Individual Differences in Neural Reward and Threat Processing: Identifying Pathways of Risk and Resilience for Psychopathology

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

The goal of this dissertation is two-fold: 1) to identify novel biological pathways implicating individual differences in reward and threat processing in the emergence of risk and resilience for psychopathology, 2) to identify novel genetic and epigenetic predictors of the inter-individual variability in these biological pathways. Four specific studies are reported wherein blood oxygen-level dependent functional magnetic resonance imaging (BOLD fMRI) was used to measure individual differences in threat-related amygdala reactivity and reward-related ventral striatum (VS) reactivity; self-report was used to measure of mood and psychopathology as well as the experience of stressful life events. In addition, DNA was derived from peripheral tissues to identify specific genetic and epigenetic markers.

Results from Study 1 demonstrate that individuals with relatively low reward-related VS reactivity show stress-related reductions in positive affect, while those with high VS reactivity remain resilient to these potentially depressogenic effects. Heightened VS reactivity was, however, associated with stress-related increases in problem drinking in Study 2. Importantly, this effect only occurred in individuals showing concomitantly reduced threat-related amygdala reactivity. Study 3 demonstrates that using a multilocus genetic profile capturing the cumulative impact of five functional polymorphic loci on dopamine signaling increases power to explain variability in

reward-related VS reactivity relative to an approach considering each locus independently. Finally, Study 4 provides evidence that methylation in the proximal promoter of the serotonin transporter gene is negatively correlated with gene expression and positively correlated with threat-related amygdala reactivity above and beyond the effects of commonly studied functional DNA-sequence based variation in the same genomic vicinity.

The results from these studies implicate novel biological pathways, namely reward-related VS reactivity and threat-related amygdala reactivity, as predictors of relative risk or resilience for psychopathology particularly in response to stressful life events. Moreover, the results suggest that genetic and epigenetic markers may serve as easily accessible peripheral tissue proxies for these neural phenotypes and, ultimately, risk and resilience. Such markers may eventually be harnessed to identify vulnerable individuals and facilitate targeted early intervention or prevention efforts.

Dedication

I dedicate this dissertation to my parents Tatyana and Stoycho. Thank you for instilling a passion for science and knowledge in me ever since I was a little child. Thank you for enrolling me in English lessons at the age of 8 and for resisting the urge to stop me when I decided to move a continent and an ocean away from home to pursue this same passion. And thank you for never missing an opportunity to send me tokens of your unconditional support (and chocolate) across the miles.

I also dedicate this dissertation to my brother Nikolay and my aunt Diana. Thank you for inspiring me to always strive to achieve my best, each in your own unique way.

This dissertation is also dedicated to my friends Lauren Williamson, Shian-Ling Keng, Masha Blokh and Alexandra Kjuchukova-Sashka. Thank you for never getting tired of listening to me talk about life, in the best and worst of times alike. Your support has meant more to me than I can ever express.

Last but not least, I dedicate this dissertation to my mentor Ahmad Hariri and all members of the Hariri lab. You have truly become my second family over the past five years. Thank you for making this journey so enjoyable and intellectually stimulating and for supporting me both within and outside the lab.

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1. General Introduction

The processing and integration of information about reward and threat in one's environment is crucial for the planning and execution of context-appropriate approach and avoidance behaviors in the service of survival and well-being. While the adaptive functioning of neural circuits dedicated to reward and threat processing facilitates the regulation of fundamental aspects of emotion and behavior, maladaptive hypo- or hyper-responsiveness of these circuits is associated with various types of psychopathology ranging from mood and anxiety disorders (Drevets *et al.*, 1992, Etkin & Wager, 2007, Russo & Nestler, 2013) to addiction (Cornelius *et al.*, 2010, Evans *et al.*, 2006), and psychopathy (Blair, 2008, Buckholtz *et al.*, 2010a). Importantly, significant differences in the functioning of reward and threat circuits exist even among healthy individuals. Moreover, this variability can be mapped onto individual differences in core aspects of behavior such as personality traits as well as relative risk and resilience for psychopathology.

In **Chapter 1** of this dissertation, I briefly describe the corticostriatal and corticolimbic circuitries, which support reward and threat processing, respectively. In doing so, I delineate their functional neuroanatomy, along with the major neurotransmitters and neuromodulators that regulate signaling within those circuits. I then move on to describe genetic markers associated with variability in the functioning of those circuits, which can potentially be used as easily accessible peripheral proxies of

neural function. In **Chapters 2-5**, I present four original research studies drawing predictive links between (epi)genetic markers, threat- and reward-related neural circuit function and relative risk and resilience for psychopathology. I conclude by outlining directions for future work in **Chapter 6**.

1.1. *Reward Processing*

1.1.1. Basic functional neuroanatomy of the corticostriatal circuit

Reward processing is subserved by a distributed corticostriatal circuitry (CSC) comprising a network of brain regions whose signals are dynamically integrated to produce goal-directed behavior in accordance with environmental reward contingencies. This network is critically regulated by the neurotransmitter dopamine (DA). Dopaminergic neurons residing in the ventral tegmental area (VTA) within the midbrain send axonal projections to the ventral striatum (VS), which receives additional input from cortical and limbic regions and serves as a hub for this distributed circuitry. Importantly, the VS comprises the Nucleus Accumbens (NAcc) – a neural region which non-human animal research has implicated in various aspects of reward processing ranging from core hedonic reactions to motivation to pursue appetitive stimuli (Berridge *et al.*, 2009).

Theoretical models building upon functional neuroanatomy studies in animal models postulate that the VS is a part of a motivational loop, wherein it is instrumental in selecting the optimal PFC-generated motor program for achieving a particular

behavioral goal by integrating reward-related dopaminergic signals from the VTA, as well as relevant sensory and affective input from the amygdala and contextual inputs from the hippocampus (Figure 1). Information about the most goal-relevant motor plan is then sent back to the PFC for execution, via the VP and the mediodorsal nucleus of the thalamus (Grace, 2000). In the presence of reward, dopaminergic input from the VTA drives medium spiny neurons of the VS to execute selective inhibition of target projection sites with the ultimate goal of maintaining behavior according to the currently selected motor plan. When a particular behavior fails to produce a reward, decreasing dopaminergic inputs form the VTA to the VS signal the need for a behavioral strategy switch, which is enacted via a motor program re-selection as part of the CSC motivational loop described above (Sesack & Grace, 2010).

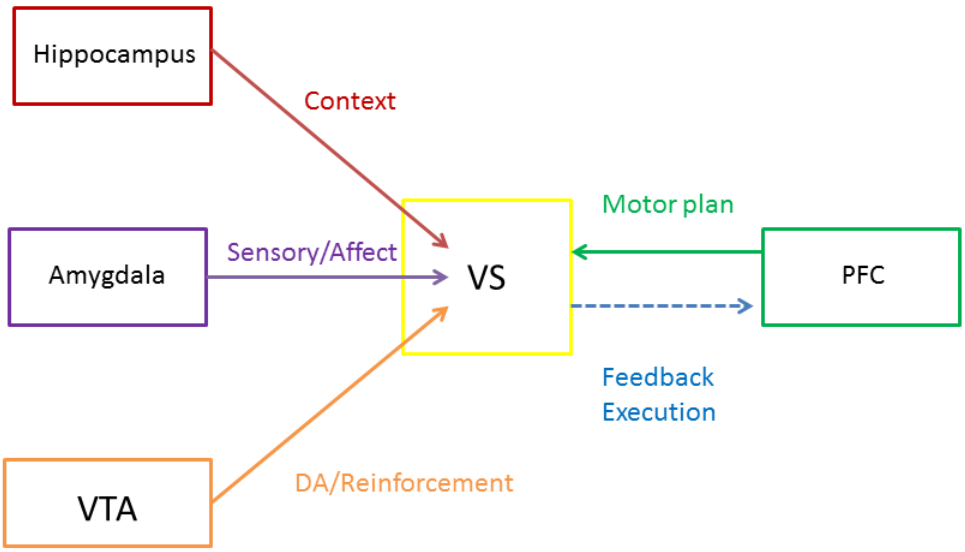

Figure 1. Diagram of information flow through the VS. Dotted line indicates indirect projections. Note that for the sake of brevity additional CSC nodes are not depicted.

Recent evidence suggests that reward processing is not a monolithic phenomenon, but can instead be parsed into distinct psychological, neuroanatomical, and neurochemical subcomponents behaviorally characterized as "wanting" (anticipatory), "liking" (consummatory), and "learning" (prediction) (Berridge *et al.*, 2009). Core hedonic or "liking" reactions have primarily been linked to opioid signaling within a subregion of the NAcc (the NAcc shell). Dopamine, in contrast, has been shown to play a primary role in mediating "wanting" and "learning" by regulating activity in

other subcomponents of the NAcc (e.g., the NAcc core) and interconnected regions of the extended CSC. Given the relative inability of currently available research methodologies to fully parse these biological and behavioral components of reward processing in humans, as well as the large body of research investigating the role of DA in regulating VS reactivity, reward learning and reward processing in general, the current review focuses on the regulatory role DA plays on CSC functioning more broadly and any aspect of reward processing subserved by this circuitry.

DA is a catecholamine monoamine neurotransmitter involved in a variety of functions ranging from lactation and movement to the regulation of crucial aspects of emotion and cognition. Of particular relevance to the topic of this review and as described above, within the CSC, DA acts as a modulator of reward-related processes. Studies have demonstrated the existence of two types of dopaminergic activity of particular importance for this regulatory role – tonic and phasic. Tonic activity refers to a constant slow "background" firing of dopaminergic neurons, while phasic refers to the quick bursts of action potentials, which occur in response to specific environmental events (Grace, 1991).

Early studies linking DA to reward in animal models did not discriminate between tonic and phasic activity and have observed that both natural and drug rewards result in an extracellular DA surge in the NAcc (Hernandez & Hoebel, 1988). Conversely, pharmacological blockade of DA signaling has been shown to result in

decreased reward-seeking behavior (Yokel & Wise, 1975). More recent studies have begun to delineate a more complex role for DA in reward-related neural function by demonstrating that DA release is not merely a correlate of reward consumption or reward seeking, but may instead be involved in coding complex information about the occurrence of appetitive stimuli in the environment. This research has demonstrated that spikes in phasic dopaminergic activity in VTA neurons occur in response to the unexpected delivery of a rewarding stimulus (or the delivery of an unexpectedly large reward), while, conversely, a dip in phasic activity occurs in response to unexpectedly low rewards or the absence of expected reward (Schultz, 2002). Furthermore, as reward-based learning progresses, these phasic responses transition from the reward itself to the stimulus predicting the reward (Schultz, 2002). This transition, coupled with the adaptive modulation of DA phasic activity in response to changes in reward contingencies (i.e., unexpectedly high/low rewards), which then feeds into the motivational CSC loop, suggest that DA may be specifically involved in reward learning and anticipation in addition to reward responsiveness more generally.

1.1.2. CSC dysfunction and psychopathology

Reward processing and motivation are crucial for the adaptive engagement in goal-directed behavior. Importantly, these fundamental cognitive and behavioral processes are not only disrupted in, but also built into the diagnostic criteria for, a range of psychiatric disorders, including perhaps most notably, major depressive disorder

(MDD) and substance use disorders (First, 1996). Extensive behavioral studies of these constructs and their role in psychopathology and psychiatric disorder risk have recently been complemented by research on the contribution of CSC function to the biological basis of these same phenotypes (Pizzagalli, 2014).

Over the past two decades, advances in blood-oxygenation-level dependent functional magnetic resonance imaging (BOLD fMRI) have afforded the opportunity to measure activity in the VS and broader CSC reliably and non-invasively in humans. BOLD fMRI tasks which activate the VS typically expose participants to primary reinforcers, such as food (Demos *et al.*, 2011, Grabenhorst *et al.*, 2010), psychoactive substances (Gilman *et al.*, 2012), and, perhaps even more commonly, secondary reinforcers such as money (Delgado *et al.*, 2000, Knutson *et al.*, 2000). The BOLD fMRI signal recorded in the VS during these paradigms is widely thought to reflect increases in phasic DA activity, occurring upon the receipt of unexpected primary rewards or in response to a conditioned stimulus previously paired with reward. Lending support to this notion, multimodal neuroimaging research combining positron emission tomography (PET) and BOLD fMRI has shown that the magnitude of the VS response to monetary reward directly correlates with the amount of DA released in the same region following pharmacological challenge (Buckholtz *et al.*, 2010a).

Consistent with the role of the CSC in reward processing, dysregulation in this circuit leading to abnormally high or low reward sensitivity has been associated with

clinically significant alterations in positive emotion, motivation and/or consummatory behaviors, as well as psychopathology risk. In line with the hedonic and motivational deficits characteristic of MDD, individuals currently experiencing a major depressive episode show blunted striatal response to reward (Epstein *et al.*, 2006, Knutson *et al.*, 2008, Pizzagalli *et al.*, 2009). Relatively reduced neural responsivity to reward is, however, also associated with depression vulnerability following childhood adversity, regardless of current diagnosis (Dillon *et al.*, 2009). On the other hand, relative VS hyper-reactivity is associated with high trait impulsivity (Buckholtz *et al.*, 2010b, Forbes *et al.*, 2009a), steeper delay discounting (Hariri *et al.*, 2006), and antisocial behavior (Buckholtz *et al.*, 2010a), all of which represent risk factors for addiction and other disorders characterized by behavioral disinhibition (Alloy *et al.*, 2009, Kreek *et al.*, 2005, Krueger *et al.*, 2007). These divergent associations between reward-related VS reactivity and disorder risk emphasize the context-specificity of risk and resilience conceptualizations within the framework of dimensional approaches to establishing predictive links between brain function and clinically relevant behavioral, cognitive and affective constructs.

1.1.3. Genetic variants affecting VS reactivity

Despite promising links between individual differences in reward-related VS reactivity and psychopathology risk, neuroimaging phenotypes of reward processing are of limited clinical utility due to the logistic constraints posed by the specifics of

human functional neuroimaging technology. BOLD fMRI is a costly and time-consuming procedure and is thus unlikely to be routinely implemented in clinical settings in the foreseeable future. Therefore, identifying easily accessible peripheral biological markers of neural circuit function is likely to provide a more practical way of determining the relative risk status of an individual. Moreover, identifying such markers is likely to yield additional insights into the molecular mechanisms underlying disorder risk, which may in turn enable the development of novel and/or individualized therapeutic and preventative strategies. By combining molecular genetics and BOLD fMRI technology, the field of imaging genetics has begun to map variability in clinically relevant brain function onto common genetic variation, which may then serve as a relatively accessible proxy of disorder risk and resilience (Hariri, 2009).

Imaging genetics studies on the functioning of the CSC have focused on the neurotransmitter DA, because of the central role it plays in regulating reward-related and motivational processing. Functional polymorphisms within genes involved in regulating each individual step of DA synthesis, signaling and synaptic clearance are apt to create individual variability in CSC reactivity and reward processing on the behavioral level. Several commonly studied functional genetic polymorphisms affecting CSC function by modulating various steps of the DA signaling cascade are reviewed below.

1.1.3.1. The dopamine transporter (DAT)

The dopamine transporter (DAT), encoded by the gene *SLC6A3*, is a protein with a key role in regulating DA neurotransmission. DAT binds DA after its release into the synaptic cleft and facilitates reuptake of the neurotransmitter into the pre-synaptic neuron. Thus, DAT helps regulate the duration and intensity of post-synaptic responses to dopaminergic inputs as well as the available presynaptic pool of DA (via recycling). DAT is expressed predominantly in the striatum, including the VS, where it plays a crucial role in modulating dopaminergic inputs from the VTA (Lewis *et al.*, 2001, Sesack *et al.*, 1998, Wayment *et al.*, 2001).

A 40-base pair (bp) variable number tandem repeat (VNTR) polymorphism termed DAT1 occurs within the 3' untranslated region (UTR) of *SLC6A3* and results in alleles of various lengths ranging from 3 to 13 repeats, with the 9- and 10-repeat alleles being most frequent in the majority of world populations studied (Doucette-Stamm *et al.*, 1995, Kang *et al.*, 1999, Mitchell *et al.*, 2000). Although not all studies have found an effect of the DAT1 40-bp VNTR genotype on DAT expression levels (Martinez *et al.*, 2001, Mill *et al.*, 2005), several studies have linked the 9-repeat allele to reduced DAT availability *in vitro* (Arinami *et al.*, 1997, Vanness *et al.*, 2005) and *in vivo* (Cheon *et al.*, 2005, Heinz *et al.*, 2000). The presence of the 9-repeat allele would then presumably lead to less efficient DA reuptake and heightened CSC reactivity through increased synaptic levels of DA. Consistent with this notion, individuals carrying at least one copy of the

low expressing 9-repeat allele have been shown to have increased VS reactivity to positive feedback in a number-guessing BOLD fMRI paradigm, relative to individuals homozygous for the 10-repeat allele (Forbes et al., 2009).

1.1.3.2. Dopamine receptors

In addition to reuptake mechanisms, DA signaling is also critically dependent on the properties of DA receptors. There are two major classes of DA receptors – D1-like receptors, which have primarily excitatory functions and include DA receptors D1 and D5; and D2-like receptors, which are primarily inhibitory and include DA receptors D2, D3 and D4 (Beaulieu & Gainetdinov, 2011).

The D1 and D5 DA receptors are encoded by the genes *DRD1* and *DRD5*, respectively. Partially due to the simpler structure of those genes (e.g., characterized by lack of introns), few association studies have investigated the effects of D1-like receptor variants on neural function. The molecular, cellular, neural and behavioral effects of common polymorphisms within the D2-like family have been studied much more extensively. Thus, the rest of the current DA signaling overview focuses on this class of receptors.

Dopamine D2 receptors, encoded by the *DRD2* gene, are most densely expressed in the VS, where they are located both pre- and post-synaptically (Beaulieu & Gainetdinov, 2011). Two alternatively spliced isoforms of D2 receptors exist – short (D2S) and long (D2L), which are expressed primarily pre- and post-synaptically,

respectively (Giros *et al.*, 1989, Monsma *et al.*, 1989). The pre-synaptic D2S functions as an autoreceptor and is part of a negative feedback regulatory mechanism of DA signaling, while D2L mediates post-synaptic inhibition.

Consistent with the inhibitory effect of D2 receptor signaling on DA neurotransmission, imaging genetics studies have linked polymorphisms resulting in relatively reduced *DRD2* expression to heightened VS reactivity. Specifically, the Deletion (Del) allele of a one-point Insertion/Deletion polymorphism (rs1799732) occurring within the 5′ UTR of *DRD2*, frequently termed *DRD2* -141C Ins/Del, has been associated with up to 78% reduction in striatal *DRD2* expression *in vitro* (Arinami *et al.*, 1997) and increased VS reactivity to positive feedback in a BOLD fMRI number-guessing paradigm (Forbes et al., 2009).

Another commonly studied *DRD2* polymorphic locus is the *DRD2* Taq1A (rs1800497), which is a single nucleotide polymorphism (SNP) located in the adjacent ankyrin repeat and kynase domain containing 1 (*ANKK1*) gene and probably affects *DRD2* function only indirectly. Its two alleles T (A1) and C (A2) have been linked to relatively decreased and increased D2 receptor availability, respectively (Jonsson *et al.*, 1999, Pohjalainen *et al.*, 1998). However, the C allele has been associated with increased striatal glucose metabolism (Noble *et al.*, 1997) and reactivity to reward (Stice *et al.*, 2008). This pattern may reflect a specific effect of the *DRD2* Taq1A polymorphism on post-synaptic D2 receptors localized on inhibitory GABA interneurons, which modulate

striatal function by inhibiting glutamatergic medium spiny neurons. Thus, the C allele may result in increased DA-mediated inhibition of GABAergic interneurons leading to disinhibition of excitatory medium spiny neurons and thus, ultimately, increased VS reactivity measured with fMRI. Alternatively, recent studies suggest that the *DRD2* Taq1A polymorphism may not affect DA signaling directly, but rather "tag" (i.e., be in linkage disequilibrium with) two intronic SNPs within *DRD2* associated with differential expression of the D2S and D2L receptor isoforms (Moyer *et al.*, 2011, Zhang *et al.*, 2007).

Similarly to the D2 receptor, the DA D4 receptor, encoded by the *DRD4* gene, mediates both autoreceptor regulation and post-synaptic inhibition of DA signaling. Unlike *DRD2*, however, *DRD4* exhibits relatively low expression in the striatum (Jaber *et al.*, 1996) and the lowest expression levels in the human brain of all DA receptors (Beaulieu & Gainetdinov, 2011, Rondou *et al.*, 2010). Nonetheless, preliminary data suggest that the D4 receptor is expressed post-synaptically on striatal neurons, as well as pre-synaptically on glutamatergic afferents from the PFC to the striatum (Jaber *et al.*, 1996, Missale *et al.*, 1998, Tarazi *et al.*, 1998). Thus, D4 receptor stimulation can inhibit striatal function either directly or indirectly, via one or both of these independent mechanisms. Based on these localization data, genetic variants associated with higher levels of D4 function are likely to result in greater DA-mediated inhibition of post-synaptic target neurons and reduced striatal reactivity.

A common 48-bp VNTR within exon 3 of *DRD4* results in alleles of different length (ranging from 2 to 11 repeats), associated with differential gene transcription and protein function (Asghari *et al.*, 1995). Specifically, the 7-repeat allele has been linked to reduced D4 receptor sensitivity and reduced postsynaptic inhibition (Asghari *et al.*, 1995). Consistent with the inhibitory role of D4 receptors on striatal DA, the 7-repeat allele has also been linked to higher VS reactivity to positive feedback (Forbes *et al.*, 2009a). Finally, in line with its putative neurochemical effects, the same allele has been associated with increased approach to reward on the behavioral level (Roussos *et al.*, 2010). Taken together, these findings demonstrate that, despite its relatively low expression levels in the striatum, *DRD4* may play an important role in regulating the reactivity of the CSC and the behaviors associated therewith.

1.1.3.3. Other variants

Additional polymorphisms implicated in the regulation of DA signaling in the VS and CSC include the catechol-O-methyltransferase (*COMT*) gene Val158Met (rs4680) SNP (Dreher *et al.*, 2009, Yacubian *et al.*, 2007), and the monoamine oxidase A (*MAOA*) rs12843268 SNP (Nymberg *et al.*, 2013). Both of these likely exert their effects on DA signaling through modulating the rate and efficiency of DA enzymatic degradation. Variants which modulate DA signaling more distally have also been implicated in the regulation of VS signaling. Notable examples include the C385A (rs324420) SNP in the fatty amino acid hydroxylase gene (*FAAH*), involved in the enzymatic degradation of

endogenous cannabinoid neuromodulators (Hariri *et al.*, 2009), A118G (rs1799971) in the mu-opioid receptor (*OPRM1*) (Ramchandani *et al.*, 2011), as well as rs2513281 within the *GAL* gene encoding the hypothalamic neuropeptide galanin (Nikolova *et al.*, 2013).

1.2. *Threat Processing*

1.2.1. Basic functional neuroanatomy of the corticolimbic circuit

The neural system which plays a pivotal role in the detection and response to threat in the environment is the brain's corticolimbic circuit (CLC, Figure 2). The CLC comprises a distributed network of cortical and subcortical (i.e., limbic) regions, within which the amygdala, a small structure in the anterior medial temporal lobe, serves as a circuit hub. The basolateral amygdala (BLA) receives rich multi-modal sensory input, including nociceptive information, from thalamic and cortical regions. Thus, it is uniquely positioned to mediate early perceptual processing of incoming environmental input and the detection of salient stimuli indicative of potential threat. Salient sensory information associated with the presence of an aversive stimulus can then be carried forward to the amygdala's output nuclei (primarily in the centromedial amygdala, CeA) in order to launch physiological and behavioral responses aimed to terminate exposure to, or minimize the impact of, noxious or intrinsically unpleasant (i.e., unconditioned) stimuli.

Direct projections from the CeA to the paraventricular nucleus (PVN) of the hypothalamus serve to trigger the body's hypothalamic-pituitary-adrenal (HPA) axis,

the activity of which mediates physiological changes associated with the body's "fight or flight" response. Additional amygdala projections to the brainstem can directly regulate breathing and heart rate. In addition, the CeA can indirectly affect activity in extensive cortical regions via projections to the Nucleus Basalis of Meynert (NBM), a group of cholinergic neurons located within the dorsal extended amygdala. Cholinergic input from the NBM to the sensory cortices and the PFC, among other regions, serves to increase attention, vigilance, and acuity in sensory processing (Sarter *et al.*, 2006).

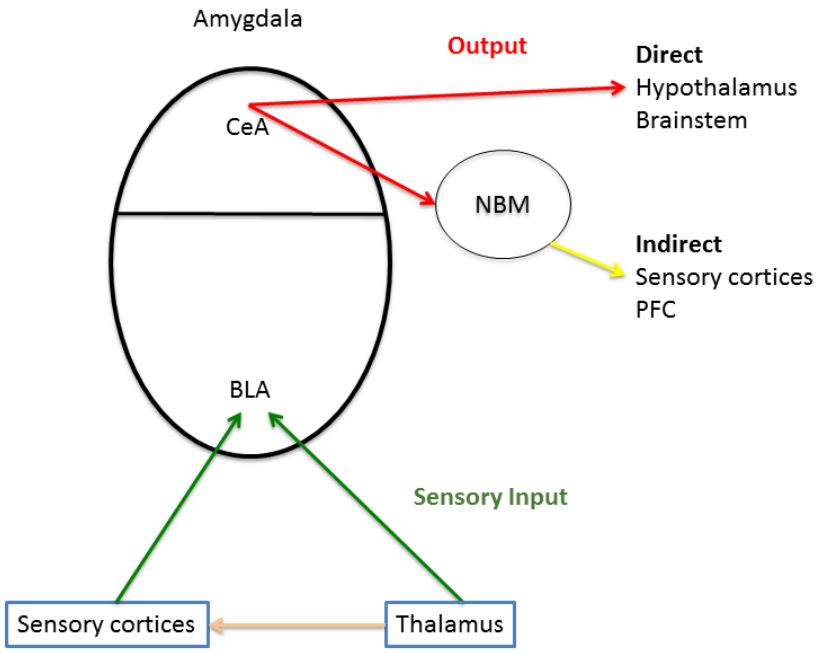

Figure 2. Diagram of information flow through the amygdala. Note that for the sake of brevity additional CLC nodes are not depicted.

In addition to triggering physiological and behavioral responses to intrinsically noxious stimuli, the amygdala is also critically involved in fear conditioning and extinction. Fear conditioning and extinction are associative learning processes whereby links between spatially and temporally co-occurring noxious (unconditioned) stimuli and neutral (conditioned) stimuli are established or modified, such that (only) conditioned stimuli consistently predictive of threat can come to elicit similar responses as the primary threatening stimuli themselves.

The physiological changes effected by the amygdala in response to both conditioned and unconditioned stimuli are primarily associated with increased vigilance and arousal and are neither directly equivalent to the conscious experience of fear, nor in fact sufficient to produce this affective state in humans (Ledoux, 2014) . Nonetheless, they are a necessary and indispensable part of the biological basis of fear and negative affect (Ochsner *et al.*, 2004, Schaefer *et al.*, 2002). Consistent with this notion, and as reviewed in the next section of this chapter, variability in the functioning of the amygdala has been associated with pathological, as well as normal-range, negative affective states and traits in humans.

1.2.2. CLC dysfunction and psychopathology

While the physiological and behavioral changes associated with the amygdala's response to environmental threat are fundamentally adaptive and aid in dealing with specific environmental challenges, they can be harmful when excessive, chronic or

context-inappropriate (Chrousos, 2009). Based on extensive functional neuroanatomy studies in animal models, many forms of pathological affective processing in humans, particularly in the context of environmental adversity, have been conceptualized as indicative of a reduced capacity for regulation of signaling within the CLC, leading to abnormally strong fear conditioning or impaired extinction, and ultimately the potentiation and prolongation of negative affective states (Garakani *et al.*, 2006, Gold & Chrousos, 2002, Rosen & Schulkin, 1998).

Similarly to the response of the VS to rewarding stimuli, the reactivity of the amygdala to threat can be measured reliably and non-invasively using BOLD fMRI technology combined with simple perceptual processing tasks. Amygdala reactivity to threat is typically probed with paradigms exposing study participants to pictures of human faces expressing anger or fear. These facial expressions engage the amygdala particularly strongly, because they represent salient social signals indicative of the presence of threat in the environment (Sergerie *et al.*, 2008, Whalen *et al.*, 2001). Faces expressing surprise as well as those with neutral expressions have also been shown to engage the amygdala (Carre *et al.*, 2013, Somerville *et al.*, 2004), most likely because of the novelty and ambiguity they convey, both of which merit an increase in vigilance. Despite the fact that these stimuli robustly engage the amygdala, substantial inter-individual variability exists in the strength of the response they elicit. Importantly, this variability has been leveraged to explain variance in emotion processing and expression,

ranging from clinical diagnoses of affective illness to normal-range state and trait factors of relevance for psychopathology risk and resilience.

Perhaps least surprisingly, amygdala reactivity to threat is typically elevated in patients diagnosed with anxiety disorders characterized by excessive or context-inappropriate fear, such as simple phobia, post-traumatic stress disorder (PTSD) and social anxiety disorder (SAD) (Etkin & Wager, 2007, Evans *et al.*, 2008). Increased amygdala activity is also observed in MDD (Abercrombie *et al.*, 1998, Arnone *et al.*, 2012, Drevets *et al.*, 1992, Victor *et al.*, 2010, Whalen *et al.*, 2002, Yang *et al.*, 2010), a disorder characterized by potentiated negative affect and frequently comorbid with anxiety (Moffitt *et al.*, 2007, Sartorius *et al.*, 1996). Some studies indicate that depression-associated amygdala hyperactivity may correlate with symptom severity (Abercrombie *et al.*, 1998, Gaffrey *et al.*, 2011, Peluso *et al.*, 2009) and persist even after remission of a major depressive episode (Arnone *et al.*, 2012, Drevets *et al.*, 1992, Victor *et al.*, 2010). The observation that this neural trait may be relatively independent of disease state is in line with the notion that amygdala hyper-reactivity to threat may be part of a pre-existent vulnerability factor for the development of the disorder.

Consistent with this proposition, an independent line of research has demonstrated that the magnitude of threat-related amygdala reactivity in healthy individuals is both relatively stable over time (Johnstone *et al.*, 2005, Manuck *et al.*, 2007) and positively correlated with anxiety- and negative-affect-related traits (Chan *et al.*,

2009, Fakra *et al.*, 2009, Indovina *et al.*, 2011, Stein *et al.*, 2007, Zhong *et al.*, 2011). Notably, these and similar traits are in turn predictive of affective illness susceptibility, particularly in the context of stress or environmental challenge (Kendler *et al.*, 2004, Sandi & Richter-Levin, 2009). Taken together, these studies suggest that individuals with CLC dysregulation leading to relatively heightened amygdala reactivity may be at elevated risk for affective illness, especially when faced with environmental adversity.

At the same time, it is worth noting that CLC dysregulation may also result in abnormally low levels of amygdala reactivity, which can in turn increase risk of other forms of psychopathology. Specifically, relatively blunted amygdala response has been observed in individuals with callous-unemotional traits (Jones *et al.*, 2009), pediatric depression (Thomas *et al.*, 2001), as well as depression risk not attributable to experiential factors (Wolfensberger *et al.*, 2008). In addition, relatively reduced amygdala reactivity to threat is present in individuals with, or at risk for, alcoholism (Glahn *et al.*, 2007, Marinkovic *et al.*, 2009). In the latter context, amygdala hypo-activity may be indicative of a reduced ability to recognize threat or detect salient environmental signals, both of which can increase risk of substance abuse. As with VS reactivity, both amygdala hypo- and hyper-reactivity can increase vulnerability to psychopathology, which similarly highlights the context-specificity of risk and resilience definitions.

1.2.3. Genetic variants affecting amygdala reactivity

As with the CSC and reward processing, peripheral proxies of amygdala reactivity, CLC dysregulation and threat processing can serve as relatively accessible indices of disorder risk and resilience. Imaging genetics studies have begun to identify genetic variants, which may help explain both normal-range variability and pathological extremes of amygdala responsiveness.

While DA clearly plays a central role in reward processing and learning, there is not a single neurotransmitter molecule that plays a similar role in the context of threat processing and fear conditioning in the amygdala and the CLC. Instead, multiple systems and neuromodulators contribute to variability in the functioning of the circuit. Signaling within and by the amygdala relies primarily on the brain's major excitatory and inhibitory neurotransmitters glutamate and GABA, respectively. However, the relative sensitivity of amygdala to glutamatergic and GABAergic inputs from additional nodes of the CLC can be modulated by monoaminergic projections originating in the brainstem.

Monoaminergic modulation of amygdala function is mediated by serotonin (5-hydroxytryptamin, 5-HT), DA, and norepinephrine (NE), which are synthesized and secreted by neurons residing in the dorsal raphe nucleus (DRN), VTA, and locus ceruleus (LC), respectively. Functional neuroanatomy research in animal models and human pharmacological challenge studies converge to suggest all three monoamines are

involved in regulating the activity of the amygdala (Bigos *et al.*, 2008, Hariri *et al.*, 2002a, Hurlemann *et al.*, 2010, Sadikot & Parent, 1990). However, the modulator whose contribution appears most prominent is 5-HT (Sadikot & Parent, 1990). Thus, the vast majority of studies which have identified genetic markers of CLC function have focused on genes involved in the regulation of 5-HT signaling.

1.2.3.1. The serotonin transporter

The serotonin transporter (5-HTT) is a pre-synaptic transmembrane protein, which is instrumental in the reuptake of 5-HT following its release into the synaptic cleft. Similarly to DAT for DA, 5-HTT is an important regulator of both the duration and the intensity of the post-synaptic responses elicited by 5-HT signaling in the CLC. A common VNTR polymorphism occurs in the promoter region of the serotonin transporter gene (*SLC6A4*). This polymorphism, commonly referred to as the serotonin transporter linked polymorphic region (5-HTTLPR), results in a short (S) and a long (L) allele of the gene, which have divergent functional properties. Critically, the S allele has been associated with reduced *SLC6A4* transcription and reduced capacity for 5-HT reuptake *in vitro* (Lesch *et al.*, 1996). Consistent with these early findings, numerous imaging genetics studies have also linked the S allele to relatively heightened amygdala reactivity *in vivo* (Hariri *et al.*, 2002b, Munafo *et al.*, 2008, Murphy *et al.*, 2013). Notably, the latter finding constitutes the most widely replicated association in imaging genetics. The link between reduced 5-HT reuptake capacity and amygdala hyper-responsivity is

also corroborated by studies demonstrating that threat-related amygdala response is potentiated in individuals with lower DRN 5-HTT binding (Rhodes et al., 2007) or following the acute administration of a selective serotonin reuptake inhibitor (SSRI) (Bigos *et al.*, 2008).

Consistent with animal studies demonstrating that stress results in local increases in amygdala 5-HT signaling (Amat *et al.*, 1998), additional BOLD fMRI research in humans indicates that the relationship between 5-HT reuptake capacity and amygdala reactivity may be potentiated by environmental adversity. Specifically, carriers of the S allele show larger increases in amygdala reactivity under acute threat (Drabant *et al.*, 2012), as well as stronger fear conditioning, particularly in the context of recently experienced stressful life events (Klucken *et al.*, 2013). This synergistic effect of exposure to environmental challenge and genetically driven variability in 5-HT reuptake capacity, is also consistent with extensive epidemiological evidence linking the S allele to increased vulnerability to MDD particularly in the wake of life adversity (Caspi *et al.*, 2003, Karg *et al.*, 2011).

1.2.3.2. The serotonin 1A receptor

The serotonin 1A receptor, encoded by the *HTR1A* gene, is another key element in the regulation of 5-HT signaling in the CLC. Serotonin 1A receptors are found in DRN projection target areas, where they mediate post-synaptic inhibition, as well as on DRN neurons, where they function as autoreceptors mediating negative feedback inhibition of

5-HT release. Multimodal PET-fMRI imaging studies have shown that there is an inverse correlation between DRN 5-HT 1A receptor binding and threat-related amygdala reactivity, consistent with a reduced capacity for autoreceptor regulation of 5-HT signaling in this region (Fisher *et al.*, 2006).

A common functional SNP (rs6295) resulting in a C->G substitution, occurs in the promoter region the human *HTR1A* gene. The G allele is associated with impaired transcriptional repression and consequently relatively increased levels of 1A receptor and enhanced capacity for autoreceptor-mediated negative feedback (Lemonde *et al.*, 2003). Consistent with these *in vitro* findings, the same allele has been associated with relatively decreased threat-related amygdala reactivity *in vivo* as well as reduced levels of trait anxiety (Fakra *et al.*, 2009).

1.2.3.3. Other variants

Additional variants impacting 5-HT signaling through divergent biochemical mechanisms have also been associated with individual differences in amygdala reactivity. Those include the tryptophan hydroxylase 2 (*TPH2*) rs4570625 SNP (Brown *et al.*, 2005, Canli *et al.*, 2005), which likely modulates 5-HT synthesis; and *MAOA* upstream VNTR (u-VNTR) (Buckholtz *et al.*, 2008), which impacts 5-HT enzymatic degradation. Additional genetic variants in systems more distally involved in the regulation of neurotransmission within the amygdala include the brain-derived neurotrophic factor (*BDNF*) rs6265 (Montag *et al.*, 2008), haplotypes in neuropeptide Y (*NPY*) (Zhou *et al.*,

2008), as well as rs1064448 within the adenylate cyclase 7 (*ADCY7*) (Joeyen-Waldorf *et al.*, 2012). In recent years, several genome-wide association studies of amygdala reactivity (Brown *et al.*, 2012, Ousdal *et al.*, 2012) have identified additional candidates of unknown functionality, whose importance has yet to be elucidated.

1.3. *The Present Studies*

The goal of this dissertation was two-fold: 1) to identify novel biological pathways implicating individual differences in reward and threat processing in risk and resilience for psychopathology (Studies 1 and 2); and 2) to identify biological markers of neural reward and threat processing, which may provide clues as to the molecular mechanisms underlying this inter-individual vulnerability and serve as easily accessible proxies of brain function (Studies 3 and 4). To this end, building on the literature reviewed above, in **Chapters 2** and **3** (Studies 1 and 2), I explore novel ways in which inter-individual variability in VS and amygdala reactivity contributes to psychopathology risk and resilience in the context of recent life stress. In **Chapters 4** and **5** (Studies 3 and 4)**,** I identify novel genetic and epigenetic contributions to those same neural phenotypes.

While prior studies have established that relatively reduced neural reactivity to reward is a concomitant of current MDD (Knutson *et al.*, 2008, Pizzagalli *et al.*, 2009) or risk thereof (Dillon *et al.*, 2009), no study has explicitly examined the potential protective effects of the opposite neural phenotype. In **Chapter 2**, I test the hypothesis that robust

reward-related VS reactivity would be associated with resilience to some of the depressogenic effects of stress (Nikolova *et al.*, 2012). Specifically, I hypothesize that the recent experience of stressful life events would be associated with reductions in self-reported positive affect only for individuals with relatively low VS reactivity, but not for those with high VS reactivity.

Similarly, prior research has shown that individuals with or at risk for alcoholism have reduced threat-related amygdala reactivity (Glahn *et al.*, 2007, Marinkovic *et al.*, 2009), but no study has investigated the effects of the opposite phenotype on resilience to harmful alcohol use patterns. In **Chapter 3**, I test the hypothesis that threat-related amygdala reactivity may confer resilience to stress-related increases in problem drinking (Nikolova & Hariri, 2012). I also explore the moderating effect of reward-related VS reactivity on this association, hypothesizing that heightened VS reactivity may be an additional risk factor for heightened alcohol use.

Earlier in this chapter, I summarized findings from imaging genetics research, which has begun to map individual differences in brain function onto differences in genetic makeup. Most of these studies, however, have each focused on the effects of a single polymorphism within a single gene on neural phenotypes pertaining to threat and reward processing. Thus, they have identified effects of relatively small size, some of which have failed to replicate, particularly in small replication samples. In **Chapter 4**, I demonstrate the utility of a novel approach for compiling multilocus genetic profile

scores reflecting the cumulative effects of multiple polymorphisms affecting dopaminergic signaling for increasing power to detect significant genetic effects on VS reactivity (Nikolova *et al.*, 2011).

Above**,** I also summarized findings demonstrating the involvement of 5-HTTLPR genotype in the modulation of amygdala reactivity (Hariri *et al.*, 2002b, Munafo *et al.*, 2008, Murphy *et al.*, 2013). However, in recent years, it has been increasingly recognized that non-sequence based variation in DNA and chromatin could affect gene expression and downstream processes above and beyond the effects of sequence-based genetic polymorphisms (Goldberg *et al.*, 2007). In **Chapter 5**, I test the hypothesis that epigenetic modifications in the promoter region of the human *SLC6A4* leading to reduced expression of the gene, would be associated with heightened amygdala reactivity and that this effect may in fact be stronger than the effect of the low-expressing S allele of 5-HTTLPR on the same neural phenotype.

Study-specific background, methods, and results are described in detail in **Chapters 2-5** below. **Chapter 6** summarizes the conclusions drawn from all four studies and delineates directions for future work.

2. Ventral Striatum Reactivity to Reward and Recent Life Stress Interact to Predict Positive Affect[1]

2.1. *Background*

Stressful life events are among the most reliable predictors of MDD (Brown, 1978, Van Praag, 2004). However, while nearly everyone is confronted with stressful life events, the majority of the population does not subsequently develop depression. Uncovering the neurobiological basis of individual differences in relative vulnerability and resilience to the depressogenic effects of stress may provide unique insights into the pathophysiology of stress-related MDD.

Potential clues to the relationship between stress and depression can be garnered from extensive non-human animal research (Anisman & Matheson, 2005, Willner, 2005) and emerging human work (Berenbaum & Connelly, 1993, Bogdan & Pizzagalli, 2006), which converge to reveal that stress can induce anhedonia, a core symptom of MDD reflecting an inability to experience pleasure or respond to rewarding stimuli, and a general reduction in positive affect (PA). Since anhedonia is associated with relative reductions in reward-related brain function (Epstein *et al.*, 2006, Keedwell *et al.*, 2005, Steele *et al.*, 2007, Surguladze *et al.*, 2005), it is reasonable to postulate that relative vulnerability to the depressogenic effects of stress is, at least in part, related to

[1] This Chapter is based on the following publication: Nikolova, Y.S., Bogdan, R., Brigidi, B.D. & Hariri, A.R. (2012) Ventral Striatum Reactivity to Reward and Recent Life Stress Interact to Predict Positive Affect. Biol. Psychiatry, 72, 157-163.

individual differences in neural responsiveness to reward. Accordingly, relatively increased responsiveness to reward, especially when robust to the detrimental effects of stress, has been hypothesized to confer relative resilience to stress-related psychiatric disorders, including MDD (Charney, 2004, Feder *et al.*, 2009).

While reduced levels of PA are a hallmark of MDD, PA can vary independently of negative effect and other depressive symptomatology (Radloff, 1977). At the same time, even subclinical reductions in PA can predict the development of full-blown depression as well as general psychological well-being, particularly in the face of stress (Folkman & Moskowitz, 2000). Consistent with the idea that PA levels reflect the extent of one's pleasurable engagement with the environment (Watson & Clark, 1988), real-world PA has been found to correlate with the relative reward-related responsiveness of the brain's mesocorticostriatal system across both healthy and depressed individuals (Forbes *et al.*, 2009b). Thus, PA and its temporal stability may serve as informative psychological markers with tractable biological substrates, which may help distinguish individuals at risk for or resilient to this disorder, particularly in the context of stress.

In the current study, we tested the hypothesis that reward-related reactivity of the ventral striatum (VS), a brain structure critically involved in reward processing and appetitive behaviors (Berridge *et al.*, 2009, Haber & Knutson, 2010), would moderate the relationship between recent life stress and state PA. Specifically, we hypothesized that individuals with relatively low VS reactivity would show lower PA in the context of

recent life stress, while those with high VS reactivity would display stable PA regardless of stress. A large cohort of non-patient young adults (N = 200) underwent BOLD fMRI during a number guessing paradigm previously demonstrated to elicit robust VS reactivity (Forbes *et al.*, 2009a, Hariri *et al.*, 2006). The experience of recent stressful life events, as well as early childhood trauma, depressive symptoms and PA (state and trait) were assessed using self-report questionnaires.

2.2. *Methods*

2.2.1. Participants

A total of 200 participants were included from the ongoing Duke Neurogenetics Study (DNS), which assesses a wide range of behavioral and biological traits among nonpatient, young adult, student volunteers. All participants provided informed consent in accord with Duke University guidelines, and were in good general health. Twenty-nine participants were excluded from analyses due to signal dropout in VS regions of interest (see below) and one participant did not have valid self-report data due to programming error, leaving a final sample of 170 individuals (104 women; mean age 19.55 ± 1.26).

All participants were free of the following study exclusions: (1) medical diagnoses of cancer, stroke, diabetes requiring insulin treatment, chronic kidney or liver disease, or lifetime history of psychotic symptoms; (2) use of psychotropic, glucocorticoid, or hypolipidemic medication; and (3) conditions affecting cerebral blood

flow and metabolism (e.g., hypertension). Diagnosis of any current *DSM-IV* Axis I disorder or select Axis II disorders (Antisocial Personality Disorder and Borderline Personality Disorder), assessed with the electronic Mini International Neuropsychiatric Interview (Sheehan *et al.*, 1998) and Structured Clinical Interview for the *DSM-IV* (SCID) subtests (First, 1996), respectively, were not an exclusion as the DNS seeks to establish broad variability in multiple behavioral phenotypes related to psychopathology. No participants met criteria for either Antisocial or Borderline Personality Disorder, and 29 participants from our final sample (N = 170) met criteria for at least one Axis I disorder (Table 1). Since the exclusion of these individuals did not substantially alter our results, we present data from the entire sample in the main text (see Table 4 for analyses excluding individuals with Axis I disorders).

Table 1. Number of participants meeting criteria for DSM-IV Axis I diagnoses.

Disorder	Number
Agoraphobia (no history of Panic Disorder)	1
Alcohol Dependence	12
Alcohol Abuse	5
Cocaine Abuse	1
Marijuana Abuse	1
Generalized Anxiety Disorder	2
Multiple Psychopathologies*	7
Total	**29**

2.2.2. Ventral Striatum Reactivity Paradigm

As described previously (Forbes *et al.*, 2009a), our blocked-design number guessing paradigm consists of a pseudorandom presentation of three blocks of predominantly positive feedback (80% correct guess), three blocks of predominantly negative feedback (20% correct guess) and three control blocks. During each task trial, participants have 3 seconds to guess, via button press, whether the value of a visually presented card is lower or higher than 5 (index and middle finger, respectively). The numerical value of the card is then presented for 500 milliseconds and followed by appropriate feedback (green upward-facing arrow for positive feedback; red downward-facing arrow for negative feedback) for an additional 500 milliseconds. A crosshair is then presented for 3 seconds, for a total trial length of 7 seconds. During control blocks, participants are instructed to simply make button presses during the presentation of an "x" (3 seconds), which is followed by an asterisk (500 milliseconds) and a yellow circle (500 milliseconds). Each block is preceded by an instruction of "Guess Number" (positive or negative feedback blocks) or "Press Button" (control blocks) for 2 seconds resulting in a total block length of 38 seconds and a total task length of 342 seconds.

Participants are unaware of the fixed outcome probabilities associated with each block and are led to believe that their performance will determine a net monetary gain at the end of the scanning session. Instead, all participants receive $10. We include one incongruent trial within each task block (e.g., 1 of 5 trials during positive feedback

blocks was incorrect, resulting in negative feedback) to prevent participants from anticipating the feedback for each trial and to maintain participants' engagement and motivation to perform well.

2.2.3. BOLD fMRI Data Acquisition and Analysis

Each participant was scanned using a research-dedicated GE MR750 3T scanner equipped with high-power high-duty-cycle 50-mT/m gradients at 200 T/m/s slew rate, and an eight-channel head coil for parallel imaging at high bandwidth up to 1MHz at the Duke-UNC Brain Imaging and Analysis Center. A semi-automated high-order shimming program was used to ensure global field homogeneity. A series of 34 interleaved axial functional slices aligned with the anterior commissure-posterior commissure (AC-PC) plane were acquired for full-brain coverage using an inverse-spiral pulse sequence to reduce susceptibility artifact (TR/TE/flip angle = 2000 ms/30 ms/60; FOV = 240 mm; 3.75 × 3.75 × 4 mm voxels; interslice skip = 0). Four initial RF excitations were performed (and discarded) to achieve steady-state equilibrium. To allow for spatial registration of each participant's data to a standard coordinate system, high-resolution three-dimensional structural images were acquired in 34 axial slices co-planar with the functional scans (TR/TE/flip angle = 7.7 s/3.0 ms/12; voxel size = 0.9 × 0.9 × 4 mm; FOV = 240 mm, interslice skip = 0).

Images for each subject were realigned to the first volume in the time series to correct for head motion, spatially normalized into a standard stereotactic space

(Montreal Neurological Institute template) using a 12- parameter affine model (final resolution of functional images = 2 mm isotropic voxels), and smoothed to minimize noise and residual difference in gyral anatomy with a Gaussian filter, set at 6-mm full-width at half-maximum. Voxel-wise signal intensities were ratio normalized to the whole-brain global mean.

Variability in single-subject whole-brain functional volumes was determined using the Artifact Recognition Toolbox (http://www.nitrc.org/projects/artifact_detect). Individual whole-brain BOLD fMRI volumes meeting at least one of two criteria were assigned a lower weight in determination of task-specific effects: 1) significant mean-volume signal intensity variation (i.e., within volume mean signal greater or less than 4 standard deviations of mean signal of all volumes in time series), and 2) individual volumes where scan-to-scan movement exceeded 2 mm translation or 2° rotation in any direction.

The general linear model (GLM) of SPM8 (http://www.fil.ion.ucl.ac.uk/spm) was used to conduct fMRI data analyses. Following preprocessing, linear contrasts employing canonical hemodynamic response functions were used to estimate differential effects of feedback (i.e., reward) from the contrast of Positive Feedback > Negative Feedback for each individual. Individual contrast images were then used in second-level random effects models accounting for scan-to-scan and participant-to-

participant variability to determine mean condition-specific regional responses using one-sample t-tests.

Because of the relatively extensive signal dropout and noise typically observed in the VS due to magnetic susceptibility associated with the region's proximity to tissue boundaries (Ojemann *et al.*, 1997), only participants with greater than 90% signal coverage (N = 170) in a bilateral VS anatomical regions of interest were included in analyses. Whole-brain analyses were then conducted on participants with adequate signal to identify reward-related VS reactivity. A statistical threshold of $p < 0.05$, FWE whole-brain corrected, and ≥ 10 contiguous voxels was applied to the contrast of Positive > Negative feedback blocks for this analysis.

Mean BOLD values from VS clusters exhibiting a main effect of task were extracted using the VOI tool in SPM8. These extracted values were then entered into regression models using IBM SPSS Statistics 19.0 (SPSS Inc., Chicago, IL). Importantly, by extracting VS BOLD parameter estimates from the functional clusters activated by our paradigm rather than clusters specifically correlated with our independent variables of interest (i.e., depressive symptoms and PA), we preclude the possibility of any correlation coefficient inflation that may result when an explanatory covariate is used to select a region of interest (Viviani, 2010). We have successfully used this conservative strategy in previous reports (Carre *et al.*, 2010, Hyde *et al.*, 2010, Nikolova *et al.*, 2011).

2.2.4. Self-Report Measures

2.2.4.1. Depressive symptoms and PA.

. Participants completed the Center for Epidemiologic Studies-Depression (CES-D) scale (Weissman *et al.*, 1977). Based on previous factor analytic studies (Leventhal *et al.*, 2008, Shafer, 2006), four subscales were computed: 1) positive affect (CES-D PA), 2) negative affect (CES-D NA), 3) somatic features (CES-D SF), and 4) interpersonal functioning (CES-D IP). The four items that have previously been identified as loading onto a PA factor, were submitted to a confirmatory factor analysis (CFA) in Mplus 6.0 (Mplus, Los Angeles, CA). This CFA model fit the data well ($\chi^2 = 301.937$, df = 164, $p < 0.05$; CFI = 0.90; RMSEA = 0.070). Note also that in separate analyses, we modeled all four factors of the CES-D and this model also fit the data acceptably, confirming past factor analytic findings (Leventhal *et al.*, 2008, Shafer, 2006) in our sample and strengthening our use of the anhedonia subscale as a separable underlying construct.

Trait PA was assessed using the Positive Emotions subscale of the Extraversion dimension of the NEO personality inventory revised edition (NEO-PI-R) (Costa & Mccrae, 1992).

2.2.4.2. Stressful life events.

To assess recent life stress we administered a modified version of the Life Events Scale for Students [LESS (Clements & Turpin, 1996); see Appendix] This modified version of the scale asks participants to indicate whether they experienced common

stressful life events within the past 12 months; in addition, for each event that occurred participants reported on the impact it had on their lives on a 1-4 scale (with 4 being the highest). The impact scores were set to zero for events that did not occur. We derived three main variables of interest from the LESS: 1) LESS Total Number of Events; 2) LESS Highest Impact, reflecting the highest impact associated with any event which occurred within the past year; and 3) LESS Average Impact, capturing the average impact of all events which occurred within the past year. To ensure the specificity of our results to current life stress, we assessed early life trauma using the Childhood Trauma Questionnaire [CTQ; (Bernstein, 2002)] and used this measure as a covariate in regression analyses.

2.2.5. Statistical Analysis

Regressions using LESS and VS reactivity as independent variables, and CES-D PA scores as a dependent variable were conducted within IBM SPSS Statistics 19 (SPSS Inc, Chicago, IL). Significant interactions were probed using the Johnson-Neyman method (Johnson & Fay, 1950), as implemented in the SPSS MODPROBE macro (Hayes & Matthes, 2009), to calculate the range of VS reactivity values for which stress is significantly correlated with PA. Rather than probing the interactions at specific values of the moderator variable (in this case, VS reactivity), the Johnson-Neyman method allows for calculating the entire range of moderator variable values for which the focal predictor (i.e., the other interacting variable; LESS) is significantly correlated with the

dependent variable (CES-D PA; please see Figure S3 for results from analyses probing the interaction at VS reactivity values of 1 SD below the mean, mean, and 1 SD above the mean).

2.3. *Results*

2.3.1. Sample Demographics

There were no significant effects of gender or age on any self-report measure. However, several trend-level effects emerged (Table 2). In addition, consistent with previous literature (Spreckelmeyer *et al.*, 2009), men had higher right VS reactivity (p = 0.032) compared to women. Finally, race/ethnicity had a significant effect on CES-D Total and all CES-D subscales except CES-D IP (Table 3). To account for the potentially confounding effects of these demographic variables, all analyses were conducted with and without age, gender and race/ethnicity (dummy coded) as covariates in addition to current Axis I diagnosis and trait PA. Analyses with trait PA as a covariate were conducted on N=169 participants because of missing NEO-PI-R scores in one individual resulting from a programming error.

Table 2. Effects of gender and age on self-report variables and VS reactivity. Bolded values indicate significant or trend-level effects.

	Gender Effects				Age Effects	
	Men (N=66)	**Women (N=104)**	**T(168)**	**p**	**b**	**p**
CES-D						
PA	8.92 (2.64)	8.67 (2.96)	-0.56	0.58	-0.032	0.68
NA*	0.79 (0.78)	0.98 (0.79)	-1.58	0.12	**0.148**	**0.054**
SF	3.83 (3.09)	4.35 (3.38)	-1.00	0.32	0.040	0.60
IP*	0.36 (0.48)	0.34 (0.49)	0.32	0.76	0.059	0.45
Total*	2.13 (0.75)	2.22 (0.77)	-0.75	0.45	0.062	0.42
Trait PA	20.09 (4.55)	21.17 (5.46)	-1.34	0.18	-0.059	0.45
CTQ						
Total*	3.50 (0.18)	3.56 (0.24)	**-1.87**	**0.064**	0.081	0.29
LESS						
Number	4.56 (3.41)	4.63 (3.12)	-0.13	0.90	-0.057	0.46
HI	2.89 (1.29)	2.91 (1.04)	-0.11	0.91	-0.115	0.13
AI	2.08 (0.92)	2.22 (0.77)	-1.08	0.28	**-0.133**	**0.083**
rVS (a.u.)	0.13 (0.18)	0.07 (0.18)	**2.17**	**0.032**	-0.046	0.55
lVS (a.u.)	0.12 (0.20)	0.07 (0.19)	1.47	0.14	-0.015	0.85

Table 3. Effects of race/ethnicity on self-report measures and VS reactivity. Bolded values indicate significant differences between groups.

	Caucasian (N=75)	African/ African American (N=31)	Asian/ Asian American (N=45)	Bi- or Multiracial (N=11)	Other (N=8)	F (4,165)	p
CES-D							
PA	**9.48 (2.66)**	**8.13 (2.99)**	**7.78 (2.87)**	**9.90 (2.21)**	8.63 (2.67)	3.60	**0.008**[a]
NA*	**0.67 (0.73)**	**1.10 (0.87)**	**1.01 (0.78)**	**1.16 (0.63)**	**1.34 (0.70)**	3.40	**0.011**[b]
SF	**3.60 (2.77)**	**5.71 (3.88)**	3.64 (3.35)	5.27 (3.50)	4.50 (2.67)	3.05	**0.019**[c]
IP*	0.31 (0.46)	0.42 (0.54)	0.31 (0.48)	0.41 (0.60)	0.48 (0.42)	0.55	0.70
Total*	**1.98 (0.75)**	**2.43 (0.79)**	2.25 (0.79)	2.47 (0.51)	2.18 (0.77)	2.88	**0.024**[c]
Trait PA	**20.75 (5.61)**	**21.65 (4.89)**	**19.02 (4.43)**	**22.90 (4.32)**	24.13 (3.04)	2.99	**0.020**[d]
CTQ*	**3.43 (0.18)**	**3.63 (0.20)**	**3.60 (0.24)**	**3.61 (0.23)**	**3.61 (0.22)**	8.58	**<0.001**[b]
LESS							
Number	4.56 (2.96)	4.94 (3.08)	3.80 (3.00)	5.64 (4.27)	6.75 (4.86)	2.00	0.097
HI	2.87 (1.12)	3.10 (0.83)	2.71 (1.34)	3.36 (1.03)	3.00 (1.15)	1.02	0.40
AI	2.10 (0.78)	2.30 (0.62)	2.03 (1.00)	2.52 (0.73)	2.49 (1.08)	1.44	0.22
rVS	0.10 (0.18)	0.06 (0.16)	0.11 (0.21)	0.08 (0.13)	0.09 (0.14)	0.35	0.84
lVS	0.08 (0.21)	0.07 (0.16)	0.09 (0.24)	0.10 (0.12)	0.11 (0.15)	0.11	0.98

[a]Caucasian>Asian/Asian American and African/African American; Bi/Multiracial>Asian/Asian American, p's<0.024, LSD corrected

[b]All groups>Caucasian, p's<0.05, LSD corrected

[c]African/African American>Caucasian, p=0.002, LSD corrected

[d]African/African American>Asian/Asian American, p=0.009, LSD corrected

2.3.2. VS Reactivity

Consistent with previous studies (Forbes *et al.*, 2009a, Hariri *et al.*, 2006), our paradigm elicited significant reward-related (i.e., Positive>Negative feedback) bilateral VS reactivity (Figure 3). Because there was a small area of contiguity between the left and right VS activation clusters, we extracted BOLD signal values from 5mm spheres built around the peak voxels in each hemisphere (left: x = -12, y = 10, z = -10; right: x = 12, y = 10, z = -8).

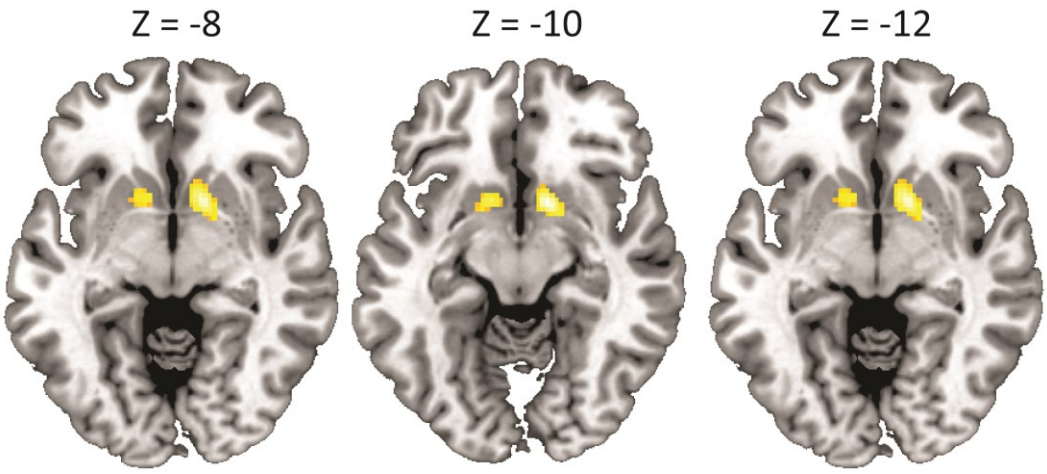

Figure 3. Reward-related VS reactivity. Statistical parametric map illustrating bilateral VS activation clusters for the contrast Positive > Negative Feedback overlaid onto a canonical structural brain image in the axial plane. MNI coordinates and statistics (p < 0.05, FWE whole-brain corrected and ≥ 10 contiguous voxels): left hemisphere: x = -12, y = 10, z = -10, t = 6.19, p = 2.12 x 10^{-9}, right hemisphere: x = 12, y = 10, z = -8, t = 7.31, p = 4.85 x 10^{-12}; k_E = 446.

2.3.3. VS Reactivity, Stress, and PA

In support of our hypothesis, there was a significant interaction between right VS reactivity and LESS Highest Impact scores (ΔR^2 = 0.045, b = 0.500, p = 0.0054; Cohen's f^2 =

0.049), such that higher LESS impact was associated with lower CES-D PA for participants with relatively low VS reactivity (bottom 28.2%, N = 48), but not for those with high VS reactivity (remaining 71.8%, N = 122); Figure 4). Importantly, the interaction term explained significant CES-D PA variance above and beyond the main effects of VS reactivity (b = 0.318, p = 0.14) and LESS (b = 0.217, p = 0.26). Furthermore, the interaction remained significant after controlling for age, gender, race/ethnicity, CTQ Total, Axis I diagnosis and trait PA (ΔR^2 = 0.033, b = 0.450, p = 0.0095, Cohen's f^2 = 0.034). In addition, the interaction remained significant when LESS Number of Events and CES-D non-PA scores, computed by subtracting CES-D PA from CES-D Total, were added to the model individually (LESS: ΔR^2 = 0.031, b = 0.441, p = 0.011, Cohen's f^2 = 0.032; CES-D: ΔR^2 = 0.026, b = 0.401, p = 0.012, Cohen's f^2 = 0.027), or simultaneously (ΔR^2 = 0.026, b = 0.403, p = 0.013, Cohen's f^2 = 0.027)). A similar pattern emerged on the left side (ΔR^2 = 0.035, b = 0.444, p = 0.014; Cohen's f^2 = 0.036), however, the interaction between LESS Highest Impact and left VS reactivity was not equally robust to the inclusions of covariates and was reduced to a statistical trend after their addition to the model (ΔR^2 = 0.016, b = 0.313, p = 0.075).

Similarly to LESS Highest Impact, LESS Number of Events also interacted with right VS reactivity (ΔR^2 = 0.030, b = 0.152, p = 0.024, Cohen's f^2 = 0.03; Figure 4) to predict significant variability in CES-D PA above and beyond the main effects of VS reactivity (b = 0.327, p = 0.13) and LESS (b = 0.100, p = 0.14). Specifically, LESS Number of Events was

associated with lower PA only for participants with relatively low VS reactivity (bottom 34.7%, N = 59), but not for those with high VS reactivity (remaining 65.3%, N = 111). The interaction remained significant when age, gender, race/ethnicity, CTQ scores, Axis I diagnosis and trait PA were added as covariates ($\Delta R^2 = 0.023$, b = 0.138, p = 0.032). As with LESS Highest Impact, we found a similar pattern on the left side ($\Delta R^2 = 0.025$, b = 0.125, p = 0.040, Cohen's $f^2 = 0.026$), which, however, was reduced to a trend when covariates were included in the model ($\Delta R^2 = 0.018$, b = 0.111, p = 0.057). Unlike LESS Highest Impact, however, the Number of Events by right VS reactivity interaction was not robust to the inclusion of covariates in the sample excluding participants with current Axis I diagnosis (Table 5). LESS Average Impact did not interact with VS reactivity to predict CES-D PA (p values > 0.20).

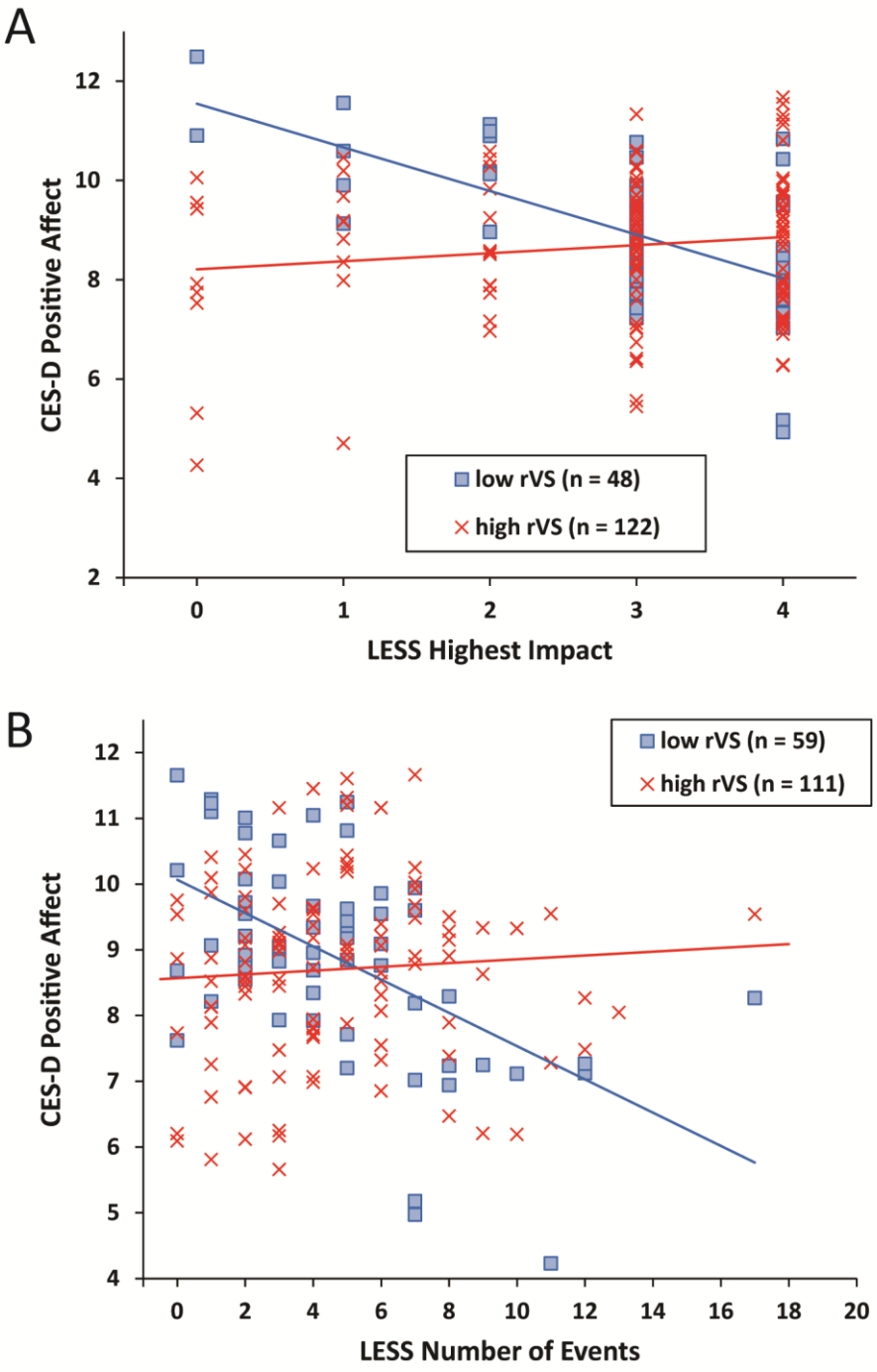

Figure 4. Reward-related VS reactivity moderates the relationship between recent life stress and current levels of positive affect. Stress (A: LESS Highest Impact, B: LESS Number of Events) was associated with lower state PA in participants with relatively low (blue line) but not high (red line) right VS reactivity. Removal of the

two participants reporting 17 life events from the analyses did not change the significance of the LESS Number of Events by VS reactivity interaction term (ΔR^2 = 0.033, b = 0.207, p = 0.018). The plotted values are adjusted for gender, age and race/ethnicity.

Table 4. Results from regressions predicting CES-D A from LESS Highest Impact and VS reactivity, when individuals with any Axis I diagnosis are excluded (N = 141).

LESS Highest Impact	ΔR^2	b	p
No Covariates			
Left VS	0.032	-0.442	**0.034**
Right VS	0.049	-0.552	**0.0079**
With Covariates			
Left VS	0.008	-0.228	0.254
Right VS	0.024	-0.407	**0.040**

Table 5. Results from regressions predicting CES-D A from LESS Number of Events and VS reactivity, when individuals with any Axis I diagnosis are excluded (N = 141).

LESS Number of Events	ΔR^2	b	p
No Covariates			
Left VS	0.017	-0.137	0.123
Right VS	0.032	-0.198	**0.031**
With Covariates			
Left VS	0.004	-0.072	0.405
Right VS	0.011	-0.126	0.158

2.3.4. Control Analyses

To ascertain the specificity of our findings to the CES-D PA subscale, we conducted a regression using LESS and VS reactivity as predictors of CES-D non-PA scores, computed by subtracting CES-D PA from CES-D Total. As hypothesized, this model resulted in a main effect of LESS (Number of Events or Highest Impact) on non-PA depressive symptoms (b coefficients > 0.090, p values < 0.001), but no significant effect of VS reactivity, or VS reactivity by LESS interaction (p values > 0.25). Further demonstrating the specificity of our findings to CES-D PA, no LESS measure interacted with VS reactivity to individually predict any specific non-PA CES-D subscale (p values > 0.05),

Underscoring the specificity of our results to recent life stress, CTQ (Total or Emotional Neglect subscales) scores did not interact with VS reactivity to predict CES-D PA or any of the other CES-D subscales (p values > 0.16). In addition, neither the LESS nor the CTQ or any of their subscales had a direct effect on VS reactivity (p values > 0.16).

2.4. *Discussion*

Consistent with theoretical predictions that robust responsiveness to reward may protect against the depressogenic effects of stress (Feder *et al.*, 2009), we provide empirical evidence that recent life stress interacts with reward-related ventral striatum

reactivity to predict self-reported state positive affect. Specifically, we show that recent life stress is associated with decreased PA only in individuals with relatively low VS reactivity. In those with relatively high VS reactivity levels of PA did not vary as a function of life stress. This interaction effect was robust to the effects of age, gender, race/ethnicity, childhood trauma, trait PA and current psychopathology.

Despite numerous studies implicating reward system dysfunction in MDD (Epstein *et al.*, 2006, Pizzagalli *et al.*, 2009, Steele *et al.*, 2007), little is known about how differences in reward-related brain function influence depressive symptomatology in the context of environmental adversity. Results from the current investigation suggest that individual differences in reward system reactivity may shape one's propensity to experience reductions in PA in the wake of recent life stress. Long-term prospective studies investigating interactions between life stress and individual differences in VS reactivity are needed to evaluate if this pattern is associated with vulnerability for developing MDD. However, the relevance of this putative risk pathway is corroborated by extant research demonstrating that reward system reactivity in non-depressed adults is shaped by various genetic and environmental factors known to modulate depression vulnerability. Specifically, VS reactivity in healthy adults is regulated by polymorphisms within dopaminergic genes (Forbes *et al.*, 2009a, Nikolova *et al.*, 2011), that have also been linked to differential depression susceptibility particularly in the context of environmental adversity (Elovainio *et al.*, 2007, Haeffel *et al.*, 2008). In

addition, both early life stress and experimentally manipulated acute stress have been linked to reductions in reward-related neural reactivity (Bogdan *et al.*, 2011, Dillon *et al.*, 2009) and increased risk for depression (Heim *et al.*, 1997). Drawing on our current results, we speculate that reward-related VS reactivity may further interact with recent life stress to modulate stress-related reductions in PA, and potentially, risk for depression.

Contrary to prior findings demonstrating that early life stress reduces neural responses to reward in adults (Dillon *et al.*, 2009), we found that neither early childhood trauma, as assessed by the CTQ, nor recent life stress, had a direct effect on VS reactivity. It is worth noting, however, that participants in the current sample were not specifically selected for childhood trauma experience and were primarily high functioning college students with little endorsed childhood trauma exposure. Thus, it is possible that only severe childhood trauma or chronic stress of a magnitude outside the range present in the current sample would result in significant reduction in adult neural responsiveness to reward.

As hypothesized, the interaction between VS reactivity and stress was most robust when predicting PA (i.e., CES-D PA), rather than general depressive symptoms as measured by the other subscales of the CES-D. This suggests that VS reactivity may be protective against decreases in PA specifically, rather than depression in general, possibly by conferring resiliency to stress-related hedonic impairments. Moreover,

because anhedonia and reductions in PA are a defining feature of other stress-related psychopathology, such as Post-Traumatic Stress Disorder (PTSD), our findings may not be specific to MDD resilience. In fact, the results we report are also consistent with studies suggesting that pre-existing individual differences in neural responsiveness to reward correlate with resilience to PTSD in the face of trauma (Vythilingam *et al.*, 2009). Nonetheless, since we used a non-clinical sample in this study, direct translation of the observed patterns into vulnerability and resilience for psychopathology cannot be assumed until confirmed by prospective longitudinal studies mapping the etiology of clinical disorders.

In addition to the specificity of our results to PA, our findings were strongest when using the LESS Highest Impact metric, rather than total Number of Events or Average Impact. Importantly, the results involving LESS Highest Impact scores remained significant when controlling for number of events, suggesting that the effects of the event with the highest impact may override the independent and/or additive effects of multiple less impactful stressful events. Moreover, the results with LESS Number of Events did not survive when individuals with current psychopathology were removed from analyses. While the additivity of stressful life events has long been the subject of debate in the literature (Paykel, 1983), some empirical support does exist for the notion that once a highly impactful event has occurred, the depressogenic effects of minor events may become negligible (Brown, 1978). Future research employing

interview-based stress measures (Duggal *et al.*, 2000) embedded within a prospective longitudinal design may be necessary to corroborate the credibility of this postulation.

Finally, while linear regression analyses conducted separately for the right and the left VS activation clusters yielded convergent results, the VS reactivity x stress interaction effect was more robust in the right hemisphere. Such asymmetries are not uncommon in the literature (Jocham *et al.*, 2009, Yacubian *et al.*, 2007) and may reflect intrinsic differences in neurotransmitter regulation of VS function across the two hemispheres (Besson & Louilot, 1995, Merali *et al.*, 2004, Sullivan & Dufresne, 2006, Young & Williams, 2010). However, the precise biological mechanisms mediating such lateralized effects are currently unknown. Alternatively, it is possible that, perhaps due to its visuospatial component, our task preferentially recruited the right VS. Although a paired-samples t test directly comparing activation in the peak two voxels in the left and right VS was not significant in the current sample ($p = 0.29$), our whole-brain voxel-wise analysis showed that the peak activation voxel on the right side was somewhat more strongly activated than the peak voxel on the left side (right: $t = 7.31$, $p = 4.85 \times 10^{-12}$; left: $t = 6.19$, $p = 2.12 \times 10^{-9}$). Consistent with this notion, we have previously found right-hemisphere specific correlations between reward-related VS reactivity, as assessed by the same task, and variability in behavioral measures of impulsivity (Forbes *et al.*, 2009a, Hariri *et al.*, 2006). Further research is necessary to determine the functional significance of these lateralized effects.

The current study is not without limitations. Most importantly and as highlighted above, while trait PA has been found to be predictive of depression risk (Folkman & Moskowitz, 2000, Sheeber *et al.*, 2009, Watson, 2005), the direct relevance of these findings to understanding depression vulnerability and resilience, particularly over long periods, of time is limited. The clinical significance of the findings is also limited by the fact that we focused on high-functioning non-patient young adult participants, who may be more resilient than the general population. This could at least partially explain why we did not find a main effect of recent life stress on either depressive symptomatology more generally or PA levels specifically. Thus, caution must be used in interpreting the broader clinical significance of these findings until replicated in the context of more severe mood pathology.

Another potential limitation of the present study lies in the instrument we used to assess tress. Specifically, we used a self-report retrospective measure of stressful life events occurring in the past 12 months. This questionnaire did not ask participants to indicate the specific time when each event occurred, leaving us unable to differentiate between more proximal and distal events (Kendler *et al.*, 1998) or evaluate potential "kindling" effects (Post, 1992). However, prior studies have shown that stressful life events can have detrimental effects on psychological well-being for up to a year following their occurrence (Caspi *et al.*, 2003, Clements & Turpin, 1996). In addition, the appraisal of an event's impact, as conveyed by LESS Highest Impact scores, at the time

of study completion (i.e., when VS reactivity and CES-D PA were assessed) provides an index of subjective importance of a life event, which may be more informative than its proximity in time. Nonetheless, given the retrospective nature of our self-report measure of stressful life events, we cannot rule out the possibility that these events affected subsequent measures of VS reactivity. However, prior research suggests that VS reactivity is a temporally stable neural phenotype. Specifically, studies have shown that VS reactivity as assessed by this task correlates with temporally stable personality and behavioral traits such as impulsivity (Forbes *et al.*, 2009a) and delay discounting (Hariri *et al.*, 2006). In addition, we have previously shown that the same neural phenotype is under the direct influence of polymorphisms within several dopaminergic genes, suggesting this phenotype may be relatively independent of environmental effects (Forbes *et al.*, 2009a, Nikolova *et al.*, 2011). Finally, systematic effects on VS reactivity would be expected to manifest as direct correlations between LESS scores and VS reactivity in the current sample and all such correlations were non-significant.

Finally, recent studies have demonstrated that reward processing may not be a unitary phenomenon (Berridge *et al.*, 2009), and our task does not allow for differentiation between brain function during different phases of reward processing (e.g., reward anticipation, outcome and learning). Relatedly, we focused our analyses on state positive affect levels, which capture overall happiness, but do not tap directly into motivational aspects of reward processing or reward learning. In light of studies

showing reduced reward responsiveness and reward-based learning in the context of stress (Bogdan & Pizzagalli, 2006, Pizzagalli *et al.*, 2007), it is possible that the relatively low levels of positive affect we observed as a function of recent stressful life events and relative hyporesponsiveness of the VS, may in fact be due to stress-related reductions in motivation to pursue rewards or a reduced ability to learn from prior reinforcement. Future studies using tasks allowing for greater specificity on both the behavioral and neural level could identify discrete components of reward-related processes that may better explain stress-related variability in positive affect alongside other relevant aspects of reward processing.

These limitations notwithstanding, the current study is the first empirical demonstration that robust neural reactivity to reward may protect against stress-related reductions in positive affect. Given that impairments in PA are cardinal features of mood disorders in general, and MDD in particular, the current work provides a useful framework for future research to investigate the relevance of these pathways in the expression of clinical dysfunction. Additional work establishing molecular adaptations in the reward system that may mediate resilience to stress-related hedonic impairments holds promise not only to enhance our understanding of vulnerability and resilience to depression, but also ultimately inform advances in treatment and prevention strategies for MDD and other stress-related psychopathology. Such research may benefit specifically from combining laboratory stress manipulations with multimodal PET/fMRI

imaging to measure reward-related brain function alongside dopamine release (Buckholtz *et al.*, 2010a), while also taking into account genetic variants affecting neurotransmission within the VS (Hariri, 2009).

3. Neural Responses to Threat and Reward Interact to Predict Stress-Related Problem Drinking: A Novel Protective Role of the Amygdala[1]

3.1. *Background*

Increased amygdala reactivity to threat has been consistently associated with heightened risk for mood and anxiety disorders (Stein *et al.*, 2007). In contrast to this heightened risk, a few studies have suggested that threat-related amygdala reactivity may buffer risk for drug abuse. Specifically, one study reported that individuals at high familial risk for alcoholism exhibit relatively reduced threat-related amygdala reactivity (Glahn *et al.*, 2007). The authors speculate that this pattern may indicate reduced sensitivity to the harmful consequences of excessive alcohol use in those at risk.

Consistent with these findings, a recent study has linked a genetic variant conferring increased risk for drug abuse (Sipe *et al.*, 2002), with relatively decreased threat-related amygdala reactivity (Hariri *et al.*, 2009). Interestingly, the same genetic risk variant was associated with heightened reward-related reactivity of the ventral striatum (VS), a neural phenotype associated with both risk for and pathophysiology of drug abuse (Evans *et al.*, 2006, Forbes *et al.*, 2009a). These data suggest a potentially synergistic effect of threat-related amygdala reactivity and reward-related VS reactivity

[1] This Chapter is based on the following publication: Nikolova, Y.S. & Hariri, A.R. (2012) Neural responses to threat and reward interact to predict stress-related problem drinking: A novel protective role of the amygdala. Biology of mood & anxiety disorders, 2, 19.

in precipitating drug abuse risk. In addition to variability in these neural phenotypes, drug abuse risk is moderated by environmental factors, such as recent life stress (Sinha, 2001). Both VS and amygdala function also are affected by stress (Rademacher *et al.*, 2008), suggesting that complex interactions between these neural circuits may contribute to variability in stress-related risk for drug abuse.

Here, we explore the interactions of recent life stress, threat-related amygdala and reward-related VS reactivity in predicting variability in self-reported problem drinking in a sample of 200 young adults. We focused on drinking because alcohol is the most commonly used and abused drug in adolescents and young adults (Young *et al.*, 2002), and its use is often triggered by stress (Sinha, 2001). Using two well-characterized BOLD fMRI paradigms, we quantified threat-related amygdala and reward-related VS reactivity. Recent life stress and problem drinking were assessed using the Life Events Scale for Students [LESS, (Nikolova *et al.*, 2012)] and the Alcohol Use Disorder Identification Test [AUDIT, (Saunders *et al.*, 1993)], respectively. Based on prior research, we predicted that higher threat-related amygdala reactivity would protect against increased problem drinking in the context of stress, particularly in those whose risk is exaggerated by higher reward-related VS reactivity.

3.2. *Methods*

3.2.1. Participants

200 participants were included from the ongoing Duke Neurogenetics Study (DNS), which assesses a range of behavioral and biological traits among young adult, student volunteers. DNS exclusionary criteria are described in detail elsewhere (Nikolova *et al.*, 2012). All participants provided informed consent in accord with Duke University guidelines, and were in good general health. Twenty-nine participants who completed the study were subsequently excluded from analyses due to poor BOLD fMRI signal in VS regions of interest (Nikolova *et al.*, 2012) and one participant did not have valid self-report data due to programming error, leaving a final sample of 170 individuals (104 women; mean age 19.55 ± 1.26). Twenty-nine of these 170 met criteria for at least one Axis I disorder (Table 1). The significance of all reported results did not change when controlling for disorder (p values < 0.030).

3.2.2. BOLD fMRI Data Acquisition and Analysis

Amygdala and the VS reactivity paradigms have been described in detail previously (Carre *et al.*, 2013, Nikolova *et al.*, 2012). Briefly, the amygdala paradigm consists of 4 blocks of a face-processing task interleaved with 5 blocks of a sensorimotor control task. During task blocks, participants view a trio of faces (with neutral, angry, fearful or surprised expressions) and match 1 of 2 faces (bottom) identical to a target face (top). During control blocks, participants match simple geometric shapes. Here, we

focus on the contrast of all task blocks versus control blocks (i.e., Faces > Shapes) as we are interested in amygdala reactivity to threat broadly, which is conveyed to varying degrees by all of our expressions (Costafreda *et al.*, 2008, Sabatinelli *et al.*, 2011).

Our VS reactivity paradigm consists of a number guessing task wherein participants receive predominantly positive feedback (80% correct guess), predominantly negative feedback (20% correct guess), or no feedback. There are three pseudorandomly presented blocks of each condition. Participants are unaware of the fixed outcome probabilities associated with each block and are led to believe their performance will determine a net monetary gain at the end of the scanning session. Instead, all participants receive $10. Here we focus on differential VS reactivity from positive > negative feedback blocks. BOLD fMRI acquisition parameters, preprocessing and analytic techniques are described in detail elsewhere (Nikolova *et al.*, 2012).

3.2.3. Statistical Analysis

Linear regressions using self-report of recent stress from the Life Events Scale for Students Highest Impact score, left and right amygdala reactivity, left and right VS reactivity, and their interactions as independent variables, and self-reported drinking from the Alcohol Use Disorders Identification Test Total scores (square root transformed to normalize distribution) as a dependent variable were conducted within SPSS (SPSS Inc, Chicago, IL). Significant three-way stress x amygdala x VS reactivity interactions where examined using two-way stress x amygdala reactivity interactions at low (-1 SD),

mean, or high (+1 SD) VS reactivity. Significant stress x VS reactivity interactions were in turn probed by examining the linear relationship of stress and drinking at low (-1 SD), mean, and high (+1 SD) VS reactivity.

3.3. *Results*

As expected (Sinha, 2001), there was a significant positive correlation between recent stress and problem drinking ($r = 0.22$, $p = 0.004$). Critically, however, this relationship was moderated by amygdala and VS reactivity. Specifically, a three-way interaction predicting problem drinking emerged between recent stress, left amygdala reactivity, and left VS reactivity ($\Delta R^2 = 0.035$, $b = -0.26$, $p = 0.012$). Among participants with low VS reactivity (1 SD below mean; Figure 1C), stress did not predict any increases in drinking, regardless of amygdala reactivity. Among participants with high VS reactivity (1 SD above mean), who are likely to be at increased risk for drug abuse (Evans *et al.*, 2006), stress predicted increased problem drinking only for those who also had low amygdala reactivity (1 SD below mean; Figure 1D). This three-way interaction remained significant after controlling for gender, age, and race/ethnicity ($\Delta R^2 = 0.031$, $b = -0.25$, $p = 0.012$). There was no such interaction for right VS or amygdala reactivity (p values > 0.10), and no significant main effects of either amygdala or VS reactivity on problem drinking (p values > 0.14).

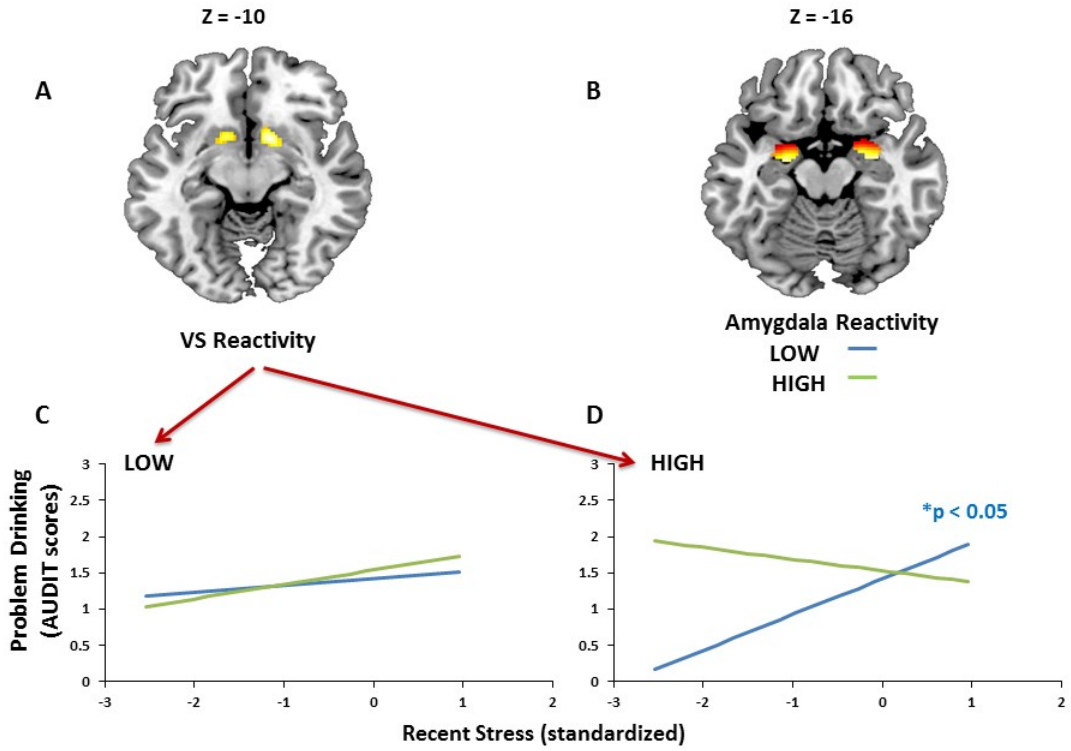

Figure 5. (A) Statistical parametric map illustrating mean bilateral threat-related amygdala reactivity (left: x = -22, y = -6, z = -18, t = 19.76, p < 0.000001, k_E = 173; right: x = 28, y = -4, z = -20, t = 20.16, p < 0.000001, k_E=199). (B) Statistical parametric map illustrating mean bilateral reward-related VS reactivity (left: x = -12, y = 10, z = -10, t = 6.19, p = 3.07 x 10^{-7}, k_E = 357; right: x = 12, y = 10, z = -8, t = 7.31, p = 1.03 x 10^{-9}, k_E = 383). Activation clusters in (A) and (B) are overlaid onto canonical structural brain images in the axial plane. (C) Among participants with low VS reactivity, (1 SD below the mean), recent stress (LESS Highest Impact) was not associated with increased problem drinking (total scores on the AUDIT; square root transformed) regardless of amygdala reactivity. (D) For participants with high (1 SD above the mean) VS reactivity, recent stress predicted significant increases in problem drinking only for those who also had relatively low (1 SD below the mean) amygdala reactivity (blue line). Plotted values are adjusted for sex, age and race/ethnicity.

Demonstrating the specificity of these findings to recent, as opposed to early, life stress, the three-way interaction remained significant when total scores from the Childhood Trauma Questionnaire were added as an additional covariate (left VS: R^2 = 0.033, b = -0.25, p = 0.011). Furthermore, childhood trauma did not interact with amygdala or VS reactivity to predict problem drinking (p values > 0.63). Finally, the same three-way interaction emerged in a subsample of participants (N = 85) who completed a three-month follow-up assessment of stress and problem drinking (without covariates: ΔR^2 = 0.085, b = -0.365, p = 0.008; with covariates: ΔR^2 = 0.063, b = -0.324, p = 0.019). The temporal stability of this interaction suggests that stress-related problem drinking reflects rather than affects the relative neural responsiveness to threat and reward.

3.4. *Discussion*

In the current study, we report novel evidence that threat-related amygdala reactivity and reward-related VS reactivity interact to predict current and future levels of problem drinking, such that higher levels of recent life stress are associated with more alcohol use only in those individuals with relatively high VS reactivity and relatively low amygdala reactivity.

An important caveat to consider when interpreting these findings is the possibility that participants drinking more alcohol may experience more stressful life

events partially as a result of their increased drinking, rather than the other way around. Since our measures of stress and problem drinking are based on retrospective self-report spanning the past 12 months, the directionality of the association between stress and drinking cannot be determined on the basis of these analyses. Thus we cannot rule out the alternative interpretation that individuals with high VS reactivity and low amygdala reactivity are more likely to experience highly impactful stressful life events in the context of problem drinking. This interpretation would be consistent with a heightened drive to pursue immediate rewards, coupled with a reduced ability to recognize and avoid threat in those individuals.

Limitations notwithstanding, we provide novel evidence that recent life stress is associated with increased problem drinking only in individuals with higher reward-related VS reactivity and lower threat-related amygdala reactivity. Consistent with the relative temporal stability of amygdala (Manuck *et al.*, 2007) and VS (Plichta *et al.*, 2012) reactivity, the interactions between these neural phenotypes and recent life stress predicted future problem drinking in a subset of participants. This finding suggests that the pattern we observe spans longer periods of time and may be useful in identifying individuals at particularly high risk for developing alcohol and possibly other substance use disorders in the wake of stress. Future research identifying factors that predict the observed variability in neural responsiveness to threat and reward (e.g., functional

genetic polymorphisms) can inform the development of biomarkers for drug abuse risk and interventions targeting these specific intermediate phenotypes.

4. Multilocus genetic profile for dopamine signaling predicts ventral striatum reactivity[1]

4.1. *Background*

Two rapidly emerging and highly complementary strategies have accelerated progress into biological mechanisms mediating individual differences in behavior and related risk for psychopathology: imaging genetics and gene-environment interactions research. Through the systematic mapping of common genetic polymorphisms affecting brain chemistry onto variability in brain structure and function, imaging genetics has established multiple fundamental mechanisms through which individual differences in behavior emerge and bias responses to the environment (Hariri, 2009). In parallel, gene-environment interactions research has demonstrated how such genetically mediated variability in behaviorally relevant brain function translates into individual risk for psychopathology upon exposure to environmental stress or adversity (Caspi & Moffitt, 2006).

Imaging genetics studies to date, however, have been almost universally limited by their reliance on single genetic loci to model variability in complex brain chemistry and, subsequently, brain function. Recent studies have begun to recognize the importance of considering the simultaneous involvement of multiple genes in the

[1] This Chapter is based on the following publication: Nikolova, Y.S., Ferrell, R.E., Manuck, S.B. & Hariri, A.R. (2011) Multilocus genetic profile for dopamine signaling predicts ventral striatum reactivity. Neuropsychopharmacology, 36, 1940-1947.

regulation of these pathways by taking into account epistatic interactions among polymorphic loci (Buckholtz *et al.*, 2007, Nicodemus *et al.*, 2010, Pezawas *et al.*, 2008). Nonetheless, studies of this kind have typically focused on no more than two genes/polymorphisms at a time and those which have taken more into account have done so within the framework of a data-driven approach (Nicodemus *et al.*, 2010, Potkin *et al.*, 2009, Seshadri *et al.*, 2007). As multiple functional polymorphisms of various effect sizes are likely to shape overall variability in brain function, one strategy for extending and expanding the utility of this research is to establish biologically founded multilocus genetic profiles that represent the cumulative effect of multiple polymorphic loci of known functionality on a specific signaling mechanism (Plomin *et al.*, 2009). Individual polymorphic loci account for a small proportion of phenotypic variance such that their independent effects are unlikely to produce statistically significant effects especially in relatively small samples. The simultaneous consideration of multiple functional loci through a multilocus genetic profile score may allow for the inclusion of otherwise non-significant polymorphisms, which only collectively account for significant proportions of variability. In turn, such genetic profiles may serve as the foundation for gene-environment interactions research that can establish trajectories of risk for psychopathology applicable at the level of the individual.

In the present study, we sought to establish the utility of multilocus genetic profiles representing the cumulative biological impact of multiple functional

polymorphic loci in mapping individual differences in brain function. The simultaneous consideration of multiple polymorphisms has already been successfully used to explain variability in antidepressant treatment response (Ising *et al.*, 2009) and to model individual differences in sensation seeking (Derringer *et al.*, 2010) and basal ganglia response to reward (Dillon *et al.*, 2010). However, no study to date has created a biologically informed multilocus genetic profile representing variability in neurotransmitter signaling across multiple genes that can be used to explain individual differences in behaviorally relevant brain function. The neural target of our study was variability in the responsiveness of the ventral striatum (VS), a central node of a distributed corticostriatal circuitry supporting reward-related and appetitive behaviors (Gan *et al.*, 2010, Tanaka *et al.*, 2004), which is also implicated in the pathophysiology of mood, impulse and substance use disorders (Buckholtz *et al.*, 2010a, Buckholtz *et al.*, 2010b, Dalley *et al.*, 2007). The genetic target of our study was dopamine (DA), which plays a key role in modulating the responsiveness of the VS (Sesack & Grace, 2010). We hypothesized that multilocus genetic profile scores representing relatively increased DA signaling, would significantly predict increased VS reactivity, and that the variance in reactivity explained by the profile scores would be significantly greater than that associated with any single locus.

All five loci included in the genetic profile were carefully selected based on their prior links with functional changes in DA transmission and/or VS reactivity. The DAT1

9-repeat allele of a 40 base pair (bp) variable number tandem repeat (VNTR) within the 3′ untranslated region (3′ UTR) of the dopamine transporter gene (*SLC6A3*) has been linked to reduced DA reuptake and increased striatal DA signaling (Heinz *et al.*, 2000, Vanness *et al.*, 2005). Similarly, the Deletion allele of an Insertion/Deletion polymorphism (*DRD2* -141C Ins/Del; rs1799732) within the promoter region of the DA receptor D2 gene (*DRD2*) has been associated with reduced expression of DRD2 (Arinami *et al.*, 1997), and has been implicated in increased VS reactivity (Forbes *et al.*, 2009a). We also considered the *DRD2* Taq1A polymorphism, a C/T SNP (rs1800497) located in the ankyrin repeat and kinase-domain containing 1 (*ANKK1*) gene. Relative to the T (A1) allele, the C (A2) allele has been associated with increased DA signaling (Noble *et al.*, 1991), increased striatal glucose metabolism (Noble *et al.*, 1997) and reactivity to reward (Stice *et al.*, 2008). The fourth polymorphism we considered was a 48 bp VNTR within the DA receptor D4 gene (*DRD4*). The 7-repeat allele of this VNTR has been previously linked to reduced DRD4-mediated postsynaptic inhibition and hence increased DA signaling (Wang *et al.*, 2004) as well as increased VS reactivity (Forbes *et al.*, 2009a). Finally, our genetic profile score incorporated a functional SNP (rs4680) within the third exon of the catechol-O-methyltransferase gene (*COMT*), which results in nonsynonymous Val/Met substitution (*COMT* $Val^{158}Met$). The Met allele has been associated with decreased enzymatic degradation of dopamine (Egan *et al.*, 2001) and increased VS reactivity (Dreher *et al.*, 2009, Yacubian *et al.*, 2007).

4.2. *Methods*

4.2.1. Participants

A total of 103 subjects were recruited from a parent study, the Adult Health and Behavior (AHAB) project, which assessed a wide range of behavioral and biological traits among nonpatient, middle-aged, community volunteers. All participants provided informed consent in accord with local guidelines, and were in good general health. The participants were free of the following study exclusions: (1) medical diagnoses of cancer, stroke, diabetes requiring insulin treatment, chronic kidney or liver disease, or lifetime history of psychotic symptoms; (2) use of psychotropic, glucocorticoid, or hypolipidemic medication; (3) conditions affecting cerebral blood flow and metabolism (e.g., hypertension); and (4) diagnosis of any current *DSM-IV* Axis I disorder (First, 1996). Given the general confounds of population stratification, we limited our analyses to sixty-nine Caucasian subjects (37 women; mean age 44.46 ± 6.66 years) with overlapping reward-related VS data and genotypes at all five loci of interest.

4.2.2. Genetic Profile Scores

We compiled individual genetic profile scores reflecting the total number of variants that have each been previously associated with relatively increased striatal DA signaling and/or VS reactivity across five functional polymorphic loci: *SLC6A3* 3′ 40bp VNTR (DAT1), *DRD2* -141C Ins/Del (rs1799732), *DRD2* Taq1A (rs1800497), *DRD4* exon 3 48bp VNTR and *COMT* Val^{158}Met (rs4680) (for **Genotyping**, see Supplementary

Methods). Across all loci, relatively "High" DA genotypes were assigned a score of 1, "Low" DA genotypes a score of 0, and "Intermediate" DA genotypes a score of 0.5. These scores at each locus were then totaled to create an individual profile score (Table 6).

Table 6. Composition and distribution of multilocus genetic profile scores. Individual genetic profile scores represent the sum of "High" DA genotypes across five functional polymorphic loci. "High" genotypes received a score of 1, "Low" genotypes a score of 0, and "Intermediate" genotypes a score of 0.5. For example, the genetic profile score for an individual with the following five polymorphisms – DAT1 9-repeat carrier, *DRD4* 7-repeat carrier, *DRD2* Taq1A T homozygote, *DRD2* -141C Del carrier & *COMT* heterozygote – is 3.5 (1 + 1 + 0 + 1 + 0.5).

Polymorphism	Genotypes	N	DA Profile Score
DAT1 VNTR	9-repeat carrier	35	High
	10/10	34	Low
DRD4 VNTR	7-repeat carrier	42	High
	All others	27	Low
DRD2 Taq1A	C/C	43	High
	C/T	23	Intermediate
	T/T	3	Low
DRD2 -141C Ins/Del	Del carrier	14	High
	Ins/Ins	55	Low
COMT $Val^{158}Met$	Met/Met	12	High
	Val/Met	41	Intermediate
	Val/Val	16	Low

Consistent with previous research suggesting a dominant role for the 9-repeat allele (Heinz *et al.*, 2000, Van De Giessen *et al.*, 2009, Vanness *et al.*, 2005), DAT1 9-repeat allele carriers were coded as having a "High" DA genotype, while 10-repeat allele

homozygotes were coded as having a "Low" DA genotype. Drawing on prior reports, we also established two genotype groups for the *DRD2* -141C locus: -141C deletion carriers (Ins/Del) and non-carriers (Ins/Ins), and designated -141C Del carriers as the "High" and non-carriers as "Low" DA genotypes. Since prior studies of *DRD2* Taq1A have used either T (A1) or C (A2) allele homozygotes as a reference group (Bakker *et al.*, 2008, Jonsson *et al.*, 1999, Kwon *et al.*, 2008, Pohjalainen *et al.*, 1998), and other research suggests additive effects for the number of *DRD2* Taq1A alleles on relative change in DRD2 expression levels (Noble *et al.*, 1991), we modeled allele load effects of the *DRD2* Taq1A on overall DA transmission with C allele homozygotes designated as the "High" DA genotype, T allele homozygotes as the "Low" DA genotype and heterozygotes as "Intermediate" DA genotype. The *DRD4* 7-repeat allele carriers were considered "High", while other allele combinations were considered "Low" DA genotypes. Finally, consistent with additive effects of the Met allele of COMT Val^{158}Met (Weinshilboum *et al.*, 1999), we established three genotype groups in relation to this locus: Val homozygotes, Val/Met heterozygotes and Met homozygotes. For the purposes of the DA profile scores, Met allele homozygotes were considered "High", Val allele homozygotes "Low", and heterozygotes "Intermediate" genotypes.

4.2.3. Ventral Striatum (VS) Reactivity Paradigm

As described previously (Forbes *et al.*, 2009a, Gianaros *et al.*, 2010, Hariri *et al.*, 2006, Hariri *et al.*, 2009) our blocked-design paradigm consisted of pseudorandom

presentation of trials wherein participants played a card guessing game and received positive or negative feedback (i.e., correct or incorrect guess) for each trial. Our task was selected primarily with the aim of robustly engaging the VS, so that individual differences in VS responsiveness could be recorded and mapped onto genetic background. Participants were told that their performance on the card game would determine a monetary reward to be received at the end of the game. During each trial, participants had 3 seconds to guess, via button press, whether the value of a visually presented card was higher or lower than 5 (index and middle finger, respectively). After a choice was made, the numerical value of the card was presented for 500 milliseconds and followed by appropriate feedback (green upward-facing arrow for positive feedback; red downward-facing arrow for negative feedback) for an additional 500 milliseconds. A crosshair was then presented for 3 seconds, for a total trial length of 7 seconds. Each block was comprised of 5 trials, with 3 blocks each of predominantly positive feedback (80% correct) and 3 of predominantly negative feedback (20% correct) interleaved with 3 control blocks. During control blocks, participants were instructed to simply make alternating button presses during the presentation of an "x" (3 seconds) which was followed by an asterisk (500 milliseconds) and a yellow circle (500 milliseconds). Each block was preceded by an instruction of "Guess Number" (positive or negative feedback blocks) or "Press Button" (control blocks) for 2 seconds resulting in a total block length of 38 seconds and a total task length of 342 seconds. Participants

were unaware of the fixed outcome probabilities associated with each block and were led to believe that their performance would determine a net monetary gain at the end of the scanning session. Instead, all participants received $10. We included one incongruent trial within each task block (e.g., 1 of 4 trials during positive feedback blocks was incorrect, resulting in negative feedback) to prevent participants from anticipating the feedback for each trial and to maintain participants' engagement and motivation to perform well.

4.2.4. BOLD fMRI Data Acquisition and Analysis

Each participant was scanned using a Siemens 3T Allegra scanner (Siemens AG, Medical Solutions, Erlangen, Germany) developed specifically for advanced brain imaging applications and characterized by increased T2* sensitivity and fast gradients that minimize echo spacing, thereby reducing echo-planar imaging (EPI) geometric distortions and improving image quality. BOLD functional images were acquired with a gradient-echo echo planar imaging sequence (repetition time [TR]/echo time [TE] = 2000/25 milliseconds, field of view [FOV] = 20 cm, matrix = 64 × 64) that covered 34 interleaved axial slices (3 mm slice thickness) aligned with the anterior commissure-posterior commissure (AC-PC) plane and encompassing the entire cerebrum and the majority of the cerebellum. All scanning parameters were selected to optimize the quality of the BOLD signal while maintaining a sufficient number of slices to acquire whole-brain data. Before the collection of fMRI data for each participant, we acquired a

reference EPI scan that we visually inspected for artifacts (e.g., ghosting) and good signal across the entire volume of acquisition, including the VS. The fMRI data from all participants included in this study were cleared of such problems. Additionally, an autoshimming procedure was conducted before the acquisition of BOLD data in each subject to minimize field inhomogeneities.

Whole-brain image analysis was completed using SPM2 (http://www.fil.ion.ucl.ac.uk/spm). Images for each participant were realigned to the first volume in the time series to correct for head motion. Data sets were then selected for their high quality (scan stability) as demonstrated by small (≤ 2mm and 2°) motion correction. Based on this criterion, data from all 69 participants were included in subsequent analyses. Realigned images were spatially normalized into a standard stereotactic space (Montreal Neurological Institute template) using a 12-parameter affine model. These normalized images were then smoothed to minimize noise and residual differences in gyral anatomy with a Gaussian filter, set at 6mm full-width at half-maximum. Voxel-wise signal intensities were ratio normalized to the whole-brain global mean. Following preprocessing, linear contrasts employing canonical hemodynamic response functions were used to estimate differential effects of feedback (i.e., reward) from the contrast of Positive Feedback > Negative Feedback for each individual. Individual contrast images were then used in second-level random effects models accounting for scan-to-scan and participant-to-participant variability to

determine mean condition-specific regional responses using one-sample t-tests thresholded at $p < 0.05$, FWE-corrected, and ≥10 contiguous voxels. Our VS region of interest was constructed using the Talairach Daemon option of the WFU PickAtlas Tool, version 1.04 (Wake Forest University School of Medicine, Winston-Salem, North Carolina). Two spheres of 10mm radius were created around MNI coordinates x = ±12, y = 12 & z = -10 to encompass the VS in the right and left hemisphere, respectively.

4.2.5. Genotyping

High molecular weight DNA was isolated from EDTA anticoagulated whole-blood samples obtained from all participants using the Puregene kit (Gentra Systems, Minneapolis, MN, USA). Each sample was genotyped using allele-specific primers and polymerase chain reaction conditions from published protocols: *SLC6A3* DAT1 VNTR (Vandenbergh *et al*, 1992); *DRD4* third exon 48 bp VNTR (Lichter *et al*, 1993), *DRD2* -141C Ins/Del (Gelernter *et al*, 1998) and *COMT* Val^{158}Met (Lachman *et al*, 1996). All genotypes were scored by two independent readers by comparison to sequence-verified standards. The *DRD2* Taq1A was genotyped on an Illumina 610-Quad BeadChip (Illimina Inc, San Diego, CA) platform. Individual genotypes for *DRD2* Taq1A were extracted from the master database using the "list" command in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/). Genotype frequencies at all five loci were in Hardy-Weinberg Equilibrium (all $\chi^2 < 2.56$, all P's > 0.10).

4.2.6. Variance Analyses

To compute the relative variance explained by the cumulative genetic profile score and each individual genotype, parameter estimates from VS clusters exhibiting a main effect of task were extracted using the VOI tool in SPM2 and entered into linear and stepwise regression models in SPSS (PASW Statistics 18; SPSS Inc., Chicago, Il). Importantly, by extracting VS reactivity values from the entire functional clusters activated by our fMRI paradigm rather than clusters specifically correlated with our independent variables of interest, we precluded the possibility of any regression coefficient inflation that may result from capitalizing on the same data twice (Viviani, 2010). We have successfully used this more conservative analytic strategy in recent studies (Carre *et al.*, 2010, Hyde *et al.*, 2010). Consistent with the standards of genetic association studies (Dahlman *et al.*, 2002) we applied a Bonferroni-like adjustment to our significance level to reflect the total number of regressions conducted (12 regressions total: 5 individual loci and 1 profile score conducted independently for the left and right VS) resulting in a threshold of $p \leq 0.004$ (i.e., 0.05/12). Cook's distance values were computed for all observations and all regression models, and no single data points were identified that biased the overall models (i.e., Cook's distance < 0.195 for all data points) (Cook, 1982).

Based on evidence for significant gender differences in reward-related VS reactivity in prior studies (Spreckelmeyer *et al.*, 2009) and in our current sample [males >

females; $t(67) = 2.55$, $p = 0.013$] as well as an observed gender association with the profile scores [males > females; $t(67) = 2.12$, $p = 0.038$], all our analyses were conducted with and without gender as a covariate. Thus, when gender was controlled for, the amount of variance explained by the genetic profile scores or individual loci was computed as the change in R^2 resulting from the addition of the genetic variables to a hierarchical regression model already containing gender as a predictor of VS reactivity.

4.3. *Results*

In the current sample, there was a significant main effect of task (i.e., Positive Feedback > Negative Feedback) in a large VS cluster in the right hemisphere (x = 14, y = 12, z = -8, $T = 5.13$, $K_E = 118$, $p = 0.00028$; Figure 6). There was also a main effect of task in a smaller cluster within the left VS (x = -16, y = 6, z = -8, $T = 5.64$, $K_E = 96$, $p = 0.000043$).

Consistent with our hypothesis, individual multilocus genetic profile scores for relatively increased DA signaling predicted higher reward-related reactivity in the right VS at our corrected threshold ($\hat{\beta} = 0.342$, $p = 0.0038$, Figure 6). Moreover, the profile scores accounted for 10.9% of all variability within this VS cluster ($\Delta R^2 = 0.109$) above and beyond the effects of gender, which accounted for 4.2% of all residual cluster-level variability ($\hat{\beta} = -0.212$; $\Delta R^2 = 0.042$, $p = 0.067$). When not explicitly controlling for gender, the dopamine profile scores predicted even greater variability in reward-related VS reactivity: 14.3% ($\hat{\beta} = 0.395$; Adj. $R^2 = 0.143$, $p = 0.001$). In contrast, none of the individual

loci predicted significant variability in reward-related VS reactivity with only one (*DRD2* -141C Ins/Del) having a marginally significant effect at an uncorrected threshold of $p \leq 0.05$ (Table 7). Controlling for gender did not affect any of the associations between VS reactivity and individual loci.

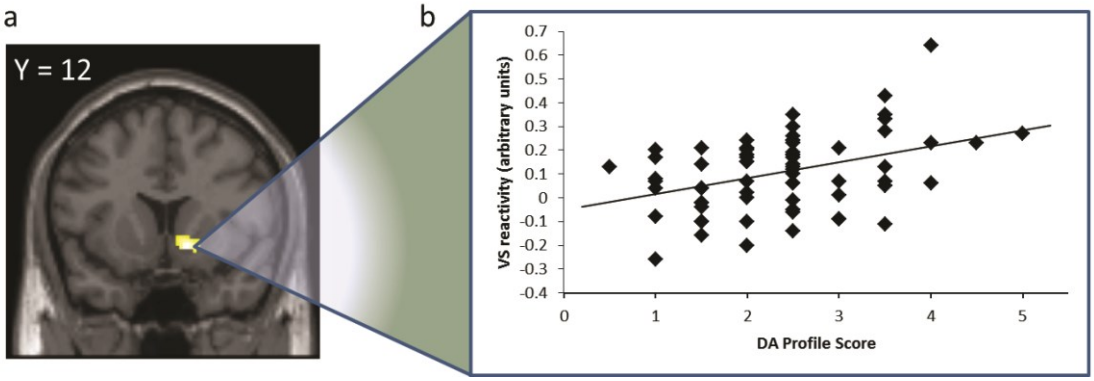

Figure 6. Multilocus genetic profile scores for DA signaling predict reward-related VS reactivity. (a) Our fMRI task produced significant activation in a large right VS cluster (x = 14, y = 12, z = -8, T = 5.13, K_E = 118, p = 0.00028). (b) Individual profile scores accounted for 10.9% of the variability within the VS activation cluster above and beyond the effects of gender (ΔR^2 = 0.109).

Table 7. Effects of individual DA loci on reward-related VS reactivity. Critically, and as noted in the main text, none of these individual loci accounted for significant variability in VS reactivity when appropriately controlling for multiple comparisons (i.e., $p \leq 0.004$).

Genotype	ΔR^2	β	p
DRD2 -141C Ins/Del	0.052	0.241	0.050
DAT1 VNTR	0.037	0.192	0.100
DRD4 VNTR	0.015	0.123	0.295
COMT Val^{158}Met	0.015	0.123	0.298
DRD2 Taq1A	0.002	0.045	0.713

Intriguingly, even though none of the individual loci with the exception of *DRD2* -141C Ins/Del accounted for significant variability in VS reactivity at an uncorrected threshold, the genetic profile scores still accounted for 6.3% of VS reactivity ($\hat{\beta}$ = 0.298; ΔR^2 = 0.063, p = 0.026) above and beyond the effects of gender and *DRD2* -141C Ins/Del. When gender was not used as a covariate, cumulative DA profile score accounted for 7.3% ($\hat{\beta}$ = 0.319; ΔR^2 = 0.073, p = 0.019) of the variance above and beyond that accounted for by *DRD2* -141C Ins/Del. The above results demonstrate the utility of multilocus genetic profiles in capturing the cumulative impact of polymorphisms, whose individual effects may be overlooked even at uncorrected statistical thresholds. Importantly, the

simultaneous consideration of all predictors in the above regression models did not pose significant multicollinearity concerns (*Tolerance* > 0.680, *Variance Inflation Factor* < 1.469).

In contrast to the patterns observed in the right hemisphere, reward-related reactivity in the left VS was not significantly associated with the profile scores or any individual polymorphism at either the corrected or uncorrected statistical thresholds. Controlling for gender did not affect any of these associations.

4.4. *Discussion*

In the current study, we demonstrate that a multilocus genetic profile score representing the cumulative impact of five functional polymorphic loci on DA signaling predicts 10.9% of the inter-individual variability in reward-related VS reactivity. In contrast, none of the individual loci predict significant variability in VS reactivity. Thus, we provide novel evidence for the utility of biologically founded multilocus genetic profiles in mapping individual differences in brain function by demonstrating that simultaneous consideration of multiple functional loci accounts for greater variability than single loci considered independently. This finding demonstrates that, given sufficient *a priori* rationale for the consideration of specific functional polymorphic loci, a multilocus profiling approach might capture the cumulative impact of polymorphisms whose individual effects may otherwise go undetected in small samples. That the genetic profile scores in the current sample accounted for a significant proportion of variability in a relatively small sample further underscores the potential for this novel

biological profiling approach to accurately predict patterns of brain function at the individual level.

Although we selected the polymorphisms investigated herein based on their prior association with DA signaling and/or VS reactivity, the precise molecular mechanisms through which each locus contributes to variability in reward-related brain function are still incompletely understood. While the DAT1 9-repeat allele and the *COMT* ^{158}Met allele are linked to reduced DA synaptic clearance (Heinz *et al.*, 2000) and enzymatic degradation (Egan *et al.*, 2001), respectively, less is known about the direct effects of the *DRD2* and *DRD4* polymorphisms on DA neurotransmission and subsequent VS reactivity. The *DRD4* 7-repeat allele has been linked to reduced postsynaptic inhibition mediated by a decreased number of D4 receptors (Asghari *et al.*, 1995). Relatedly, the *DRD2* -141C Del allele has been associated with reductions in the expression of the D2 receptor, which typically acts to inhibit DA signaling pre- or post-synaptically (Arinami *et al.*, 1997). Intriguingly, while prior research has linked the *DRD2* Taq1A T (A1) allele to similarly reduced D2 receptor binding (Jonsson *et al.*, 1999, Pohjalainen *et al.*, 1998), studies investigating the effect of the polymorphism on regional blood flow and glucose metabolism have reported decreased striatal reactivity in T (A1) allele homozygotes relative to C (A2) allele carriers (Noble *et al.*, 1997, Stice *et al.*, 2008). Since our VS reactivity phenotype is more closely related to the neuroimaging measures employed in the latter studies, we chose to code the C (A2) allele as the relatively "high"

DA allele. It is important to note, however, that the decreased D2 receptor density and the reduced glucose metabolism associated with the T allele need not be mutually exclusive. Given the diverse distribution of D2 receptors on multiple neuronal subtypes (Beaulieu & Gainetdinov, 2011), it is conceivable that reductions in D2 receptors associated with the T allele may be specific to a subpopulation of D2 heteroreceptors located on GABAergic interneurons, which modulate striatal function through inhibition of glutamatergic medium spiny neurons. Thus, the T allele may result in reduced DA-mediated inhibition of GABAergic interneurons leading to greater inhibition of excitatory medium spiny neurons and, ultimately, reduced VS reactivity measured with fMRI. Given the limitations of currently available neuroimaging methodologies, future studies incorporating non-human animal models will be required to determine the effects of each polymorphism on the cellular and systems levels with greater precision.

We previously reported that the 9-repeat allele of DAT1, the 7-repeat allele of *DRD4* and the deletion allele of *DRD2* -141C Ins/Del are all significantly associated with relatively increased VS reactivity in a sample that partially overlaps with our current study (Forbes *et al.*, 2009a). However, the previous sample was racially heterogeneous, as it included approximately 10% non-Caucasians distributed equally across all genotype groups. More importantly, in our prior report we used a less conservative approach whereby we only quantified VS reactivity as a function of genotype within functional clusters selected on the basis of their correlation with each polymorphic locus,

rather than the entire functional cluster activated by our fMRI paradigm. Moreover, we did not apply statistical thresholds that properly accounted for multiple comparisons reflecting the number of individual genotypes tested. Importantly, we replicated the associations between VS reactivity and all three loci (i.e., DAT1, *DRD2* -141C Ins/Del, *DRD4* VNTR) when applying more liberal statistical thresholds consistent with our prior report (Table 8).

Table 8. Effects of individual DA loci on reward-related VS reactivity at $p < 0.05$, uncorrected, cluster threshold of ≥ 1 voxel. Critically and as noted in the main text, this approach is much less conservative than the one employed in the main analyses. Importantly, none of these individual loci accounted for significant variability in VS reactivity when appropriately controlling for multiple comparisons within SPM (i.e., $p \leq 0.004$; cluster extent = 10 voxels). All cluster maximal voxel coordinates reported in MNI space. KE = cluster extent.

Genotype	x	y	z	K_E	T
DAT1 VNTR	16	4	-10	36	3.55
	12	18	-10	24	2.47
	-18	12	-4	18	2.43
***DRD4* VNTR**	16	4	-6	27	3.45
	22	12	-10	10	2.61
	-12	20	-10	7	1.95
***DRD2* Taq1A**	4	14	-8	8	2.84
	18	18	-12	4	2.15
	18	10	-12	2	1.68
	-18	16	-12	2	1.97
	-2	16	-8	2	1.82
	-14	20	-10	1	1.73
***DRD2* -141C Ins/Del**	8	8	-2	73	2.93
	-14	16	-2	28	2.28
	16	14	-6	18	2.11
***COMT* Val^{158}Met**	-10	22	-10	1	2.58
	-14	4	-6	1	1.8

In addition to the three polymorphisms investigated in our prior report, the cumulative genetic profile scores used in the current analysis also incorporated *DRD2* Taq1A and *COMT* Val^{158}Met. Previously, we did not find a main effect of *COMT* Val^{158}Met on reward-related VS reactivity (Forbes *et al.*, 2009a). However, other imaging genetics studies have reported significant associations between the ^{158}Met allele and increased VS reactivity (Dreher *et al.*, 2009, Schmack *et al.*, 2008, Yacubian *et al.*, 2007). Thus, while COMT Val^{158}Met did not by itself predict significant variability in VS reactivity in either our prior (Forbes *et al.*, 2009a) or current analysis, it did significantly contribute to the predicted utility of the cumulative profile scores. Removal of the *COMT* Val^{158}Met genotype from the profile score resulted in non-significant effects using our corrected threshold.

A possible limitation to our biological profiling approach is the assumption that the selected polymorphisms act additively, as opposed to interactively, to influence DA signaling. Importantly, however, our prior investigation of three polymorphisms considered herein (i.e., DAT1, *DRD4* VNTR, and *DRD2* -141C Ins/Del) did not find any two- or three-way interactions among these polymorphisms in predicting VS reactivity (Forbes *et al.*, 2009a). Unlike Yacubian *et al* (2007) and Dreher *et al* (2009), we also did not find a *COMT* Val^{158}Met-by-DAT1 interaction in the current sample (p = 0.757). By assigning a score of "1" to "High" DA alleles at all loci, we also assumed all loci had an equal impact on VS reactivity. We believe a more sophisticated approach to compiling

genetic profiles is warranted whereby potential multiplicative relationships are taken into account and polymorphisms are weighted according to predicted effect size. However, given the relatively small sample size (N = 69) and insufficient knowledge regarding potential interactions among the targeted polymorphisms and the relative magnitude of their impact on VS reactivity (but see Yacubian *et al*, 2007 and Dreher *et al*, 2009), we regard the current investigation as a useful starting point for compiling informative multilocus genetic profile scores. Future studies replicating the current findings would lend additional credibility to this approach.

The DA profile we compiled in this study accounted for significant variability in reward-related reactivity of the right but not the left VS. Such asymmetrical findings are not uncommon in imaging genetics research in general (Hariri *et al.*, 2002b, Meyer-Lindenberg *et al.*, 2006) or studies focusing on the VS specifically (Jocham *et al.*, 2009, Yacubian *et al.*, 2007). Although a number of studies have reported asymmetries in monoaminergic modulation of cortical and subcortical circuits (Besson & Louilot, 1995, Merali *et al.*, 2004, Sullivan & Dufresne, 2006, Young & Williams, 2010), the biological mechanisms mediating such lateralized effects, particularly in the VS, are difficult to ascertain on the basis of the existing literature. It is possible that our specific task differentially recruits the right VS and, subsequently, results in greater DA modulation of reactivity in this hemisphere, which is reflected in the right-lateralized significant associations. Consistent with this suggestion, we have previously found right-

hemisphere specific correlations between reward-related VS reactivity and variability in behavioral measures of impulsivity (Forbes *et al.*, 2009a, Hariri *et al.*, 2006). Future research incorporating this genetic profile within a multimodal neuroimaging strategy (Fisher *et al.*, 2009, Fisher *et al.*, 2006, Kienast *et al.*, 2008) whereby fMRI is used to measure reward-related VS reactivity and PET is used to measure DA release within the same sample could shed light on the nature of these functional asymmetries.

While this proof-of-principle study focused on a single neural target and modeled the additive effects of multiple functional loci through a single genetic profile, future research can refine genetic profile scores by assigning differential weights to loci of potentially different effect sizes and consider functional interactions among loci within a profile as well as between profiles for different pathways. The extension of genetic profiling in this manner, particularly in larger samples, offers the opportunity to generate increasingly complete information regarding variability in behaviorally relevant brain function and related gene-environment interactions.

5. Beyond genotype: Epigenetic regulation of the human serotonin transporter predicts behaviorally and clinically relevant brain function[1]

5.1. *Background*

The systematic integration of human molecular genetics and *in vivo* neuroimaging have contributed to our increasing understanding of how DNA sequence-based genetic variation shapes individual differences in brain function, complex behavioral traits, and related risk for psychopathology (Hariri, 2009). Parallel research in animal models has highlighted a critical role for non-sequence-based epigenetic variation in the emergence of individual differences in brain function and risk-related behavior (Meaney & Szyf, 2005). The importance of similar epigenetic mechanisms for behaviorally and clinically relevant brain function in humans has yet to be fully explored.

We used bisulfite sequencing to determine percent methylation of the proximal promoter region of *SLC6A4* in saliva-derived DNA from a Discovery cohort of 80 young adults and blood-derived DNA from an independent Replication cohort of 96 adolescents. We targeted *SLC6A4* because it encodes the serotonin transporter, which

[1] This Chapter is based on the following manuscript currently under review at Nature Neuroscience: Nikolova Y.S., Koenen K.C., Galea S., Wang C.M., Seney M.L., Sibille E., Williamson D.E., Hariri A.R. Beyond genotype: Epigenetic regulation of the human serotonin transporter predicts behaviorally and clinically relevant brain function.

plays an important role in modulating brain function and behavior by regulating the duration and intensity of synaptic serotonin signaling. Dysfunction of the serotonin transporter is also implicated in the pathophysiology of mood and anxiety disorders (Caspi *et al.*, 2010), and pharmacologic blockade of this molecule is the primary mode of treating these same disorders.

We focused our analyses on the 20 CpG sites closest to the transcription start site (TSS) of *SLC6A4* exhibiting substantial variability across individuals (see Table 11 and Table 12). Additional proximal promoter sites were excluded due to virtually no variability across individuals (Table 11). In light of recent work suggesting methylation immediately downstream of the TSS may also impact transcription (Brenet *et al.*, 2011), we sampled additional CpG sites up to 119 bp downstream of the TSS, spanning exon 1 and intron 1 (Table 11), whose effects were investigated in separate control analyses.

We evaluated the relationship between *SLC6A4* proximal promoter methylation and amygdala reactivity to emotional facial expressions conveying threat assayed using blood oxygen level dependent (BOLD) functional magnetic resonance imaging (fMRI). We selected this neural phenotype as a measure of behaviorally and clinically relevant brain function because it is clearly involved in the emergence of both normal and pathologic emotional behaviors (Abercrombie *et al.*, 1998, Fakra *et al.*, 2009). Importantly, these behaviors include responsiveness to environmental and social stress, which is associated not only with epigenetic modification (Mehta *et al.*, 2013) but also

variability in serotonin signaling (Chaouloff, 2000). Moreover, there is now ample evidence linking variability in serotonin signaling with individual differences in amygdala reactivity (Fakra *et al.*, 2009, Fisher *et al.*, 2006, Hariri *et al.*, 2002b).

5.2. *Methods*

5.2.1. Participants

5.2.1.1. Discovery cohort

The first 91 Caucasian participants (47 women; mean age 19.66±1.36) to complete the ongoing Duke Neurogenetics Study (DNS) were selected for inclusion in analyses involving methylation assays. The DNS assesses a range of behavioral and biological traits among young adult, student volunteers. All participants provided informed consent in accord with Duke University guidelines, and were in good general health. Two participants' samples displayed wrong sequence patterns for our *SLC6A4* promoter assays due to unknown mutations or equipment dispensation error and were thus excluded for this analysis. Nine additional participants were excluded due to task non-compliance or response box failure leaving a final sample of 80 individuals (42 women; mean age 19.74 ± 1.33).

All participants were free of the following study exclusions: (1) medical diagnoses of cancer, stroke, diabetes requiring insulin treatment, chronic kidney or liver disease, or lifetime history of psychotic symptoms; (2) use of psychotropic, glucocorticoid, or hypolipidemic medication; and (3) conditions affecting cerebral blood

flow and metabolism (e.g., hypertension). Diagnosis of any current *DSM-IV* Axis I disorder or select Axis II disorders (Antisocial Personality Disorder and Borderline Personality Disorder), assessed with the electronic Mini International Neuropsychiatric Interview (Sheehan *et al.*, 1998) and Structured Clinical Interview for the *DSM-IV* (SCID) subtests (First, 1996), respectively, were not an exclusion as the DNS seeks to establish broad variability in multiple behavioral phenotypes related to psychopathology. However, all participants were medication-free at the time of the study. No participants met criteria for either Antisocial or Borderline Personality Disorder, and 16 participants from our final sample (N = 80) met criteria for at least one Axis I disorder. Since the exclusion of these individuals did not substantially alter our results, we present data from the entire sample in the main text (see Table 9 for specific diagnoses). In addition, all analyses were conducted both with and without current diagnosis as a covariate (dummy coded: 0 = no psychopathology, 1 = meeting criteria for one or more psychiatric disorders).

Table 9. Number of participants meeting criteria for DSM-IV Axis I diagnoses in the Discovery cohort (DNS).

Disorder	Number
Social Anxiety Disorder	1
Alcohol Dependence	7
Alcohol Abuse	2
Cocaine Abuse	0
Cannabis Abuse	1
Generalized Anxiety Disorder	0
Multiple Psychopathologies	5
Total	**16**

5.2.1.2. Replication cohort

Our Replication cohort was drawn from among children and adolescents (N = 323, 11-15 years old) participating in the Teen Alcohol Outcomes Study (TAOS) at the University of Texas Health Science Center at San Antonio (UTHSCSA). This ongoing longitudinal study aims to investigate how individual differences in genetic background, environmental experience, and neural function contribute to the emergence of psychopathology, with an emphasis on alcohol use disorders. The current analysis focused on 96 participants (48 girls, mean age 13.62 ± 0.99) who were of Caucasian origin and who had high quality fMRI data in the first wave of neuroimaging. The study was approved by the institutional review board at UTHSCSA. Consent for study participation was obtained from participants' parents or guardians. Under age participants provided assent.

5.2.1.3. Postmortem cohort

The demographic and clinical parameters of the cohort and technical parameters of the samples have been described in detail previously (Sibille *et al.*, 2009). All procedures involving this cohort were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research. Consent was obtained from each subject's next of kin. For all subjects, consensus DSM-IV diagnoses of MDD were made by an independent

committee of experienced clinical research scientists at a case conference utilizing information obtained from clinical records, toxicology exam and a standardized psychological autopsy (Glantz & Lewis, 1997). The latter incorporates a structured interview, conducted by a licensed clinical psychologist with family members of the index subject, to assess diagnosis, psychopathology, medical, social and family histories, as well as history of substance abuse. All subjects died suddenly without prolonged agonal periods. For consistency across samples and to minimize population stratification and pharmacologic confounds, we focused our analysis on Caucasian individuals and excluded those with ascertained medication use at time of death (ascertained by toxicology screen on peripheral tissue), leaving a final sample of 35 (10 women, mean age 49.57 ± 11.87, range 22-69). Brains were analyzed for adequate pH (> 6.0) and RNA integrity by optical density (RNA ratio; OD ≥ 1.3) and Agilent bioanalyzer analysis (Agilent Technologies, Palo Alto, CA; RIN expert scoring system ≥ 7) as described (Sibille *et al.*, 2009). Gender, age, MDD vs. control status, pH, RNA ratio and post mortem interval were controlled for in all analyses involving this sample. A square root transformation was applied to *SLC6A4* mRNA levels to normalize its positively skewed and kurtotic distribution (pre-transformation skewness = 1.92, kurtosis = 6.45, post-transformation skewness = 0.65, kurtosis = 1.43).

5.2.2. BOLD fMRI Data Acquisition and Analysis

As described previously (Carre *et al.*, 2013), the amygdala reactivity paradigm used in the Discovery cohort (DNS) consists of 4 blocks of a face-processing task interleaved with 5 blocks of a sensorimotor control task. During task blocks, participants view a trio of faces (with neutral, angry, fearful or surprised expressions) and match 1 of 2 faces (bottom) identical to a target face (top). During control blocks, participants match simple geometric shapes. In the Replication cohort (TAOS), the task consisted only of Angry and Fearful Faces. Thus, for consistency between samples, we focused our analyses on the Anger + Fear > Shapes contrast in our Discovery cohort. Performance was monitored and participants with accuracy <75% were excluded from analysis.

Participants in the Discovery cohort were scanned using a research-dedicated GE MR750 3T scanner equipped with high-power high-duty-cycle 50-mT/m gradients at 200 T/m/s slew rate, and an eight-channel head coil for parallel imaging at high bandwidth up to 1MHz at the Duke-UNC Brain Imaging and Analysis Center. A semi-automated high-order shimming program was used to ensure global field homogeneity. A series of 34 interleaved axial functional slices aligned with the anterior commissure-posterior commissure (AC-PC) plane were acquired for full-brain coverage using an inverse-spiral pulse sequence to reduce susceptibility artifact (TR/TE/flip angle = 2000ms/30ms/60; FOV = 240 mm; 3.75 × 3.75 × 4 mm voxels; interslice skip = 0). Four initial RF excitations

were performed (and discarded) to achieve steady-state equilibrium. To allow for spatial registration of each participant's data to a standard coordinate system, high-resolution three-dimensional structural images were acquired in 34 axial slices co-planar with the functional scans (TR/TE/flip angle = 7700ms/3.0 ms/12; voxel size = 0.9 × 0.9 × 4 mm; FOV = 240 mm, interslice skip = 0).

Participants in the Replication cohort were scanned on a Siemens 3T Trio Scanner at the UTHSCSA. BOLD fMRI data were acquired with a gradient-echo echo planar imaging (EPI) sequence (TR/TE/flip angle = 2000ms/25ms/70; FOV = 256 mm, 2.00 x 2.00 x 3.00 mm voxels, interslice skip = 0) covering 34 interleaved 3 mm-thick axial slices. As in the Discovery cohort, high-resolution three-dimensional structural images were acquired in 34 axial slices co-planar with the functional scans (TR/TE/flip angle = 5610ms/72ms/150; voxel size = 0.8 × 0.8 × 3 mm; FOV = 220 mm x 320 mm, interslice skip = 0).

The same data preprocessing steps were applied to both the Discovery and the Replication cohort. Briefly, images for each subject were realigned to the first volume in the time series to correct for head motion, spatially normalized into a standard stereotaxic space (Montreal Neurological Institute template) using a 12-parameter affine model (final resolution of functional images = 2 mm isotropic voxels), and smoothed to minimize noise and residual difference in gyral anatomy with a Gaussian filter, set at 6-

mm full-width at half-maximum. Voxel-wise signal intensities were ratio normalized to the whole-brain global mean.

Variability in single-subject whole-brain functional volumes was determined using the Artifact Recognition Toolbox (http://www.nitrc.org/projects/artifact_detect). Individual whole-brain BOLD fMRI volumes meeting at least one of two criteria were assigned a lower weight in determination of task-specific effects: 1) significant mean-volume signal intensity variation (i.e., within volume mean signal greater or less than 4 standard deviations of mean signal of all volumes in time series), and 2) individual volumes where scan-to-scan movement exceeded 2 mm translation or 2° rotation in any direction.

The general linear model (GLM) of SPM8 (http://www.fil.ion.ucl.ac.uk/spm) was used to conduct fMRI data analyses. Linear contrasts employing canonical hemodynamic response functions were used to estimate differential effects of condition from the contrast of Faces > Shapes for each individual. Individual contrast images were then used in second-level random effects models accounting for scan-to-scan and participant-to-participant variability to determine mean condition-specific regional responses using one-sample t-tests. Regions of interest (ROIs) masks for the bilateral amygdala were constructed using the automatic anatomical labeling (AAL) within WFU PickAtlas Tool, version 1.04. A statistical threshold of $p<0.05$, FWE corrected, and ≥10 contiguous voxels was applied to amygdala analyses within each hemisphere. BOLD

values from voxels within the amygdala exhibiting strongest main effect of task were extracted using the VOI tool in SPM8.

These extracted values were then entered into regression models using IBM SPSS Statistics 20.0 (SPSS Inc., Chicago, IL). Importantly, by extracting amygdala BOLD parameter estimates from the voxels activated by our paradigm rather than clusters specifically correlated with our independent variables of interest, we preclude the possibility of any regression coefficient inflation that may result from capitalizing on the same data twice (Viviani, 2010). We have successfully used this conservative strategy in previous reports (Carre *et al.*, 2012, Nikolova *et al.*, 2011, Nikolova & Hariri, 2012) ENREF 5.

5.2.3. RNA Processing and Quantitative Real-time PCR

Total RNA was isolated from TRIzol homogenates of the amygdala in all postmortem subjects. The samples were purified using RNeasy spin columns (Qiagen; Valencia, CA), and RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Walbronn, Germany). cDNA was generated by mixing 1 μg total RNA with oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) per the manufacturer's protocol. PCR products were amplified in quadruplets on a Mastercycler real-time PCR machine (Eppendorf, Hamburg, Germany) using universal PCR conditions, as described previously (Tripp *et al.*, 2012). Results were calculated as the geometric mean of threshold cycles of *SLC6A4* transcript amplification

normalized to three validated internal controls (actin, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin G). Although performed in serotonergic projection areas, *SLC6A4* transcripts are readily detectable by qPCR.

5.2.4. DNA Extraction and 5-HTTLPR/rs25531 Genotyping

Saliva samples from Discovery cohort participants were collected using Oragene kits and DNA was extracted in accordance with the manufacturer's guidelines (Oragene, Genotek, Toronto, Ontario). In the Replication cohort, DNA was extracted from whole blood. Postmortem brain DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA), using a protocol that was modified from manufacturers' instructions (additional proteinase K and RNase A).

The same 5-HTTLPR/rs25531 genotyping protocol was applied to DNA samples from all three cohorts. Primer sequences for genotyping 5-HTTLPR are described previously (Gelernter *et al.*, 1997), the forward primer having the sequence (5'-ATGCCAGCACCTAACCCCTAATGT-3') and the reverse (5'-GGACCGCAAGGTGGGCGGGA-3'). PCR was conducted using the following cycling conditions: initial 15-min denaturing step at 95°C, followed by 35 cycles of 94°C for 30 sec, 66°C for 30 sec and 72°C for 40 sec, and a final extension phase of 72°C for 15 min. Reactions were performed in 10X reaction Buffer IV (ABgene), 1.5mM MgCl2, 50ng of genomic DNA, 5pmols of each primer, 0.3mM dNTPs and 1 unit of Native Taq (Promega). PCR products were subsequently digested by MspI restriction enzyme for 4

hours at 37°C. The digestion products were separated on a 3% agarose gel (MultiABgarose, ABgene) supplemented with Ethidium bromide (0.03%, BDH) and visualised by ultraviolet transillumination. Genotype calls were made by three independent raters, who reached consensus on 100% of the Discovery and Replication cohort samples. Genotype could not be determined accurately for one postmortem sample. Thus, it was removed from analysis, leaving a final sample of 34 individuals (10 women, mean age 49.44 ± 12.02).

5.2.5. DNA Methylation Analyses

DNA methylation levels of the proximal promoter of the serotonin transporter gene in the Discovery cohort were determined using quantitative bisulfite Pyrosequencing by EpigenDx Inc (Worcester, MA). Briefly, the human *SLC6A4* proximal promoter methylation assays analyze 20 CpG dinucleotides in the promoter region from -213 to -69 bps of the transcriptional start site (TSS), based on Ensembl Gene ID ENSG00000108576 and the Transcript ID ENST00000394821. The *SLC6A4* promoter assays (ADS580-FS1 and ADS580-FS2) are targeted to the antisense sequence of *SLC6A4* gene. The target sequences (genomic DNA and bisulfite converted DNA) from the Pyrosequencing assays are listed in Table 10. Table 11 and Table 12 present the targeted CpG loci (with respect to TSS, the translational start site, and genomic location) by Pyrosequencing for this gene.

For each analysis, the bisulfite conversion was performed with 500 ng provided genomic DNA using the EZ DNA methylation kit (ZymoResearch, Inc., CA). The PCR reaction was performed based on recommended assay conditions (EpigenDx, MA) using 0.2 μM of each primer with one of the PCR primers being biotinylated in order to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed and denatured using 0.2 M NaOH solution and rewashed using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Qiagen) as recommended by the manufacturer. 10 μl of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Pyrosequencing, Qiagen) following the manufacturer's instructions (Pyrosequencing, Qiagen). The methylation status of each CpG site was analyzed individually as an artificial T/C SNP using QCpG software (Pyrosequencing, Qiagen).

Table 10. *SLC6A4* proximal promoter methylation assays for the Discovery cohort.

Assay ID	Genomic Target Sequence	Bisulfite Converted Target Sequence	Pyrosequencing Dispensation order
ADS580-FS1	cgccgccaaagagctcttgaagaatttttgcgtcactttgaggcgaataaacttaatgcttccc	YGTYGTTAAAGAGTTTTTGAAGAATTTTTGYGTTATTTTGAGGYGAATAAATTTAATGTTTTTT	GTCTGTCGCTAGAGTTGAGATTAGTCGTATTGATGTCGAT
ADS580-FS2	cttcccgggggcggcggctcggcgctccggctggatggggttgcgctgccaggggaggggccgcgcctaggggggggggtggcggccgaccccagagccaggaggggaggga	TTTTTTYGYGCGTYGYGGTTTYGYGTTTTYGTTGGATGGGGTTGYGTTYGTTAGGGAGGGGTYGYGTTAYGGGYGGGGTGYGYGTTYGATTTTAGAGTTAGGAGGGGAGGGA	GTTCTGTCAGTCTGTCAGTTCTGTCAGTTCGTCGATGGTAGTCTGTCGCTAGAGGTCAGTCGTGATCGGTCGGTAGTCTGTCAGTCGAT

Table 11. Coordinates and mean percent methylation levels for *SLC6A4* CpG sites assayed in the Discovery cohort. Number of participants with 0% methylation is indicated for each site. All promoter sites are in the proximal promoter.

	From TSS	GRCh37/hg19 chr17	Region	Mean	SD	Participants with 0% methylation
ADS1818FS2	+497	28562219	intron 1	19.48	5.43	0 (0.00%)
	+495	28562221	intron 1	28.28	7.73	0 (0.00%)
ADS1818FS1	+451	28562265	intron 1	18.62	5.2	1 (1.25%)
	+426	28562290	intron 1	27.69	6.66	1 (1.25%)
	+381	28562335	intron 1	8.19	2.89	1 (1.25%)
ADS579FS1	+119	28562597	exon 1	0.038	0.34	79 (98.75%)
	+111	28562605	exon 1	2.19	1.38	21 (26.25%)
	+106	28562610	exon 1	7.82	2.02	1 (1.25%)
	+93	28562623	exon 1	8.41	2.01	0 (0.00%)
ADS579FS2	+73	28562643	exon 1	6.95	2.51	5 (6.25%)
	+71	28562645	exon 1	6.06	2.54	8 (10.00%)
	+56	28562660	exon 1	1.79	2.39	50 (62.50%)
	+43	28562673	exon 1	10.12	1.93	1 (1.25%)
	+32	28562684	exon 1	7.66	1.72	1 (1.25%)
	+30	28562686	exon 1	4.17	2.36	17 (21.25%)
	+24	28562692	exon 1	1.32	2.27	59 (73.75%)
	+15	28562701	exon 1	1.76	2.45	52 (65.00%)
	+12	28562704	exon 1	3.09	2.94	37 (46.25%)
	+9	28562707	exon 1	0.45	1.3	71 (88.75%)
	-3	28562718	promoter	1.58	2.27	53 (66.25%)
	-11	28562726	promoter	0	0	80 (100.00%)
	-14	28562729	promoter	0.21	1.1	77 (96.25%)
	-17	28562732	promoter	0	0	80 (100.00%)
	-19	28562734	promoter	0	0	80 (100.00%)
	-23	28562738	promoter	0	0	80 (100.00%)
	-35	28562750	promoter	0.07	0.64	79 (98.75%)
	-37	28562752	promoter	0	0	80 (100.00%)

Table 12. Coordinates and Discovery cohort mean percent methylation levels for proximal promoter *SLC6A4* CpG sites assayed in all cohorts. Number of participants with 0% methylation is indicated for each site.

	From TSS	GRCh37/hg19 chr17	Region	Mean	SD	Participants with 0% methylation
ADS580FS1	-69	28562784	promoter	1.79	0.54	1 (1.25%)
	-72	28562787	promoter	1.44	0.81	3 (3.75%)
	-99	28562814	promoter	1.06	0.54	11 (13.75%)
	-112	28562827	promoter	1.55	0.63	7 (8.75%)
ADS580FS2	-133	28562848	promoter	2.87	0.45	1 (1.25%)
	-135	28562850	promoter	1.1	0.33	1 (1.25%)
	-139	28562854	promoter	1.21	0.36	2 (2.50%)
	-141	28562856	promoter	0.92	0.34	5 (6.25%)
	-147	28562862	promoter	2.24	0.45	1 (1.25%)
	-149	28562864	promoter	1.02	0.34	3 (3.75%)
	-155	28562870	promoter	1.89	0.38	1 (1.25%)
	-170	28562885	promoter	3.57	0.82	1 (1.25%)
	-174	28562889	promoter	1.59	0.47	2 (2.50%)
	-188	28562903	promoter	1.74	0.38	1 (1.25%)
	-190	28562905	promoter	0.76	0.53	22 (27.50%)
	-195	28562910	promoter	0.74	0.62	30 (37.50%)
	-200	28562915	promoter	0.88	0.67	26 (32.50%)
	-207	28562922	promoter	1.25	0.48	7 (8.75%)
	-209	28562924	promoter	0.66	0.6	34 (42.50%)
	-213	28562928	promoter	0.97	0.65	22 (27.50%)

The methylation level at each CpG site was calculated as the percentage of the methylated alleles over the sum of methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of all measured CpG sites

within each targeted region. For quality control, each experiment included non-CpG cytosines as internal controls to verify efficient sodium bisulfite DNA conversion. We also included low, medium, high methylated standards (EpigenDx, MA) as controls in each run. In light of the low methylation values observed at some CpG sites (< 2%) in the Discovery cohort, additional PCR bias testing was performed using pyrosequencing by mixing the unmethylated DNA control and *in vitro* methylated DNA at different ratios (0, 20, 40 up to 100%) followed by bisulfite modification, PCR and pyrosequencing analysis. There was a high correlation between the percent methylation obtained from the mixing study and expected methylation percentages ($r^2 = 0.99$), which confirms the quality of our data.

In the Replication and postmortem cohorts, methylation analysis on the same *SLC6A4* proximal promoter 20 CpG sites as targeted by the ADS580FS1 and ADS580FS2 assays (Table 10**)** was carried out at the Core for Advanced Translational Technologies (UTHSCSA). The protocol used was the same as in the Discovery cohort, except with independently designed PCR and sequencing primers (see **Table S10**). Results were analyzed using PyroMark Q96 MD and PyroMark CpG 1.0 software (Qiagen, Valencia, CA).

Control analyses conducted by EpigenDx only in the Discovery cohort used additional methylation assays in exon 1 and intron 1 of *SLC6A4* (**Table S2**), as well as

the promoter region of the *COMT* gene (**Table S3**). Detailed assay information is available upon request.

5.2.6. Self-Report Measures

To assess recent life stress we administered a modified version of the Life Events Scale for Students [LESS; (Nikolova *et al.*, 2012)]. This modified version of the scale asks participants to indicate whether they experienced common stressful life events within the past 12 months; in addition, for each event that occurred participants reported on the impact it had on their lives on a 1-4 scale (with 4 being the highest). The impact scores were set to zero for events that did not occur. Based on prior research (Nikolova *et al.*, 2012, Nikolova & Hariri, 2012), we focused on the LESS Highest Impact metric, reflecting the highest impact associated with any event which occurred within the past year. We assessed early life trauma using the Childhood Trauma Questionnaire [CTQ; (Bernstein, 2002)].

5.2.7. Statistical Analysis

Percent methylation was computed as the ratio of methylation cytosines over the sum of all methylation and unmethylated cytosines. Our main analyses focused on two *SLC6A4* promoter assays covering a total of 20 CpG sites sampled across all three cohorts (Table 12). No single nucleotide polymorphisms (SNPs) resulting in CpG site gain or loss were identified within the assayed regions. In addition to analysis using average percent methylation across the 20 CpG sites in our region of interest, we applied

principal component analysis (PCA) to these 20 CpG sites in both the Discovery and Replication cohorts. The unrotated correlation matrix was analyzed to output principal component scores. An eigenvalue greater than 1 indicates that PCs account for more variance than accounted by one of the original variables in standardized data. PCA resulted in five PCs with eigenvalues>1 in both samples. The directionality and significance of the effects of the first PC on amygdala reactivity were the same as those associated with *SLC6A4* promoter methylation values averaged across all 20 CpG sites in both the Discovery and the Replication cohort and are thus not visualized here. In light of the smaller number of datapoints (n = 34 final sample), no PCA was performed in the postmortem cohort, where CpG sites were analyzed individually.

Linear regression models, as implemented in IBM SPSS Statistics 20.0 (Chicago, IL), were used to investigate the linear effect of methylation values (independent variable) on amygdala reactivity or *SLC6A4* mRNA levels (dependent variables). Results from two-tailed tests are reported for all analyses.

5.3. *Results*

In our Discovery cohort, percent methylation of the *SLC6A4* proximal promoter was positively correlated with threat-related amygdala reactivity in the left hemisphere (Adj. $R^2 = 0.067$, $b = 0.282$, $p = 0.011$**;** Figure 7). This effect was observed at a trend level in the right hemisphere (Adj. $R^2 = 0.032$, $b = 0.211$, $p = 0.060$). Percent methylation continued to account for significant variability in left amygdala reactivity even when controlling

for possible effects of gender, age, early and recent life stress, and current psychiatric disorder (left hemisphere: $\Delta R^2 = 0.084$, $b = 0.292$, $p = 0.009$; right hemisphere: $\Delta R^2 = 0.045$, $b = 0.214$, $p = 0.060$). Similar results were obtained when using the top principal component (PC) capturing 24% of the methylation variance in the same region (Table 13). In an exploratory follow-up analysis we probed the effects of individual CpG site methylation levels on these same phenotypes and found that CpG 14 (188 bp upstream of TSS) showed strongest association effects across both hemispheres (Table 14).

Table 13. Results from regression analyses using amygdala reactivity as a dependent variable and the first principal component capturing 24.06% of all *SLC6A4* proximal promoter methylation variance as an independent variable in the Discovery cohort. Covariates include age, gender, LESS Highest Impact, CTQ Total, 5-HTTLPR/rs25531 genotype, and current Axis I diagnosis (dummy coded: 0=no, 1=yes).

SLC6A4 promoter methylation (PC 1)	ΔR^2	b	p
No Covariates			
Left amygdala	0.070	0.264	**0.018**
Right amygdala	0.042	0.204	0.070
With Covariates			
Left amygdala	0.079	0.284	**0.012**
Right amygdala	0.043	0.210	0.066

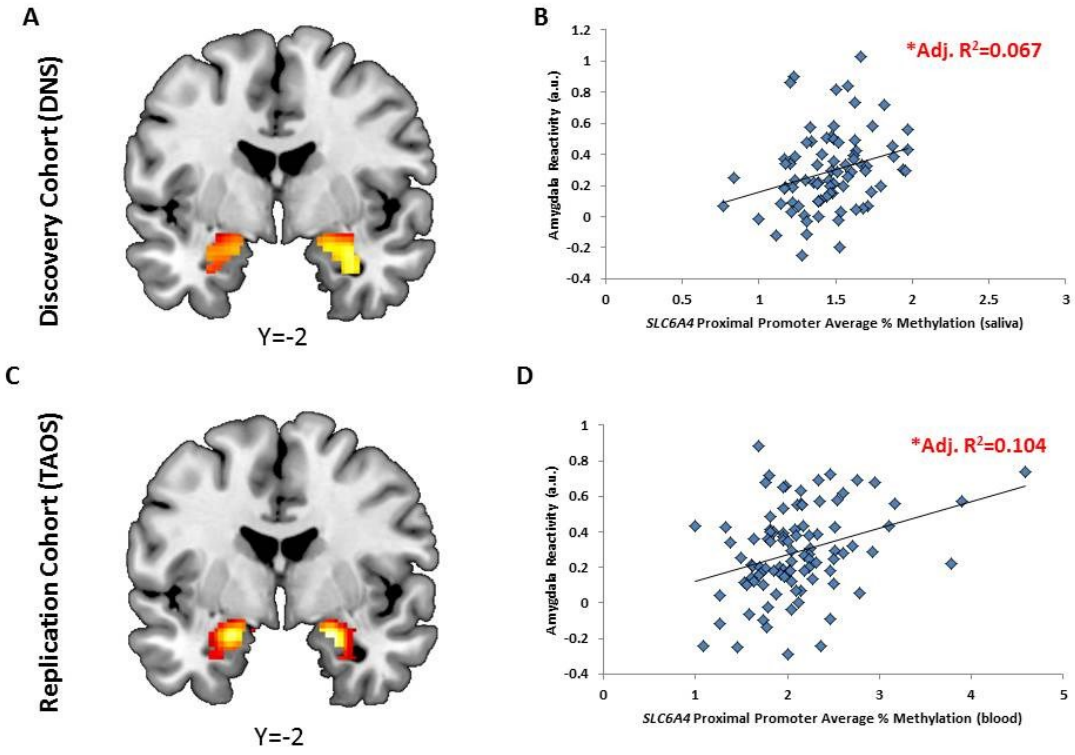

Figure 7. Effects of *SLC6A4* promoter methylation on amygdala reactivity. Statistical parametric map illustrating mean bilateral threat-related amygdala reactivity across all participants in the (A) Discovery cohort (left: x = -24, y = -8, z = -16, t = 10.29, p = 2.0095 x 10^{-14}, k_E=180; right: x = 30, y = -4, z = -20, t = 11.13, p < 0.00001, k_E = 203) and (C) Replication cohort (left: x = -20, y = -4, z = -18, t = 11.29, p < 0.00001, k_E = 197; right: x = 20, y = -4, z = -16, t = 11.80, p = 1.29 x 10^{-13}, k_E = 205). Activation clusters are overlaid onto canonical structural brain images in the coronal plane (Y = -2). Average percent *SLC6A4* proximal promoter methylation was positively correlated with reactivity of the left amygdala in both the Discovery (B) and Replication cohort (D).

a.u.= arbitrary units.

***p<0.05**

Table 14. Summary of results from linear regression models predicting *in vivo* amygdala reactivity and amygdala tissue *SLC6A4* mRNA from percent methylation levels at each of the 20 individual proximal promoter CpG sites sampled across the Discovery, Replication and postmortem cohorts. Results from the *in vivo* imaging cohorts are not adjusted for covariates. In light of gender, age, pH, postmortem interval, and RNA ratio effects in the postmortem cohort, the results for the postmortem findings are adjusted for covariates. The CpG site numbering scheme reflects the ordering of CpG site within this proximal promoter region and has no relation to any unique CpG site numerical identifiers. *CpG site with strongest association for each phenotype.

		Discovery Cohort (DNS)				Replication Cohort (TAOS)				Postmortem	
		Left Amygdala		Right Amygdala		Left Amygdala		Right Amygdala		Amygdala *SLC6A4* mRNA	
CpG site	From TSS	*b*	*p*	*b*	*p*	*b*	*p*	*b*	*p*	*b*	*p*
1	-69	0.163	0.149	0.180	0.11	0.112	0.278	-0.061	0.556	-0.073	0.707
2	-72	0.263	**0.018**	0.125	0.269	0.207	**0.043**	0.068	0.511	-0.043	0.837
3	-99	0.094	0.409	0.052	0.647	0.119	0.248	0.148	0.15	0.118	0.540
4	-112	0.224	**0.046**	0.279	**0.012**	0.107	0.297	0.130	0.208	-0.079	0.752
5	-133	0.102	0.368	0.138	0.223	0.229	**0.025**	0.070	0.500	-0.128	0.601
6	-135	0.224	**0.046**	0.260	**0.02**	0.129	0.209	-0.106	0.306	0.104	0.630
7	-139	0.069	0.544	0.191	**0.089**	0.164	0.109	-0.019	0.856	-0.271	0.169
8	-141	0.026	0.818	0.048	0.672	0.010	0.921	0.020	0.846	-0.006	0.975
9	-147	0.128	0.258	0.058	0.608	0.196	**0.056**	-0.056	0.587	0.022	0.920
10	-149	0.280	**0.012***	0.225	**0.045**	0.134	0.193	0.085	0.411	0.070	0.708
11	-155	0.186	**0.099**	0.240	**0.032**	0.214	**0.037**	0.133	0.195	0.088	0.683
12	-170	0.162	0.151	0.144	0.204	0.264	**0.009**	0.070	0.496	-0.223	0.301
13	-174	0.245	**0.028**	0.219	**0.051**	0.273	**0.007**	0.213	**0.037**	0.071	0.702
14	-188	-0.029	0.799	0.287	**0.010***	0.305	**0.003***	0.219	**0.032***	-0.378	**0.039***
15	-190	0.157	0.164	0.164	0.146	0.218	**0.033**	0.097	0.345	-0.013	0.943
16	-195	0.094	0.407	-0.103	0.363	0.226	**0.027**	0.037	0.722	0.177	0.407
17	-200	0.006	0.955	-0.107	0.343	0.082	0.424	0.004	0.965	0.166	0.495
18	-207	0.148	0.189	0.091	0.423	0.226	**0.027**	0.014	0.893	-0.032	0.865
19	-209	0.119	0.294	-0.059	0.602	0.235	**0.021**	0.029	0.782	-0.082	0.671
20	-213	0.100	0.376	-0.057	0.616	0.115	0.263	-0.116	0.261	-0.729	0.472

Given prior work establishing predictive links between genetic variation and amygdala reactivity (Hariri, 2009), we next compared the effect of *SLC6A4* proximal promoter methylation on amygdala reactivity to that of the serotonin transporter linked polymorphic region (5-HTTLPR) and rs25531, which together define a functional tri-allelic polymorphism previously associated with variability in amygdala reactivity as well as responsiveness to stress (Caspi *et al.*, 2010). *SLC6A4* methylation continued to predict amygdala reactivity even when 5-HTTLPR/rs25531 genotype was accounted for alongside all other covariates (left hemisphere: $\Delta R^2 = 0.086$, $b = 0.296$, $p = 0.009$; right hemisphere: $\Delta R^2 = 0.043$, $b = 0.211$, $p = 0.066$). This suggests preponderance of epigenetic variation over sequence-based variation in regulatory regions of the same gene.

As a negative control, we examined the correlation between amygdala reactivity and methylation in other regions of *SLC6A4* as well as the *COMT* gene, which codes for an enzyme responsible for regulating catecholamine but not serotonin signaling. As expected, there were no significant correlations between left or right amygdala reactivity and percent methylation of either intron 1 or exon 1 of *SLC6A4* ($p > 0.10$). Similarly, there were also no significant associations between reactivity and percent promoter methylation of *COMT* ($p > 0.50$). Thus, these data reveal a specific effect of *SLC6A4* proximal promoter methylation on amygdala reactivity. Given the specificity of these

findings, we followed up the effects of these same 20 CpG sites in our Replication cohort.

Consistent with the findings from our Discovery cohort, we found that percent promoter methylation of *SLC6A4,* this time assayed in DNA derived from peripheral blood, was positively correlated with left amygdala reactivity in our Replication cohort (Adj. $R^2 = 0.113$, $b = 0.336$, $p = 0.001$; Figure 7). In fact, this effect of percent methylation was larger than that accounted for in our Discovery cohort (11.3% vs 6.7%). The effect was again weaker in the right hemisphere (Adj. $R^2 = 0.007$, $b = 0.084$, $p = 0.42$). Such hemispheric asymmetries are not uncommon in the imaging genetics literature (Fisher *et al.*, 2006, Hariri *et al.*, 2002b) and may reflect task-specific characteristics (Baas *et al.*, 2004) or intrinsic differences in monoamine signaling between the two hemispheres (Young & Williams, 2010).

Notably, the effects in our Replication cohort remained unchanged when controlling for age, gender, early life stress, risk for psychiatric disorder, and 5-HTTLPR/rs25531 genotype (left hemisphere: $\Delta R^2 = 0.090$, $b = 0.312$, $p = 0.005$; right hemisphere: $\Delta R^2 = 0.002$, $b = 0.049$, $p = 0.662$). These results were confirmed using the first PC capturing 30.41% of all methylation variance in the region (Table 15). Finally, as in the Discovery cohort, the single site showing strongest associations with amygdala reactivity across hemispheres was CpG 14 (Table 14).

Table 15. Results from regression analyses using amygdala reactivity as a dependent variable and the first principal component capturing 30.41% of all *SLC6A4* proximal promoter methylation variance as an independent variable in the Replication cohort. Covariates include age, gender, psychopathology risk, CTQ Total, and 5-HTTLPR/rs25531 genotype.

SLC6A4 promoter methylation (PC 1)	ΔR^2	b	p
No Covariates			
Left amygdala	0.101	0.318	**0.002**
Right amygdala	0.009	0.096	0.354
With Covariates			
Left amygdala	0.076	0.287	**0.009**
Right amygdala	0.003	0.055	0.623

The direction of the effects we observe in our *in vivo* data (higher reactivity with greater percent methylation) is remarkably consistent with that of prior imaging genetics (Fakra *et al.*, 2009, Hariri *et al.*, 2002b), multi-modal PET/fMRI (Fisher *et al.*, 2006, Rhodes *et al.*, 2007), and pharmacologic fMRI (Bigos *et al.*, 2008, Di Simplicio *et al.*, 2013) studies linking relatively increased serotonin signaling with increased amygdala reactivity, as well as observations that methylation within or near promoter regions generally inhibits gene transcription (Brenet *et al.*, 2011). In light of these data and to gain further mechanistic insight into our *in vivo* findings, we examined the impact of percent methylation of the same 20 CpG sites sampled in our imaging cohorts on serotonin

transporter mRNA levels in postmortem amygdala tissue from a third independent cohort of 34 individuals.

As expected, clinical and biochemical parameters (i.e., diagnostic status, pH, and RNA ratio) influenced mRNA levels (p values < 0.091). Thus, those were included as covariates alongside age, gender and postmortem interval in all analyses involving postmortem data. When controlling for the effects of these parameters, there was no significant association between overall percent methylation and mRNA in amygdala tissue (p = 0.699). However, a site-specific investigation revealed a significant negative correlation between mRNA levels and percent methylation at CpG 14, which exhibited the strongest association with amygdala reactivity in both our imaging cohorts (Table 14, Figure 8). As with our *in vivo* imaging data, this epigenetic effect was further independent of 5-HTTLPR/rs25531 genotype (p = 0.031), despite nominally lower mRNA levels in S/L$_G$ allele carriers (p = 0.291).

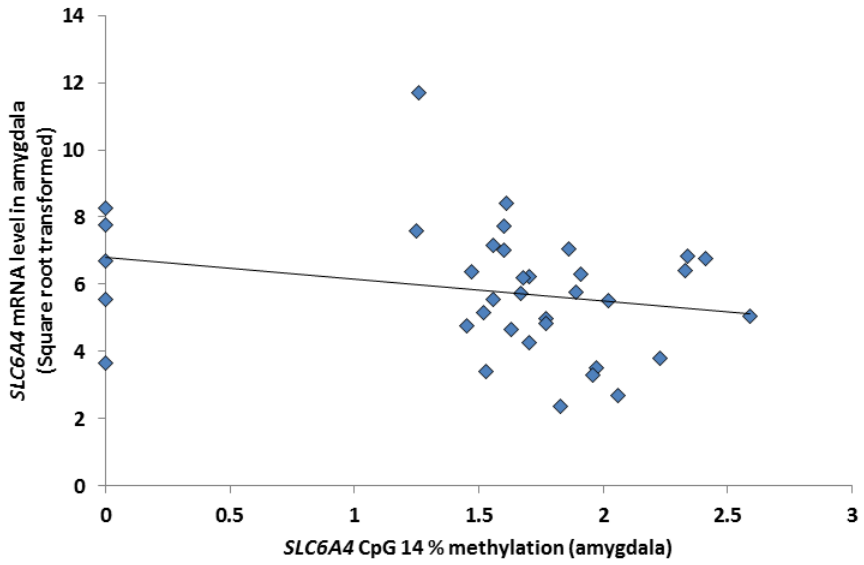

Figure 8. Scatterplot depicting the negative correlation between percent methylation at CpG 14 and *SLC6A4* mRNA levels in amygdala tissue.

5.4. *Discussion*

Collectively, our results converge to provide evidence that methylation of the proximal promoter of human *SLC6A4* predicts threat-related amygdala reactivity possibly reflecting decreased serotonin transporter gene expression and, consequently, reduced regional serotonin reuptake. Moreover, theses epigenetic effects are independent of, and greater than, the effects of the 5-HTTLPR/rs25531 functional polymorphism near the same genomic region. Further demonstrating the independence of these genetic and epigenetic effects, none of the 20 proximal promoter CpG sites surveyed across the three cohorts overlap the 5-HTTLPR, which is located 1,400 base pairs upstream of the TSS and primarily impacts the distal promoter. In addition, 5-HTTLPR/rs25531 genotype had no effect on proximal promoter methylation in any cohort (p's > 0.30).

While we do not directly map methylation in peripheral tissues onto methylation levels in brain within the same cohort, cross-cohort convergence among tissues (saliva, blood, brain) is consistent with recent work demonstrating a significant correlation between the blood and brain methylomes (Tylee *et al.*, 2013). Furthermore, this cross-tissue convergence provides evidence that two distinct types of readily assayed peripheral tissues (blood and saliva) could potentially be used as equally valid proxies of neural tissue. A notable limitation of the current work is that, due to practical

constraints, the postmortem tissue analysis was limited to the amygdala, while informative differences in *SLC6A4* transcript levels are more likely to emerge in the dorsal raphe nucleus, where the serotonin transporter is more densely expressed. This limitation notwithstanding, our current results demonstrate that meaningful associations between the human epigenome and brain can be mapped using DNA derived from readily assayed peripheral tissues. In addition to encouraging careful consideration of the effects of promoter methylation in *SLC6A4* on behaviorally and clinically relevant brain function, we hope that our current work will advance broader research on epigenetic mechanisms in the emergence of individual differences in human behavior and related risk for psychopathology.

6. General Discussion and Future Directions

Neural circuits for reward and threat processing are dysregulated in a range of psychiatric disorders. Moreover, individual differences in neural reward and threat processing may reflect stable trait-like constructs which transcend current diagnostic state and can therefore be harnessed to identify vulnerable and resilient individuals. In this dissertation, I implicate reward-related VS and threat-related amygdala reactivity in novel biological pathways of risk and resilience for psychopathology and identify novel genetic and epigenetic predictors of inter-individual variability within those pathways.

Extensive prior research suggests that stress-induced deficits in reward processing may be part of a mechanism underlying the causal link between stress and depression (Pizzagalli, 2014). Parallel theoretical models have advanced the notion that reward processing robust to such stress-related disruptions, may be part of a resilience mechanism against psychopathology in the wake of adversity (Charney, 2004). In support of this proposition, **Chapter 2** demonstrates that individuals with relatively robust reward-related VS reactivity show stable levels of state positive affect in the context of recent life stress. Those with low VS reactivity, by contrast, show stress-related reductions in positive affect (Nikolova *et al.*, 2012). These findings suggest that robust VS reactivity to reward may be part of a neural mechanism conferring resilience against stress-related affective illness.

Highlighting the context-specificity of risk and resilience conceptualizations, **Chapter 3** demonstrates that this same neural phenotype is associated with increased problem drinking in the wake of stress, thus predisposing to disorder risk of a different kind. Importantly, this effect only occurred in individuals who also had relatively low amygdala reactivity. These findings suggest that a heightened reward drive coupled with reduced threat sensitivity may predispose to risky behavior, particularly following recent stress, and that high threat-related amygdala reactivity may serve a protective role against some forms of addiction. These findings are consistent with developmental models of addiction vulnerability (Doremus-Fitzwater *et al.*, 2010) and prior imaging genetics research (Hariri *et al.*, 2009). Moreover, **Chapter 3** provides evidence that this combination of neural traits can predict stress-related problem drinking three months following initial assessment (Nikolova & Hariri, 2012). The latter finding lends support to the notion that neural reactivity to threat and reward could indeed be leveraged to predict both current and future vulnerability and resilience.

Such predictions of risk and resilience would be made easier if BOLD fMRI imaging can in fact be supplanted by more easily accessible peripheral measures of neural function, which can serve as proxies of reward and threat processing. While prior research has begun to link specific genetic variants to variability in both amygdala and VS reactivity (Hariri, 2009), few studies had investigated the combined effects of multiple variants. In **Chapter 4**, I demonstrate the utility of multilocus genetic profile

approaches for accounting for variability in neural function using a specific example capturing variability in DA signaling and VS reactivity (Nikolova *et al.*, 2011). Specifically, I show that a multilocus genetic profile reflecting the cumulative effects of five distinct polymorphic loci on DA signaling not only predicts VS reactivity more robustly than each locus taken individually, but it accounts for nearly 11% of all variability in VS reactivity. Results from this study have spurred multiple follow-up investigations extending this approach to additional phenotypes and systems (Davis *et al.*, 2013, Kohannim *et al.*, 2012, Stice *et al.*, 2012). Ideally, similar scores capturing variability in other neurotransmitter systems could be used to allow for a more comprehensive account of genetically driven inter-individual variability in neural function and, ultimately, clinically informative measures of risk and resilience attainable without dependence on BOLD fMRI.

While genetic factors are likely to account for a large proportion of the measurable variability in brain function, they are unlikely to account for all of it for two inter-related reasons: 1) there are multiple intervening steps between the assembly of a DNA sequence and its expression as a functional protein, all of which are subject to intricate non-DNA-sequence based regulation; and 2) environmental factors may moderate the effects on genetic variation on many behaviorally relevant neural phenotypes (Klucken *et al.*, 2013). Epigenetic modifications of DNA and chromatin may help account for both sources of variability, as these modifications are not only involved

in regulating gene expression without altering the basic DNA sequence, but are also heritable and subject to environmental modulation (Goldberg *et al.*, 2007). Thus, the study of the epigenetic landscape may yield much needed insight into the mechanisms that link genetic variation to complex phenotypes.

In strong support of this notion, in **Chapter 5**, I report data demonstrating that relatively high levels of methylation in the proximal promoter region of *SLC6A4* predict greater amygdala reactivity in two independent samples. Providing additional cues as to the molecular mechanisms underlying this effect, methylation in the same region was associated with reduced gene expression in postmortem amygdala tissue. Critically, all of these effects persist even after accounting for 5-HTTLPR/rs25531 genotype.

These findings critically implicate epigenetic modifications in the regulation of brain function and suggest their effects may in fact override those of functional DNA-sequence based variation. Thus, epigenetics should ideally be considered in all studies aiming to identify genetic influences on neural functioning, particularly in cases of suspected or established environmental moderation. With the increasing availability of methods for assaying the human epigenome in its entirety and reasonable blood-peripheral tissue methylome convergence (Tylee *et al.*, 2013), such considerations would hopefully become increasingly easy to meet.

The current studies provide intriguing evidence for the involvement of threat and reward processing in novel biological pathways of risk and resilience for

psychopathology. However, they are not without limitations. First and foremost, the studies included here assess disorder risk using non-clinical continuous trait or state measures extending into the normative range. Moreover, they largely focus on healthy and high-functioning populations with few individuals meeting diagnostic criteria for a current or lifetime psychiatric disorder. While such a dimensional approach is likely to be more biologically valid than one focused on diagnostic categories (Morris & Cuthbert, 2012), the possibility should be acknowledged that the data reported here may not have captured the extreme ends of the continuum along which the behaviors and traits of interest are assessed. Future studies preferentially recruiting at-risk individuals (e.g., due to family history of MDD) or individuals with current or past psychiatric diagnoses may be needed to generalize these results beyond the circumscribed behavioral and clinical phenotype range afforded by the samples used here.

Equally importantly, the results reported here are correlational in nature. True risk and resilience can only be fully appreciated in the context of prospective longitudinal designs with the power to delineate vulnerability and resilience trajectories over time. Thus, prospective longitudinal studies would be necessary to confirm the predictive power of these and other neural and genetic markers of risk and resilience. Repeated-sampling designs would also be particularly well-suited for elucidating the epigenetic correlates of environmental experience, which could in turn provide insight into the molecular basis of risk and resilience. Such knowledge could not only facilitate

the early identification of vulnerable individuals, but also foster the development of novel individually tailored modes of intervention.

An exciting avenue for future research into the molecular basis of risk and resilience lies in uncovering the relative contributions of heritable and experiential factors in shaping the epigenetic landscape across the lifespan. Using an animal model of fear conditioning, a prominent recent study demonstrated that novel epigenetic modifications associated with fear learning may in fact be transmitted to offspring, along with acquired conditioned responses (Dias & Ressler, 2014). This raises the intriguing possibility that there may be similar epigenetic mechanisms of transgenerational transmission of risk and resilience in humans. This possibility can be explored in future studies sampling DNA not only from study populations of interest, but also from parents or children of study participants.

Finally, the BOLD fMRI measures of VS and amygdala reactivity used here reflect cumulative signaling in those neural regions and can serve as useful indices for the overall functioning of the CSC and CLC, respectively. However, by themselves, they provide little direct insight into the specific molecular mechanisms that may underlie differences in reward and threat-related brain function. Future studies combining molecular genetics tools with multimodal PET and fMRI technology would be better suited to provide insight into the precise molecular mechanisms of risk and resilience. Ideally, these studies should attempt to integrate multilocus genetic and epigenetic data

into a repeated-measures prospective longitudinal design aimed to delineate personal and perhaps transgenerational risk and resilience trajectories with the ultimate goal of developing targeted early interventions to prevent psychopathology in vulnerable individuals.

Appendix

Life Events Scale for Students (LESS)

The responses to the occurrence IN THE LAST YEAR of the following LESS items were used in analyses:

1 Death of a parent (1- No, 2- Yes)

2 Major personal injury or illness (1- No, 2- Yes)

3 Major argument with parents (1- No, 2- Yes)

4 Beginning an undergraduate program at university (1- No, 2- Yes)

5 Moving away from home (1- No, 2- Yes)

6 Failing a number of courses (1- No, 2- Yes)

7 Minor violation of the law (e.g., speeding ticket) (1- No, 2- Yes)

8 Getting kicked out of college (1- No, 2- Yes)

9 Pregnancy (either yourself or being the father) (1- No, 2- Yes)

10 Minor car accident (1- No, 2- Yes)

11 Major violation of the law (e.g., sentenced with Jail term (self))

12 Moving out of town with parents (1- No, 2- Yes)

13 Spouse or boy/girlfriend died (1- No, 2- Yes)

14 Establishing new steady relationship with partner (1- No, 2- Yes)

15 Finding a part-time job (1- No, 2- Yes)

16 Sex difficulties with boy/girlfriend (1- No, 2- Yes)

17 Failing a course (1- No, 2- Yes)

18 Major change of health in close family member (1- No, 2- Yes)

19 Major car accident (car wrecked, people injured) (1- No, 2- Yes)

20 Death of your best or very close friend (1- No, 2- Yes)

21 Serious illness of your best or very close friend (1- No, 2- Yes)

22 Serious personal crisis experienced by family member or very good friend (1- No, 2- Yes)

23 Major Housing Problems (1- No, 2- Yes)

24 Breaking up of parent's marriage/divorce (1- No, 2- Yes)

25 Losing a part-time or a full-time job (1- No, 2- Yes)

26 Major and/or chronic financial problems (1- No, 2- Yes)

27 Major argument with boy/girlfriend or spouse (1- No, 2- Yes)

28 Parent losing a job (1- No, 2- Yes)

29 Switch in program within same college or university (1- No, 2- Yes)

30 Losing a good friend (1- No, 2- Yes)

31 Change of job (1- No, 2- Yes)

32 Break-up with boy/girlfriend or spouse (1- No, 2- Yes)

33 Minor financial problems (1- No, 2- Yes)

34 Major Conflict with a Family Member or very close friend (1- No, 2- Yes)

35 Assault, rape, or mugging (1- No, 2- Yes)

36 Major Difficulties at work (1- No, 2- Yes)

37 Lost driver's license (1- No, 2- Yes)

38 Pet died (1- No, 2- Yes)

39 Was robbed (1- No, 2- Yes)

40 Infidelity on the behalf of the spouse / significant other (1- No, 2- Yes)

41 Birth of a child (1- No, 2- Yes)

42 Abortion (1- No, 2- Yes)

43 Miscarriage (1- No, 2- Yes)

44 Found out that cannot have children (1- No, 2- Yes)

45 Child died (1- No, 2- Yes)

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Biography

Yuliya Stoycheva Nikolova was born in Pleven, Bulgaria, on November 11, 1985. She graduated from Harvard College, Cambridge, MA, in June 2009 with a degree of Bachelor of Arts in Psychology and a Language Citation in Swedish. She attended Duke University in Durham, NC, from August 2009 until May 2014, when she received the degree of Doctor of Philosophy in Psychology and Neuroscience. While at Duke University, she published the following articles and book chapters as lead author: 1) "Multilocus genetic profile for dopamine signaling predicts ventral striatum reactivity" (*Neuropsychopharmacology, 2011*), 2) "Ventral striatum reactivity to reward and recent life stress interact to predict positive affect" (*Biological Psychiatry, 2012*), 3) "Neural responses to threat and reward interact to predict stress-related problem drinking: A novel protective role of the amygdala" (*Biology of Mood & Anxiety Disorders, 2012*), 4) "Neurogenetics approaches: Insights from studies of dopamine signaling and reward processing" (*Handbook of Cognition & Emotion, 2012*), 5) "Reward-related ventral striatum reactivity mediates gender-specific effects of a galanin remote enhancer haplotype on problem drinking" (*Genes, Brain, and Behavior, 2013*). Yuliya was also a recipient of the James B. Duke Fellowship from the Duke University Graduate School from 2009 to 2013, as well as the Howard Hughes Medical Institute International Student Research Fellowship from 2012 to 2014.