally significant effects of rs1137100 genotype on AMPH-induced DA release in any of the other ROIs (p-value range: 0.06-0.53). We did not observe an impact of rs1137101 on any of these additional striatal ROIs (p-value range: 0.08-0.76).

Whole brain exploratory analysis

To examine unhypothesized effects of *LEPR* genotype outside of our a-priori VS ROIs, we performed a whole-brain regression analysis in SPM5. At a liberal, uncorrected exploratory statistical threshold ($p < 0.005$ with a cluster-extent threshold of 20 voxels), we did not observe any extrastriatal voxels in which AMPH-induced DA release was significantly predicted by either rs1137100 or rs1137101 genotype. In addition, we investigated the impact of *LEPR* genetic variation on baseline D2/D3 binding. At the same exploratory threshold noted above, we found no significant effect of variability at the LEPR loci on D2/D3 binding potential anywhere in the brain.

BMI Correlation Analyses

Across both the rs1137101 and the rs1137100 samples, BMI ranged from 17.5-30.6, with means (standard deviations) of 23.38 (3.39) and 23.21 (3.35), respectively. Further, the distribution of BMI values was significantly right-skewed in both samples (Figure 4), indicating that that the majority of participants were of normal weight. Despite this fact, 12 participants in the rs1137100 group and 13 participants in the rs1137101 group met criteria for overweight (BMI $\geq$ 25 kg/m$^{2)}$ and 1 person (the same individual) in each group met criteria for obesity. Therefore, we were interested in determining whether BMI variation in this range was associated with either *LEPR* genotype or AMPH-induced VS DA release. Partial correlation analysis (controlling for sex and PET scanner – all participants with BMI data were blind to AMPH administration) showed that AMPH-induced DA release was not associated with DA release in either left or right VS in both the rs1137101 ($p = 0.54$, left VS; $p = 0.91$, right VS) and rs1137100 groups ($p = 0.45$, left VS; $p = 0.83$, right VS). In addition, neither rs1137101 or rs1137100 genotypes were significant predictors of BMI (p's = 0.61 and $p = 0.74$, respectively; adjusted for sex and ethnicity).

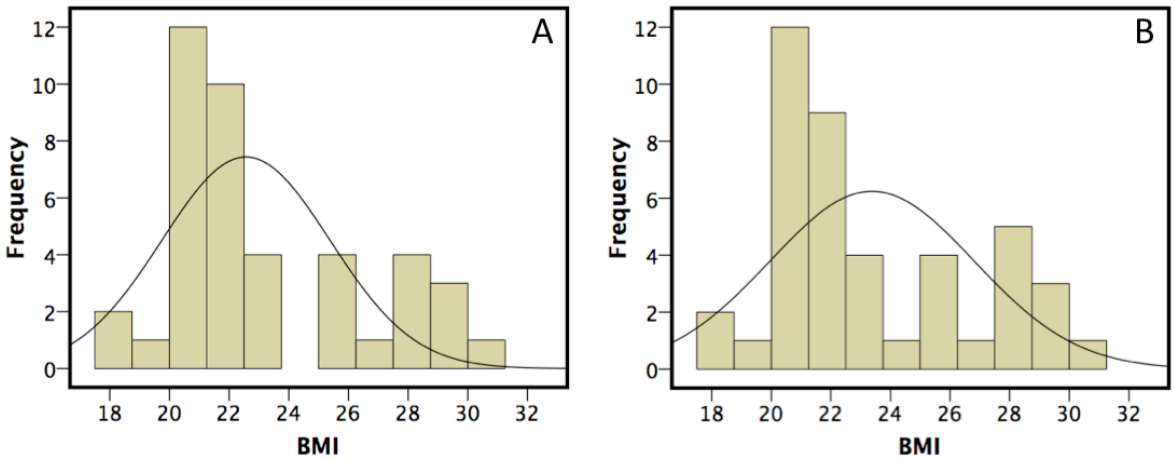

Figure 4. Distributions of BMI values for the rs1137100 (A; n = 42) and rs1137101 (A; n = 43) samples.

LEPR genotype and Impulsive Temperament

Prior work has demonstrated a shared propensity toward impulsive and novelty seeking traits in both obese and substance-abusing individuals. These same traits have been shown by our group and by others to be linked to enhanced stimulant-induced striatal DA release. Thus, the present finding that *LEPR* rs1137100 G-carriers showed relatively exaggerated AMPH-induced DA release within VS raised the possibility that *LEPR* rs1137100 genotype is also associated with impulsive traits. To test this notion, we examined the association between *LEPR* rs1137100 genotype and scores on the Barratt Impulsiveness Scale -11 (BIS-11) and Psychopathic Personality Inventory, two measures that are both sensitive to risk for addiction and which independently predict individual variation in striatal DA reactivity in response to AMPH. *LEPR* rs1137100 genotype was a significant predictor of variability in BIS-11 total scores ($p = 0.02$) and PPI impulsive-antisocial (PPI-IA) factor scores ($p = 0.001$). In both cases, G-carriers showed higher scores on these measures. Intriguingly, post-hoc control analyses

revealed that the relationship between *LEPR* genotype and impulsive temperament was somewhat specific to impulsive-antisociality: while the relationship between rs1137100 genotype and PPI-IA scores remained significant after controlling for BIS-11 scores ($p = 0.02$), rs1137100 genotype was no longer a significant predictor of BIS-11 scores after controlling for variation in PPI-IA scores, ($p = 0.44$).

LEPR Genotype, Striatal DA release and Impulsivity: Mediation Analyses

As mentioned above, we have shown previously that the magnitude of AMPH-induced striatal DA release is a significant positive predictor of impulsivity and impulsive-antisociality as measured by the BIS-11 and PPI[63, 71]. Here, we find that *LEPR* rs1137100 G-carriers show increased impulsivity and impulsive-antisociality, and increased AMPH-induced DA release in ventral striatum. Taken together, these findings raise the intriguing possibility that genetic variability in leptin signaling (as indexed by *LEPR* rs1137100 genotype) modulates impulsive temperament through an impact on striatal DA reactivity. As an initial test of this hypothesis, we performed correlation analyses to replicate in the rs1137100 sample our prior finding of an association between AMPH-induced VS DA release and impulsive traits. Consistent with our prior work (in sample that partially overlaps the one reported in this chapter), we found a significant correlation between ventral striatal DA release following AMPH administration and BIS-11 scores ($r = 0.32$, $p = 0.045$, left VS; $r = 0.43$, $p = 0.006$, right VS) and PPI-IA scores ($r = 0.39$, $p = 0.013$, left VS; $r = 0.53$, $p = 0.001$, right VS); all p-values

adjusted for sex and PET scanner. To confirm our hypothesis that enhanced striatal DA release mediates the relationship between genetic variation in *LEPR* and impulsive traits, we next performed a series of mediation analyses.

First, we tested the significance of the indirect effect of *LEPR* genotype on BIS-11 scores through left and right VS DA release[322]. For left VS, the point estimate of the indirect effect was 1.227; however, the 95% confidence interval (C.I.) for this effect was -0.587 – 5.389, and we therefore cannot conclude that this effect is not significantly different from zero. For right VS, the point estimate of the indirect effect was 2.31, with a 95% C.I. of 0.366 – 6.884, confirming that this effect was significantly non-zero. We then performed the same tests of mediation employing PPI-IA scores as the dependent measure. For left VS, the point estimate of the indirect effect was 0.4672; however, the 95% confidence interval (C.I.) did cross zero (-0.022 – 1.596), and we therefore cannot conclude that this effect is not significantly different from zero. For right VS, the point estimate was 0.842, with a 95% C.I. of 0.107 – 1.991, permitting us to claim that this effect is significantly non-zero. Thus, the analyses indicate that the effect of *LEPR* rs1137100 genotype on impulsive and impulsive-antisocial traits is due, in part, to the sensitizing effect of carrying a G-allele on striatal DA reactivity (though this effect – while statistically compelling for right VS – is merely suggestive for left VS) (Figure 5).

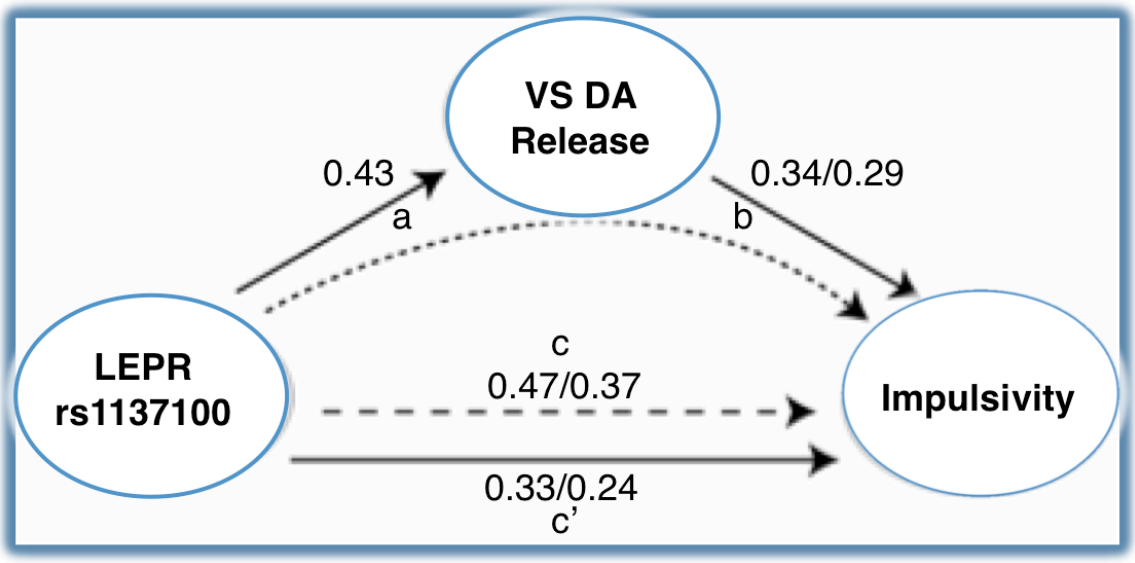

Figure 5. Striatal dopamine sensitization mediates the impact of *LEPR* genotype on impulsivity. Path a shows coefficients for the effect of *LEPR* rs1137100 genotype on right VS AMPH-induced DA release. Path b shows the coefficients for the effect of striatal DA on impulsive traits (PPI-IA/BIS-11). Paths c and c' show coefficients for the total (dashed line) and direct (solid line) effects of *LEPR* genotype on impulsivity. All coefficients standardized.

Discussion

Prior research has strongly implicated genetic variation in leptin – a critical biological mediator of feeding-related activity – as a susceptibility factor for obesity and obesity-related morbidity (e.g. Type-II diabetes and hypertension)[299-318]. While such associations have typically been presumed to be a consequence of altered leptin signaling on homeostatic feeding processes mediated by the hypothalamus[281], an alternative perspective is offered by recent preclinical work showing strong effects of leptin on mesolimbic DA circuitry for reward and motivation[264]. Here, we report the novel finding that genetically mediated variability in leptin signaling in humans predicts individual differences in impulsivity by

sensitizing striatal DA responses to a psychostimulant drug of abuse. This finding supports the notion that leptin's involvement in human feeding behavior is mediated through an impact on mesolimbic DA reward circuitry, as suggested by prior functional imaging studies of food reward[298]. Critically, we show for the first time that genetic variation in leptin affects human DA function, and does so in response to a non-food reinforcer – in particular, to a drug of abuse. Insofar as impulsive traits and exaggerated DA responses to stimulant drugs are markers of risk for addiction[63, 68, 71], the present findings raise the intruiging suggestion that the *LEPR* variant studied herein may act pleiotropically to influence both addiction and obesity, and may do so through a common sensitizing influence on the mesolimbic DA reward pathway.

Leptin was first recognized as a regulator of feeding behavior by genetic dissection of spontaneously obese (*ob*/*ob* and *db*/*db*) mice, which were found to possess highly penetrant mutations in the murine *Lep* and *Lepr* genes[281, 283, 287-289]. Typically, these mutations produce a truncated leptin receptor protein that lacks a functional intracellular domain[281, 287, 288]. Analogous human mutations – also producing a truncated form of the leptin receptor – have been identified by sequencing in families with a multigenerational phenotype of severe, early-onset obesity: approximately 10 such rare mutations in the human *LEPR* gene have been reported, each causing congenital leptin deficiency[293-295]. Notably, a functional magnetic resonance imaging (fMRI) study in two *LEPR* mutation carriers showed a heightened striatal response to food during a leptin deficient state, which was normalized by the acute administration of leptin[298]. Further of

note, striatal hyper-reactivity due to leptin deficiency was, in that study, linked to exaggerated subjective craving for food that persisted even after feeding[298]. Together, these studies suggest that genetically determined differences in leptin signaling affect feeding behavior in humans by altering brain responses to food stimuli within neural circuitry subserving reward and motivation.

In contrast to the few highly penetrant, rare mutations described above – many of which are only found in a small number of consanguineous families – many common variants in *LEPR* have been identified, and several of these have reasonably consistent associations to obesity and obesity-related morbidity[298]. In the present work, we studied two of the most thoroughly examined variants, rs1137101 and rs1137100, both of which are nonsynonymous coding SNPs located within the *LEPR* gene (Figure 6). The human *LEPR* gene is comprised of 20 exons, and spans approximately 70kb of DNA on chromosome 1p31[319, 323]. rs1137101 is an A→G SNP at position 668 in exon 6, causing an arginine to be substituted for a glutamine at codon 223 (Q223R; Gln223Arg)[319, 323]. rs1137100 is an A→G transition at position 326 in exon 4, resulting in lysine to arginine substitution at codon 109 (K109R;Lys109Arg) [319, 323] (Figure 5).

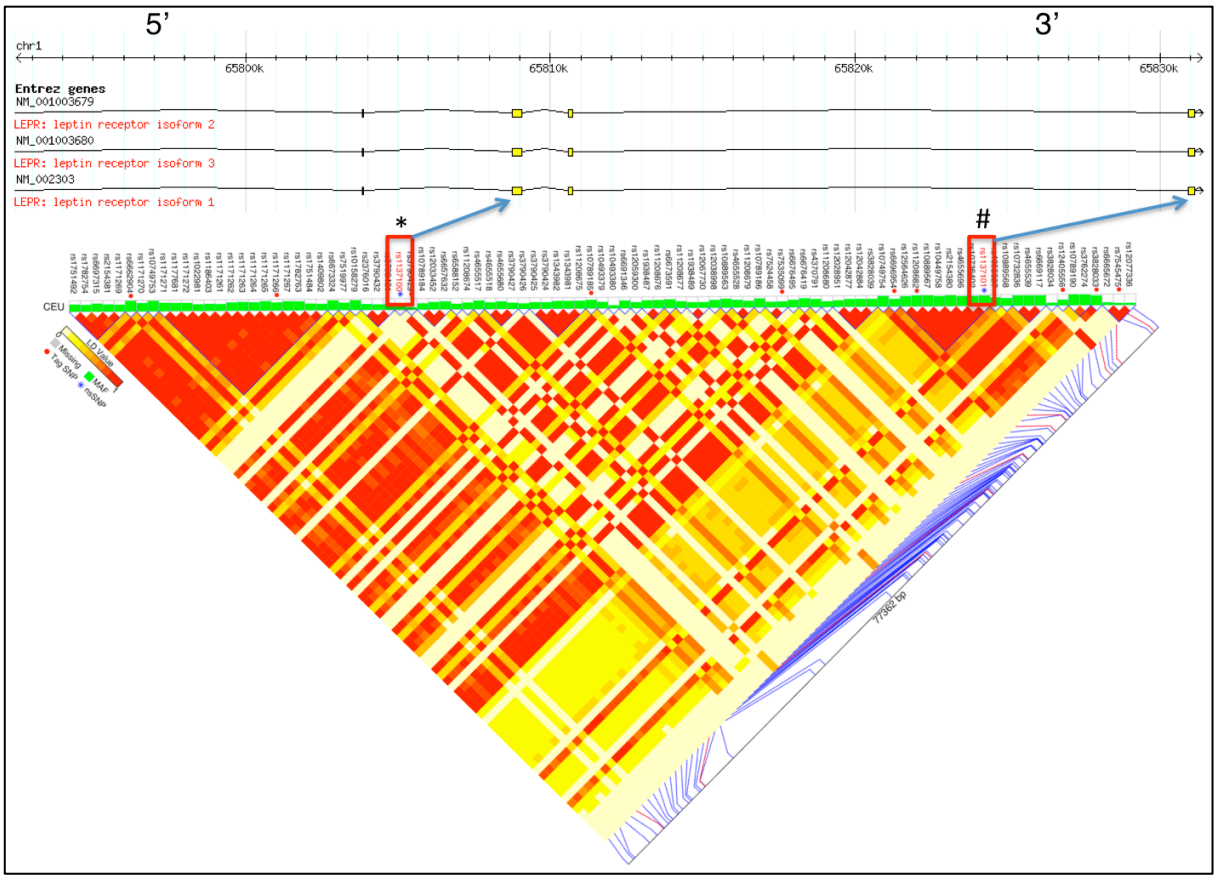

Figure 6. Segment of the *LEPR* gene on chr.1 that includes exons 4-6. The positions of the rs1137100 and rs1137101 are highlighted (* = rs1137100, # = 1137101), and their genomic position is indicated with a blue arrow. The linkage disequilibrium structure of this region is depicted in the lower part of figure.

A number of studies have previously reported statistically significant associations between both of these SNPs and variation in BMI and obesity; related physiological variables such as fat mass, insulin levels, blood pressure; and related morbidity including type-II diabetes and early athersclerosis[301, 306, 309, 324-329]. However, this literature is far from consistent, and – like most association studies of complex, multifactorial phenotypes – is rife with nonreplications and inconsistencies with respect to which allele confers risk. That said, there appears to be a trend in the literature (especially in studies with younger and relatively

healthier subject [306]) toward association of the G alleles at these SNPs (i.e. 109Arg and 223Arg) with risk.

This pattern of association (i.e. G as risk allele) would be consistent with a recent genome-wide study of soluble leptin receptor levels by Sun and colleagues (2010). Several leptin receptor isoforms have been identified: these share common extracellular and transmembrane domains, but differ in the length of the intracellular domain[330, 331]. A long form is expressed primarily in brain – most notably in hypothalamus, but also in tyrosine hydroxylase containing neurons of the dopaminergic midbrain[290, 291] – while several short forms are variably expressed in peripheral tissue[330, 331]. In addition, a soluble isoform exists (sOB-R), which lacks both the transmembrane and intracellular domains[332]. Notably, sOB-R levels strongly predict cell-surface expression of the leptin receptor (in periphery)[333], and are inversely correlated with adiposity, insulin resistance, and other risk factors for Type-II diabetes[334-338]. In their genome-wide association study with sOB-R levels, Sun and colleagues (2010) found strong evidence for association between a number of *LEPR* SNPs and sOB-R levels, most notably rs1137101 ($p = 1.91 \times 10^{-10}$) and rs1137100 ($p = 1.71 \times 10^{-10}$)[339]. In both cases, the minor (G) allele was associated with dose-dependent decreases in sOB-R levels. Thus, for both rs1137101 and rs1137100, arginine substitution at these loci may lead to lower sOB-R and lower cell-surface expression of membrane-bound leptin receptors, which may explain in part the associations to obesity and related morbidity in G-allele carriers.

Crucially, if the putative link between *LEPR* genotype and cell-surface expression of leptin receptor holds in brain, this could account for our observation that 109Arg carriers have sensitized ventral striatal DA responses to stimulant administration and higher levels of impulsive traits. There is considerable evidence that leptin acts via midbrain leptin receptors to diminish the reward value of reinforcers[291, 340, 341], and that this effect may be due in part to alterations in striatal DA output[292, 340]. A series of landmark studies provide a compelling demonstration of leptin's role in attenuating reward responsiveness: leptin administration was found to reduce brain stimulation reward[342], can block stress-induced reinstatement of heroin-seeking behavior[343], inhibits the development of conditioned place-preference for palatable food and self-administration of sucrose-reward[344], and attenuates basal and feeding-evoked DA levels within the NAcc[345].

Furthermore, the impact of leptin on reward motivation appears to be specifically mediated by its actions on functional leptin receptors located in the dopaminergic midbrain. Leptin receptor expression and tyrosine hydroxylase (a marker of dopamine neurons) are extensively colocalized within the VTA[291], and specifically in VTA DA neurons that project to the nucleus accumbens[290]. In one elegant study, Hommel and colleagues showed that direct intra-VTA administration of leptin inhibited feeding without affecting locomotor activity generally, and established – in vitro and in vivo – that leptin decreases VTA neuron excitability (i.e. lowered firing rate and action potential frequency). Critically, using a VTA-specific conditional knockdown approach, these authors showed that selectively reducing VTA leptin receptor expression potentiates feeding behavior by

enhancing the animal's preference for high-fat food consumption[291]. Subsequent work using this same approach showed that midbrain-selective knockdown of LEPR expression enhanced effort-based, instrumental responding for sucrose reward (i.e. increased breakpoint on animals maintained on a progressive ratio for sucrose)[346]. Of note, midbrain LEPR knockdown also rescued the reduction in NAcc DA levels seen in animals maintained on a high-fat diet, suggesting that midbrain leptinergic signaling may have functional effects on mesoaccumbens DA circuitry[346]. It must be said that, in some regards, these effects are contrary to those reported in prior studies in congenitally leptin-deficient mice. For example, Fulton (2006) reported that ob/ob have reduced VTA TH levels, attenuated locomotor responses to amphetamine and significantly diminished sensitization following a course of amphetamine treatment, all of which were reversed by leptin administration[290]. Moreover, these same mice were found in vitro to have lower levels of synaptic DA release in NAcc following electrical stimulation. However, these findings may result from compensatory changes in leptin receptor levels in these leptin deficient animals (leptin receptor expression is upregulated in ob/ob mice[347-349]), and moreover, a subsequent study found no changes in stimulant-induced locomotion or either baseline or stimulant-induced NAcc DA levels in vivo in ob/ob mice[350].

On the whole, the findings provide support for the idea that lowered LEPR expression in mesolimbic DA circuitry may enhance the incentive salience of rewards. Though speculative, it is possible that the effects of allelic variation at the human rs1137100 locus – previously associated with lower soluble leptin receptor

levels[339], a marker for cell-surface leptin receptor expression (at least in periphery)[333] – on NAcc DA function that we report here are due to leptin receptor-mediated alterations in mesoaccumbens DA reactivity. If the LEPR 109Arg variant is in fact associated with lower midbrain leptin receptor expression, one could predict that carriers of the 109Arg allele might show relatively impaired midbrain leptinergic signaling, resulting in a relative release from the inhibitory influence of leptin on midbrain neuron excitability. This enhanced VTA excitability could potentiate DA efflux in terminal field regions – including ventral striatum – following exposure to a pharmacological or natural reward. This relative augmentation in striatal DA release could, in analogy to the finding of increased instrumental responding for reward in Lepr knockdown animals[346], enhance reward sensitivity and promote maladaptive reward-seeking behavior, accounting for our finding of higher impulsivity in 109Arg individuals. However, empirical support for this hypothesis will require significant future study.

In this chapter, we offer the first report that genetic variation in leptin, a key regulator of feeding and obesity, affects striatal DA responses to a stimulant drug of abuse and is associated with individual differences in trait risk factors for addiction. Insofar as these data demonstrate that genetic risk for obesity is linked to markers of risk for addiction (i.e. striatal hyper-reactivity and impulsive traits), this work provides support for the notion that the striking psychological and neurobiological commonalities between addiction and obesity may have its roots in shared genetic diathesis. We believe that the pathophysiological overlap between addiction and obesity, in concert with the current data, suggest a model of risk

whereby shared genetic susceptibility factors sensitize dopaminergic circuitry for incentive motivation to promote reward-seeking behavior. This sensitization may catalyze the transition from pleasure-based consumption of an hedonically valued substance (a palatable food or drug) to a form of stimulus-driven, compulsive and habitual intake that is insensitive to devaluation and which persists despite significant adverse consequences (i.e. addiction).

Given the known importance of dopamine for feeding[351], recent evidence of its involvement in obesity[271, 352-354], and the findings of neurobiological overlap between obesity and addiction described above[247], some investigators have begun to look at a role for addiction-associated variants in DA genes in predisposing maladaptive feeding behavior and obesity. We believe that the current data validate this approach, and further, serves to nominate genetic risk factors for obesity as candidates for enhanced phenotypic investigations in addiction.

CHAPTER V

SUMMARY AND CONCLUSION

The purpose of this final chapter is threefold. First, I will summarize the aims and principal findings of each chapter (II-IV), and will contextualize these findings within current neurobiological models of substance abuse. Second, I will detail some key specific limitations of the work presented herein, and some general limitations associated with the overall experimental methodology employed in this dissertation. Finally, I will discuss future directions for this line of research, and will address this discussion particularly towards overcoming some of the limitations that are inherent in current experimental approaches to understanding the neurogenetic architecture of psychiatric illness.

Chapter II: Allelic Variation in the Dopamine Regulating Gene CSNK1E Sensitizes Stimulant-Induced Striatal Dopamine Release

Addiction involves a transition from initial "recreational" or pleasure-based use of a drug, to a state of compulsive and habitual drug taking that persists despite severe adverse consequences. While many people are exposed to drugs of abuse, relatively few continue using drugs for long enough that they move beyond that initial pleasure-based stage to addiction[4]. One determinant of whether or not an individual makes the transition from early-stage recreational drug use to compulsive drug abuse appears to be their initial subjective

experiences of the drug – in particular, the degree to which they rate the drug effects as pleasurable or positive[72, 75]. This accords nicely with a wealth of preclinical data: rats also show significant individual variability in whether they develop addiction-like behaviors after being exposed to a drug of abuse. While animals cannot provide direct information about their subjective experiences, one strong predictor of liability to addiction is their behavioral responses to the initial administration of a drug[21, 22], mirroring human findings. Moreover, in both animals and humans, subjective and behavioral responses are under the influence of genetic factors[78, 80, 107], suggesting that individual differences in drug responsivity may contribute to the known heritability of addiction.[5, 6].

In this chapter, I examined one specific genetic factor – an allelic variant in the Casein Kinase 1 (epsilon) gene – with a prior positive association to positive subjective responses to psychostimulants. Given previous work by myself and by others indicating that subjective responses to stimulants are linked to enhanced dopaminergic transmission within the ventral striatum[71, 124], we hypothesized that the allele linked to greater subjective positive responses to amphetamine would be associated with a higher magnitude ventral striatal DA release following amphetamine administration. This hypothesis was confirmed: rs135745 C-allele carriers showed significantly stronger DAergic responses to AMPH within the ventral striatum. Of note, the effect of genotype on DA release was selective for VS and did not extend into more dorsal regions of the striatum. In addition, we tested for an effect of *CSNK1E* rs135745 genotype on subjective responses to AMPH, but were unable to replicate the prior findings of Veentra-VanderWeele,

likely due to the significantly smaller sample size here. However, we did demonstrate that AMPH-induced DA release within VS significantly predicted subjective craving responses for AMPH, indirectly linking *CSNK1E* to risk for addiction. These results suggest that genetic variation in *CSNK1E* – by sensitizing striatal DA reactivity to drugs, leading to stronger subjective responses – may play a role in promoting the transition to addiction from early stage drug use.

Chapter III: Genetic Variability in HPA-axis Function Alters Human Striatal Dopaminergic Reactivity

Stress is one of the most robust environmental risk factors for substance abuse. Individuals with high levels of early life stress use drugs earlier and go on to develop addiction at higher rates[150, 355, 356], and acute stress is the single most important predictor of relapse in abstinent drug users[357]. However, there is significant individual variability in stress-reactivity, and in the predisposing effects of stress on addiction. A large body of preclinical research suggests that the effects of stress on addiction risk are due to a sensitizing impact on mesolimbic DA circuitry[227, 228], and are mediated by the effect of stress-linked alterations in hypothalamic-pituitary-adrenal (HPA) stress hormones (e.g. corticotropin releasing hormone) on midbrain DA neuron firing and DA levels in terminal field projection sites, such as the nucleus accumbens (NAcc)[237, 238]. This implies that genetic variation in HPA-axis factors could confer risk for addiction by affecting the responsivity of the mesolimbic DA system.

In this chapter, I tested this hypothesis by examining the impact of an allelic variant in the corticotropin releasing hormone gene (*CRH*) on DA responses to AMPH. We found that an allele that has been previously reported to be under-transmitted to individuals with behavioral inhibition was associated with stronger DA responses to AMPH within the ventral striatum. Given the prior report of *under*transmission to behaviorally inhibited subjects, we sought to test for an association between this allele and behavioral *dis*inhibition. Though we did not find compelling evidence for such an association, enhanced VS DA responses to AMPH positively predicted scores on two trait measures of impulsive temperament. These findings support the notion that variability in HPA signaling may affect risk for substance abuse [200, 201, 225], suggest that such associations may be mediated through the sensitization of mesolimbic DA responses to drugs of abuse, and nominate *CRH* for more thorough investigation as a specific genetic risk factor for addiction.

Chapter IV: Obesity-linked Genetic Variability in Leptin Signaling Predicts Alterations in Mesolimbic Dopamine Neurochemistry

Though traditionally considered a disease of nutrition and metabolism, obesity is increasingly recognized to have a strong psychological component. In particular, much of the recent focus on obesity as a psychological disorder centers on the striking psychopathological and neurobiological overlaps between obesity and addiction. For example, obese individuals show patterns of brain chemistry and function that are reminiscent of those seen in drug addicts[247, 258].

This observation has led to several recent findings which suggest that previously identified genetic risk factors for addiction – particularly variants in genes that participate directly in DA signaling – also play a role in the development of obesity, and may do so by impacting striatal reward circuitry[270, 358-360]. However, the possibility that obesity-linked genetic factors may affect dopaminergic function – thereby nominating them for enhanced phenotypic characterization in addiction – remains completely unexplored.

In this chapter, I examine the impact of two such variants – SNPs in the gene encoding a receptor for the energy-regulating hormone leptin (*LEPR*) – on mesolimbic dopamine system function. Prior work suggests that leptin downregulates the activity of midbrain dopamine neurons by acting directly on midbrain-expressed leptin receptors, thereby reducing DA outflow in terminal field regions such as the striatum. Based on these findings I hypothesized that individuals carrying the G-allele at two *LEPR* SNPs, which have been linked previously to obesity and to reduced soluble leptin receptor expression, would show higher levels of striatal DA release in response to AMPH. This hypothesis was confirmed for G-allele carriers at one SNP (rs1137100), but not the other (rs1137101). Individuals possessing a G at the rs1137100 locus (causing a lysine to arginine substitution at codon 109) showed greater stimulant-induced DA release in ventral striatum. Further, these same individuals demonstrated higher levels of impulsive and impulsive-antisocial traits that have been shown previously to be associated with risk for addiction. These traits were correlated with the magnitude of stimulant-induced DA release within VS in this sample.

Finally, using path analysis, I was able to show that the impact of genetic variation at the rs1137100 locus on individual differences in impulsive traits is mediated by the sensitizing effect of carrying G allele at that locus on stimulant-induced DA release, which in turn positively predicts trait impulsiveness and impulsive-antisociality. Taken together, these findings suggest that genetically-mediated individual differences in leptin signaling may lead to obesity by impacting mesolimbic DA circuitry for reward and motivation. Critically, given the associations to trait risk factors for substance abuse, these data also argue that genetic variation in *LEPR* may also contribute to risk for addiction.

General Discussion

The experiments detailed in this dissertation provide compelling evidence that the impact of genetic variation in diverse brain signaling pathways linked to risk for addiction converge to affect the reactivity of a final common neurobiological pathway: the mesolimbic dopamine system. Furthermore, there is a consistency in the directionality of the effect of the putative risk allele at each of the genetic loci under study herein. In each case, the allele that we hypothesized might confer risk for addiction – because of prior associations to enhanced subjective responses (*CSNK1E*), to lower levels of behavioral inhibition (*CRH*) or to obesity and to diminished leptin receptor expression (*LEPR*) – was associated with enhanced striatal DA responses following the administration of a stimulant drug.

These findings are potentially important given a current debate among addiction researchers on the nature of altered dopaminergic function in addiction. On the one hand, several investigators have reported diminished dopaminergic reactivity in addicted invididuals (DA *hypo*function). For example, a number of studies have shown lowered D2 receptor availability and blunted stimulant-induced DA release in cocaine, alcohol, and heroin dependence[273-275, 361-366]. Findings of lowered dopaminergic function have been taken by some as evidence for a primary deficit in reward processing in addicted individuals. According to this theory, diminished sensitivity to rewards due to dopaminergic hypofunction leads at-risk individuals to excessively seek out rewards – especially, drug rewards – to compensate for natively depressed dopaminergic tone.

Lowered dopaminergic function as a consequence of neuroplastic adaptations associated with chronic substance abuse may well be responsible for drug-seeking behavior in active addiction or drug craving in recent abstinence. However, it is difficult to make inferences about etiopathophysiology from these data, as it's impossible to know whether PET markers of dopaminergic hypofunction are a cause or consequence of chronic substance abuse, given that chronic dosing with many drugs of abuse causes dramatic synaptic remodeling within mesolimbic reward circuitry[367, 368]. Moreover, individuals with no history of drug abuse, but who nevertheless possess certain trait risk factors that robustly predict the development of addiction – such as novelty seeking and impulsivity – consistently show enhanced dopaminergic

function[63, 67, 69-71, 369]. Such findings accord well with animal data showing that the development of addiction is associated with striatal DA sensitization (with attendant enhancement of the incentive salience of the drug of abuse, as detailed in the introduction), and with work demonstrating heightened striatal DA in animals at risk for developing addiction-like behaviors[370, 371]. In light of these data, and in consideration of the fact that traits like impulsivity and novelty seeking show significant heritability[372-374], some have proposed that genetic risk for addiction is conveyed through dopaminergic *hyper*- (rather than hypo-)sensitivity[375].

Importantly, to the extent that we show that three specific putative genetic risk factors for addiction lead to ventral striatal DA hyper-reactivity in individuals with no history of substance abuse, our data supports the DA hypersensitivity model of addiction liability. It is noteworthy that we are able to demonstrate that putative genetic risk factors in three distinct neurobiological pathways (DA signaling, HPA-axis, energy-regulating hormone) have a common sensitizing effect on mesolimbic DA responses. Moreover, one of these putative risk variants (*LEPR* rs1137100) has strong support as a susceptibility factor for obesity, raising the intruiging suggestion that pleiotropic effects of leptin genetic variation on diverse forms of disinhibitory psychopathology (addiction and obesity) may be mediated by a common influence on mesolimbic DA system function. Taken together, the present data provide critical translational evidence favoring a model of addiction etiology that holds enhanced striatal DA responses to reinforcers as its cornerstone[375].

How might genetically mediated DA hypersensitivity act to confer risk for addiction? It may be that a sensitized striatal DA response during the early stage of recreational or pleasure-based drug use in risk-allele carriers promotes an excessive, inflexible, and long-lasting attribution of salience to drugs and drug cues. In this way, striatal hyperdopaminergic could cause a hyper-attribution of salience to reward predicting cues through a pathologically accelerated form of Pavlovian learning, as would be predicted from Berridge and Robinson's Incentive Salience theory of addiction. Further, after this learning has taken place, exposure to such a cue may establish an inflexible attentional focus on that cue, leading to the execution of behavioral routines that are associated with obtaining the drug. The resiliency of this attentional focus to interruption and updating by subsequent motivationally relevant (but goal-irrelevant) information may be maladaptive. This account accords with the suggestion of Gruber and colleagues that *excessive* striatal DA might result in perseverative responding through an over-stabilization of task-relevant WM representations (i.e. at the expense of flexibly updating WM representations to respond appropriately to subsequent salient events)[376]. Such a mechanism could explain how excessive striatal DA release in response to a motivationally significant cue could lead the overstabilization of cue-linked prefrontal goal representations centered on drug-seeking. This overstabilization may impair the flexible redeployment of limited-capacity attentional resources to other motivationally salient but task-irrelevant information that might otherwise adaptively modify behavior (e.g. punishment-predicting cues, or cognitive action-outcome associations derived from prior

experiences with drug-seeking behavior). In this manner, heightened striatal DA responses during early stage drug use in genetically at-risk individuals may facilitate the acquisition of maladaptive drug-related stimulus-response habits. The acquisition of such habits could promote compulsive drug use even after the development of tolerance to the positively-valenced subjective effects of the drug lead would otherwise lead to devaluation of the drug as a reinforcer.

In general, our articulation of possible systems and synapse-level mechanisms that might underpin the impact of the genetic variants detailed herein on stimulant-induced DA release assume a “steady-state” effect of these variants on DA signaling. However, it is equally likely – even probable – that our observed effects are the result of enduring, compensatory neurobiological changes that are the result of a brain that matures in the altered developmental context induced by a risk variant. That is, the specific impact of a risk variant on VS DA release may not in fact be due to the way that such a variant alters the acute response of mesolimbic circuitry at the moment of amphetamine administration. Rather, the observed enhancement of striatal DA responses in risk-allele carriers may be a consequence of developmental changes in mesolimbic circuitry that fundamentally alter the response properties of that system.

One well-known example of this phenomenon involves a variable number of tandem repeats (VNTR) polymorphism that resides in the upstream region of the serotonin transporter gene (commonly referred to as the 5HTTLPR). The “short” repeat allele at this locus is associated with lower transporter expression,

corticolimbic dysregulation, and increased risk for mood disorders such as depression. However, it was striking – and perplexing – that the allele that was associated with increased risk for depression led to a functional effect (enhanced synaptic serotonin, via reduced transporter cell-surface expression) that mirrored the primary neuropharmacological effect of first-line antidepressant drugs (i.e. serotonin-selective reuptake inhibitors). Insight into this apparent discrepancy is gained from studies by Gingrich and colleagues, who demonstrated a developmentally specific effect of increased serotonin levels on emotional behavior. Specifically, these studies showed that increasing serotonin levels pharmacologically in early development results in delayed and persistent changes in fear and anxiety behaviors in adult animals. Similar pharmacological manipulations in adult animals had no effects on these behaviors[377, 378]. These findings imply that the 5HTTLPR short allele affects serotonin signaling during a specific ontogenic window to affect the development and maturation of neural circuits that are implicated in mood and anxiety disorders, and that these developmental effects cause changes in the functional response of these circuits to affectively salient stimuli in adulthood[379, 380].

Similarly, the polymorphic variants studied in the current thesis may produce a striatal hyper-DA phenotype through developmental compensations. For example, as discussed in Chapter IV, leptin loss-of-function mutant (ob/ob) mice show a neurobehavioral phenotype that differs markedly from that generated by acute adult downregulation of leptin signaling. One noteworthy form of apparent developmental compensation in ob/ob mice is upregulation of

membrane-bound leptin receptor levels[347], which may explain some of alterations in reward behavior and striatal DA function in these mutants. In anology, changes in the function or expression of *CSNK1E*, *CRH*, or *LEPR* could sensitize striatal DA function by inducing developmental compensations that lead to a lower reactive set-point for DA release following drug administration. Given that neurotrophic factors – in particular, TrkB – appear to play a criticai role in regulating mesolimbic DA circuit development[381, 382], function[383, 384], and responses to stimulant drugs[385, 386], it is tempting to speculate that some of the effects of genetic variation in, for example, *CSNK1E*, may be mediated by intermediate effects on neurotrophin signaling. Given that *CSNK1E*, by impacting PP1 phosphorylation of voltage-gated ion channels and excitatory amino acid receptors, could influence striatal MSN membrane excitability, it seems possible that genetic variability at the *CSNK1E* locus could lead to enhanced MSN activity following exposure to a stimulus that leads to synaptic DA release. Enhanced MSN activity could, in turn, stimulate the expression of neurotrophic factors (such as brain-derived neurotrophic factor or BDNF), which may bind to presynaptically localized TRKB receptors. Retrograde neurotrophic signaling could then lead the propagation of this enhanced post-synaptic signal back through NAcc projecting axons of the VTA to influence mesolimbic circuit development. In this way, CSNK1E-mediated changes in post-synaptic MSN activity could, throughout the course of mesolimbic system development, lead to circuit and/or synapse-level-remodeling that may explain the differential sensitivity to drug reward in risk allele carriers.

Another aspect of our mechanistic proposals for the actions of polymorphic variation in *CSNK1E*, *CRH*, and *LEPR* on DA signaling is the assumption of "local" effects that only impact specific nodes within mesolimbic DA circuitry (i.e. VTA and NAcc). However, it is also possible that these variants exert primary effects at sites distal to our measured neurobiological end-point, and that our observed changes in mesolimbic DA are due to the ramification of these proximal effects outward onto mesolimbic circuit nodes. As one example, several lines of evidence point to the hippocampus as an important mediator of the effects of CRH signaling on behavior. For instance, CRH-expressing interneurons are prominently expressed in hippocampus, and – following exposure to a stressor – these neurons release CRH into intercellular hippocampal space, where it can bind to CRH1 receptors that are found abundantly on hippocampal pyramidal cell dendrites[387-390]. CRH action within the hippocampus is particularly intriguing in the context of our current findings given that this region plays a critical role in modulating dopamine neuron firing. In a series of elegant studies, Grace and colleagues have shown that only the pool of spontaneously active VTA neurons can be driven to burst firing by excitatory input, and that the size of this pool is determined largely by excitatory efferents from ventral hippocampus[391-393]. Thus, the hippocampus is well positioned to regulate VTA reactivity in response to rewarding stimuli. Of note, prior work demonstrated that stimulation of the ventral hippocampus leads to increased DA neuron activity, which is in turn correlated with increased extracellular DA within NAcc. Therefore, risk-allele associated alterations in CRH signaling within

hippocampal pyramidal neurons could impact glutamatergic inputs to DA midbrain neurons, which in turn may enhance the reactivity of these neurons, increasing DA release within terminal field regions (such as NAcc) in response to stimulation. Overall, significant future work is required to definitively identify the proximal causal site of action of the polymorphisms under study in this thesis. In the case of CRH, regionally specific conditional over-expression in concert with pharmacological stimulation and amperomic/voltametric recording of NAcc DA release events may be one fruitful avenue of research.

Limitations and Future Directions

The experiments presented in this dissertation share several significant limitations, and addressing these limitations presents an opportunity for considering future directions for this research. First, the precise cellular and molecular-level consequences of variation at the three loci reported in this dissertation are essentially unknown. In the case of the *CSNK1E* and *CRH* SNPs, their positions in the 3' UTR of those regions suggest the possibility that those base pair substitutions may affect miRNA target sites, which could conceivably affect protein expression levels by disrupting translational repression mechanisms. However, *in silco* prediction tools do not suggest that these specific substitutions would impact predicted human miRNA target sites [394]. In the case of *LEPR,* rs1137100 is a nonsynonymous coding SNP, suggesting that its functional effect on DA release is due to alterations in the protein structure of the leptin receptor. However, this amino acid substitution – located in the extracellular domain of the receptor – is conservative (does not result in a

change in charge), and is not predicted to have a dramatic or deleterious impact on function (PolyPhen: http://genetics.bwh.harvard.edu/pph/). Another interpretation is that these SNPs are monitoring haplotype blocks that harbor true causative variants: this interpretation is particularly salient for the *LEPR* and *CRH* SNPs, which reside within segments of chromosome that are characterized by high linkage disequilibrium over several kilobases. On the whole, it must be said that a much finer genetic mapping of our association signals is required. Future studies might employ targeted sequencing of the *CSNK1E*, *CRH*, and *LEPR* to identify the true causative variants that may be driving our reported gene-brain associations.

Our use of an ethnically heterogeneous sample also requires comment. In general, genetic associations studies that employ unrelated individuals face a problem in interpreting significant association signals when the sample includes individual of different genetic ancestry. Ethnically heterogeneous samples are problematic because differences in allele frequencies may exist between population segments that differ with respect the prevalence of a disorder or trait. Such differences may create spurious association signals if the effects of this population admixture are not accounted for in datasets where there is ethnic stratification across disease status or across levels of a quantitative trait[395]. Due to funding body requirements for racially inclusive subject recruitment, our study recruitment was blind with respect to ethnicity. Further, given the time and expense of acquiring dual-scan radioligand PET data (8k-10k/subject), we were unable to acquire a sample that was large enough to permit us to exclude non-

Caucasians from analysis. However, in each chapter, self-reported ethnicity was included as a nuisance covariate in all of our analyses, and all reported associations remained significant even after reanalyzing without non-Caucasian participants. That said, while some evidence does exist to support the notion that self-reported ethnicity matches genetically inferred ancestry[396], there is important population substructure that is not easily resolvable at the level of self-report, and which could contribute to spurious associations[397]. Thus, we cannot definitively rule out the possibility that our findings may be influenced by the presence of cryptic population admixture. Future studies in ethnically homogenous samples, or those using ancestry informative markers (AIMS) and/or panels of unlinked markers (genomic controls) to detect and correct for cryptic structure will useful in confirming the validity of the current findings.

One potentially important consideration in interpreting the present data is our specific measure of DA release. While the drug-induced radiotracer displacement approach that we employ has been in wide use for nearly 20 years, it is fundamentally an indirect or proxy measure of stimulated DA release. As such, this measurement is vulnerable to influence by confounding factors that are unrelated to the principal phenomenon under study. For example, it is conceivable that the measures of individual differences in D2/D3 receptor used to compute our percent-change DA release images is contaminated by individual difference in tonic DA levels. Further, some have suggested that the reduction in [18F]fallypride binding potential following AMPH administration – presumed here to reflect displacement of [18F]fallypride by stimulant-induced increase in

endogenous synaptic DA – in fact results from an AMPH-induced internalization of D2-like receptors[398]. Thus, individual differences in receptor trafficking, rather than increased striatal DA release, may play some role in determining our DA release phenotype measure. In addition, it is striking that the effects of genetic variation in the three signaling systems under study in this thesis appear to have a relatively selective impact on ventral striatum. We do not have a compelling explanation for why this is the case, although it is noteworthy that ventral striatum receives stonger input both from VTA (which may have denser expression of CRH1 and LEPR receptors relative to the neighboring substantia nigra) and from limbic regions such as the amygdala. It is possible that differential input from limbic nuclei into ventral striatum may account for the higher level of individual variability in ventral striatal DA release, but this notion remains speculative. Finally, it must be noted that a specific mechanism to account for why, in the first instance, individual differences in this measure are observed at all, remains elusive. While we have shown previously that midbrain D2/D3 binding (presumably reflecting autoreceptor levels) can affect striatal DA release, other sources – including striatal DA transporter levels, presynaptic autoreceptor levels, changes in DA synthetic or catabolic enzymes – may certainly account for a significant proportion of the cross-subject variance in striatal DA release. Taken as a whole, it must be said that significantly more work needs to be done in order to effectively parse the origin of individual differences in our AMPH-induced [18F]fallypride displacement signal.

In considering the implications of our findings, it worth reflecting on the fact that the genetic variants under study in these experiments accounted for a relatively small proportion of the variance in ventral striatal DA responses to AMPH (12-20%). Thus, even if we take as granted that individual differences in DAergic function account for some of the variance in determining who becomes addicted, and assuming that subsequent studies of genetic association confirm a role for these variants in substance dependence, variability at the loci examined in this dissertation are likely to account only for a very small proportion of the variance in susceptibility to addiction. This is akin to the situation in other psychiatric disorders, like schizophrenia, that have a moderate-high heritability, but for which common risk variant only account for a small proportion of variation in disease liability. This general issue has been termed the problem of "missing heritability,"[399, 400] and would appear to challenge the common disease-common variant (CDCV) hypothesis that underpins many approaches to the genetic dissection of complex psychological traits and disease, including those geared toward associating common genetic variants to neurobiological endophenotypes, as in this dissertation. Two possible explanations for this "missing heritability" are particularly worthy of mention here.

Many investigators have noted that an approach that is geared toward examining the effects of single variants in isolation, without taking into account the larger biological context in which this variation takes place, will necessarily fail to account for a significant degree of variability in any phenotype, regardless of the penetrance of a genetic effect to that phenotype[401, 402]. Any single variant

in any one chromosome in any one individual occurs on a complex background of genetic variability, and that background may differ from individual to individual. One technique for resolving this background is to investigate epistatic interactions between multiple variants[403]. Epistatic interactions have been observed in risk for multiple somatic disorders, implying that they may be at play in risk for psychiatric phenotypes as well[404, 405]. Indeed, one recent study of schizophrenia used machine-learning algorithms to construct multi-marker genetic risk profiles from variation in three genes that have each been weakly associated with psychosis, and whose gene products are known to participate in the same biological signaling pathway. The authors showed that epistatic interactions between the three markers resulted in a dramatic increase in the amount of variance in risk explained, compared to any of the SNPs in isolation, and used brain imaging to confirm the presence of epistatic interaction effects on a measure of brain function linked to illness[406, 407]. These and other findings[408-410] strongly point towards the study of epistatic interactions within and between the biological pathways highlighted in this dissertation as an important direction for future research on the neurogenetic architecture of addiction.

Another approach towards uncovering the missing heritability of complex diseases involves broadening the CDCV paradigm to account for the potential influence of rare, but highly penetrant, alleles on disease. Some investigators have even gone so far as to suggest that any observed association of a common variant to a disease or disease-linked phenotype is due to the fact that such common variation is incompletely tagging, or acting as a low-fidelity proxy

association signal for, causative rare mutations[411, 412]. To circumvent this issue, they recommend whole-genome sequencing to identify rare, causative mutations for psychiatric disorders in as yet unidentified genes[413].

However, others have taken a compromise approach to this issue by suggesting that the known pathobiology of a disorder is essential in guiding the selection of genes for targeted sequencing for rare variation. For example, Blakely and colleagues have found a number of highly functional coding variants in the dopamine transporter gene, which has long been implicated in the pathophysiology of disinhibitory psychopathology. Notably, these variants associate with attention deficity/hyperactivity disorder, a disinhibitory spectrum syndrome, confirming that an approach geared toward identifying rare, causative mutations in genes that are of a-priori interest based on known systems-level pathobiology may be a viable strategy for complementing studies of common variation in these disorders[414-416]. Furthermore, there are considerable opportunities for leveraging the strengths of this approach with those inherent to human neuroimaging. For example, the quantitative stimulant-induced DA release phenotype discussed in this dissertation can be used as a means of selecting participants for sequencing: we have evidence that a small proportion of individuals demonstrate anomalous striatal DA responses to AMPH that are exactly what would be predicted from carriers of some of these rare mutations[415]. On the whole, integrating the study of rare variation with human imaging phenotypes holds significant promise for elucidating a more complete picture of genetic risk for addiction.

One potentially promising future avenue of research involves using quantitative neuroimaging endophenotypes in the service of discovering novel genetic risk factors for addiction. To date, there are relatively few robust, reliable, and well-replicated associations between specific genetic variants and addiction. I believe that the nonreplications and inconsistencies that plague psychiatric genetics are likely due to the manner by which we have traditionally tested for genetic associations. In identifying risk genes for disorders, the primary approach has always been some form of statistical association between specific genetic variants to taxonomic clinical diagnostic phenotypes. This can be done in a simple univariate fashion, as in candidate gene studies, or in a massively univariate or multivariate fashion, as in genome-wide association studies.

In such studies – which can be either family-based or involve unrelated cases and controls – individuals are grouped according to diagnosis (most commonly, “affected” and “normal”), and the frequency of a genetic marker is compared between the diagnostic groups. A statistically significant difference in the frequency of the transmission of a genetic marker to an ill sibling (in family based studies) or in the frequency of that genetic marker between the groups (in case-control studies) is taken as evidence of a positive genetic association. The strength of that association is can be considered in terms of (as one example) an odds ratio, where a “risk” genotype is considered to confer a certain degree of increased susceptibility to that disorder, relative to the “non-risk” genotype. The problem here lies in the use of taxonomic clinical diagnosis as a phenotype. At the risk of stating the obvious, genes do not encode DSM-IV diagnoses: rather,

genes encode proteins, which form signal transduction pathways, which are wired together into circuits, which form large-scale neural systems, which process incoming sensory information and integrate and reconcile that information with internally generated goals to output adaptive behavior. Therefore, the proximal effect of a risk-associated genetic variant is not on a specific disorder – the conceptualization and boundary conditions of which are largely going to be socially constructed – but on protein structure, function and/or expression.

Critically, it seems readily apparent that when we move away from gene from to protein to cell to systems to disease along the path outlined above, both our genetic complexity and our phenotypic complexity increase dramatically. For example, at the level of a single DSM-IV taxonomic disorder, there are likely to be multiple genes that interact, with each other and with environmental factors, to affect risk (Figure 1).

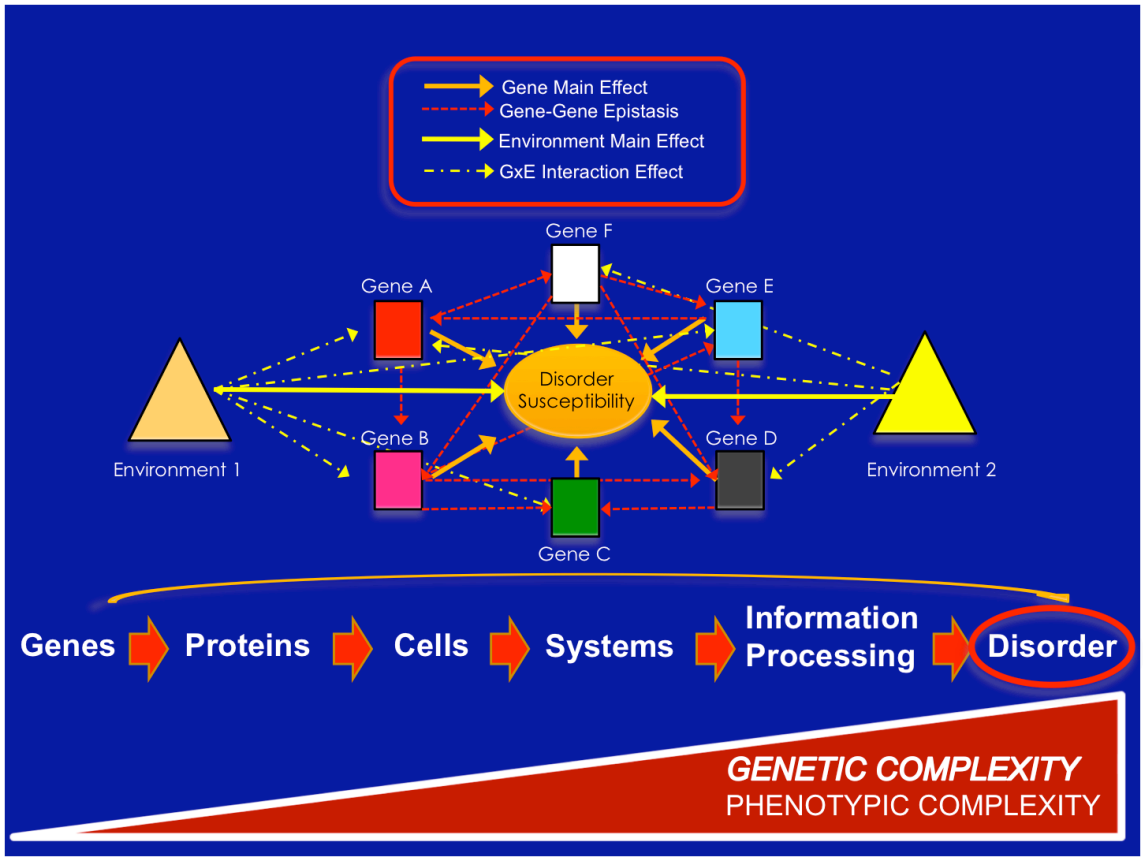

Figure 1. Genetic complexity of taxonomic disorders.

Further, there may be distinct patterns of genetic linkages to heterogenous sub-phenotypes that exist within a single broad diagnostic taxon. That is, different sets of genes may be linked to risk for relatively distinct (and possibly, cryptic or latent) disorder subtypes that exist within a broad taxon. The net effect of this complexity is necessarily going to be diminished power to detect potentially meaningful genetic associations, as each step away from the direct biological impact of a genetic variant on protein production is going to weaken the strength of the potential association signal. However, the penetrance of genetic variant – the likelihood that any given individual with a certain genotype

will express the associated phenotype – varies depending on the kind of phenotype being examined. It is thought that by studying intermediate neurobiological phenotypes, which are closer to the direct biological impact of the genetic variant under study, we are able to increase our ability to find an effect of genotype. Indeed, meta-analyses of imaging data confirm that the use brain imaging endophenotypes affords us increased genetic penetrance, larger effect sizes, and thus an increased likelihood of finding gene effects[9, 417].

Importantly, the fact that larger effect sizes are found for brain imaging endophenotypes suggests that we can use brain imaging as a deep phenotype to aid in gene discovery. Combining highly penetrant quantitative trait data with genome-wide genotyping opens up the possibility of using the genome-wide association study (GWAS) as a tool for both variant identification and pathomechanism characterization. Importantly, the enhanced effect size associated with imaging endophenotypes suggest that the number of subjects required for genome-wide significance will be dramatically fewer than the 5000-10000 typically required for genome-wide significant associations to diagnosis. A recent study by Stein and colleagues demonstrates this point. Their GWAS identified a common glutamate receptor variant (GRIN2b) that was associated with temporal lobe volume and increased risk for Alzheimer's disease using structural MRI scans. Using an imaging phenotype, the authors identified variation in a gene that was not previously under consideration as a risk factor for Alzheimer's, and characterized a mechanism through which it might affect risk[418, 419]. Potkin and colleagues took a similar approach to gene identification using a

working memory task, and reported a novel association to prefrontal function with SNPs in the MYLIP gene. Given that prefrontal function in this task has been associated previously with risk for schizophrenia, this strategy nominates MYLIP as a novel genetic risk factor for prefrontal dysfunction in that disorder[420, 421].

These reports suggest a fruitful path forward in identifying novel risk factors for addiction: genome-wide studies with stimulant-induced striatal DA release as a phenotype measure. Sample sizes will likely need to be higher than the ones reported in this dissertation, but as the utility of PET imaging disease phenotypes are recognized, one might envision future studies of 100-200 PET subjects each at multiple sites with synchronized scanning protocols. In analogy to current efforts underway to identify Alzheimer's risk genes, data from 1000-1500 participants (a likely lower bound on sample size required for genome-wide significance with imaging data, based on current effect size estimates) could be collected from these multiples sites over the course of five years. Such large-scale, well-coordinated efforts will be required to advance target selection for medication development, a major goal for the neuroscientific study of addiction.

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