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# Influence Of Stress On Substance Use: Age-Associated Variability In Molecular And Behavioral Outcomes

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# INFLUENCE OF STRESS ON SUBSTANCE USE: AGE-ASSOCIATED VARIABILITY IN MOLECULAR AND BEHAVIORAL OUTCOMES

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**DEDICATION**

To Sean D'Arcy, my inspiration, my dearest love, and my best friend:

Without you pushing along beside me, this tome of Sisyphus would have flattened me in the dust.

# INFLUENCE OF STRESS ON SUBSTANCE USE: AGE-ASSOCIATED VARIABILITY IN MOLECULAR AND BEHAVIORAL OUTCOMES

by

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## ABSTRACT

Stress introduces a number of neurological and neurohormonal changes in response to a number of stimuli, and can influence our relationship to rewarding experiences. As such, it holds the potential of promoting vulnerability to addiction to substances such as methamphetamine. While methamphetamine use and abuse has been steadily declining, the fact remains that it and closely related drugs are used in the treatment of conditions such as narcolepsy, weight loss, and attention deficit disorder. In an effort to develop better patient assessment metrics in aiding the physician in decisions of course of treatment, we ask the following questions: Do chronic or acute stresses increase vulnerability to addiction? Does the age at which stress is experienced contribute to risk? Using a restraint paradigm for stress in a rodent model we answer this question through behavioral self-administration of methamphetamine and attempt to deconstruct neurological mechanisms that may account for stress-attributable differences in responding to drug. The answer: Yes, chronic predictable emotional stress does increase methamphetamine escalation if the stress is experienced during adulthood, but adolescent stress does not result in either vulnerability to nor protection from addiction.

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# PREFACE

The late 1990's and early first decade of the 2000's saw the height of use and abuse of the psychoactive drug methamphetamine (METH) or the closely related compound, 3.4-methylenedioxymethamphetamine (MDMA) (ONDCP, 2004). A portion of that surge in usage was attributed to the ease with which METH could be synthesized from precursors like ephedrine or pseudoephedrine found in over-the-counter sinus and cold medications. This made manufacture of high-purity (~90%) METH inexpensive and enabled the establishment of not only large-scale manufacturers in the United States, Canada, and Mexico, but also provided a ripe breeding ground for small-scale "homegrown" operations. Thus, with ample supply of drug on the market, economic forces made METH affordable in addition to accessible and allowed this drug to gain favor over other psychoactive drugs such as cocaine.

Figures posted by the Office of National Drug Control Policy (ONDCP) at the peak of METH use in 2005 list the monetary cost to society in crime, medical treatment, and lost productivity as $108 billion (ONDCP, 2014). This figure fails to tally the intangible and arguably longer-term damage resulting from METH abuse (e.g., child neglect, depression and suicide, emotional and physical damage from engaging in risky or impulsive behaviors). In the United States, policies were enacted that restricted sales of pseudoephedrine and elevated vigilance within law enforcement agencies, resulting in the decline of METH reporting (metrics of reporting include drug-related emergency room visits, quantity of drugs seized, purity of drugs seized). However, it is important to point out that while METH reporting has declined over the past decade, most recent available data still cites monetary costs incurred in 2011 as $23.4 billion dollars, and balancing the decreases seen in METH use are increases in the use of other illicit substances like heroin or off-prescription use of oxycodone (US DEA, 2011).

It should also be noted that while widely known to the public as an illicit drug, METH derivatives and closely-related chemicals are also prescribed by doctors to treat disorders of narcolepsy, severe obesity, and, most commonly, attention deficit hyperactivity disorder (ADHD). According to 2010 data provided by the Centers for Disease Control and Prevention (CDC) and

the National Institutes of Health (NIH), among children and adolescents ages 4-17, roughly 9.5% were diagnosed with ADHD, and approximately half of those diagnosed were prescribed stimulant medication to treat the disorder. In one study, as many as 35% of college students surveyed reported abusing or misusing ADHD stimulant medication (Wilens et al, 2008). Prescription labels for amphetamine-derived drugs like Adderall and Vyvance list the possibility of forming dependence upon the drug, and the finding that the formation of drug tolerance requires dosage escalation (not dissimilar to hallmarks of drug addiction), has also been acknowledged by the pharmaceutical companies. Furthermore, accompanying these drugs are dosing instructions to prescribing physicians that recommend increasing the dosage until the desired effect is achieved. These observations are particularly worrisome given several studies in animal models that demonstrate a number of molecular changes in the brain's reward system among animals administered escalating doses that are not observed in animals receiving static "binge" doses of METH (Chauhan et al, 2014; Kosheleff et al, 2012; Groman et al, 2012). Given the potential for methamphetamine and amphetamine derivative medications to act as gateway drugs for addiction, it is necessary to shape a rubric for patient risk assessment.

While biomarkers for addiction proclivity are still under investigation, patient histories taken through standard medical office practice can gather information identifying pre-existing physiological and psychological conditions, thus providing doctors with a potential metric for risk assessment if correlations are known. For example, certain early life stressors such as childhood sexual abuse and childhood neglect have been linked to drug and alcohol abuse (Dube et al, 2003; Boyd, 1993; Marco et al, 2007), but a causal or mechanistic link between stress and addiction remains to be identified. By self-report, chronic emotional stressors would appear to be a common experience in modern society, but what level of severity or consistency is required to evoke such responses toward drug? Furthermore, are these stressors more significant if experienced during key developmental stages such as adolescence? With the established role of stress as an integral component of relapse in recovering addicts (or as a means of re-instating extinguished drug seeking behaviors in animal models), the type and perceived severity of patient stress experienced prior

to drug exposure may emerge as a risk factor for drug addiction (Bahi and Dreyer, 2014; Williems et al; 2014; Graf et al, 2013; Morita et al, 2013; Brecht et al, 2012; Buffalari et al, 2012; Cruz et al, 2012; Preston and Epstein, 2011; Buffalari and See, 2009; Funk et al, 2006; Wang et al, 2007; Wang et al, 2005; Shepard et al, 2004).

To begin addressing these concerns, the present study employs a rat model of a strong emotional stress (restraint) administered during mid-adolescence (PND 36-38) or during adulthood (PND 60-64) under both chronic (daily for 14 days) and acute (single stress event) conditions. Responses to methamphetamine are measured in these animals over 21 days of 6-hour intravenous self-administration (IVSA) sessions to assess initial intake, overall intake, initiation of escalation, and drug response patterns during the access periods.

# 1. INTRODUCTION

## 1.1 Structures and Signals of Stress Influence Reward

Neurological mechanisms of reward and addiction are a tangle of interconnected and co-regulated systems that incorporate functions of memory, emotion, metabolism, motor planning, and executive processing. Of the legion of reward-associated and reward-modulatory neurotransmitters, including dopamine, norepinephrine, epinephrine, 2-arachidonoylglycerol, γ-aminobutyric acid (GABA), glutamate, serotonin, the endorphins, the enkephalins, and dynorphin, dopamine (DA) is classically viewed as preeminent in motivational aspects of behavior to which we simplistically ascribe the concept of reward.

From a neuroanatomical perspective, the classic reward system is the dopaminergic mesolimbic pathway (Figure 1.1). The key structures of this pathway are: 1) the ventral tegmental area (VTA) which acts as a central integration station for myriad systems of the brain and initiates dopaminergic signals; 2) the nucleus accumbens (NAcc) which contains dopaminergic terminals from the VTA and is essential in experiencing rewarding effects of drugs or natural behaviors (palatable foods, sex, nurturing, etc.), in distinguishing pleasurable from aversive stimuli, in motor planning and physical response to stimuli, and in processing environmental cues; and 3) the

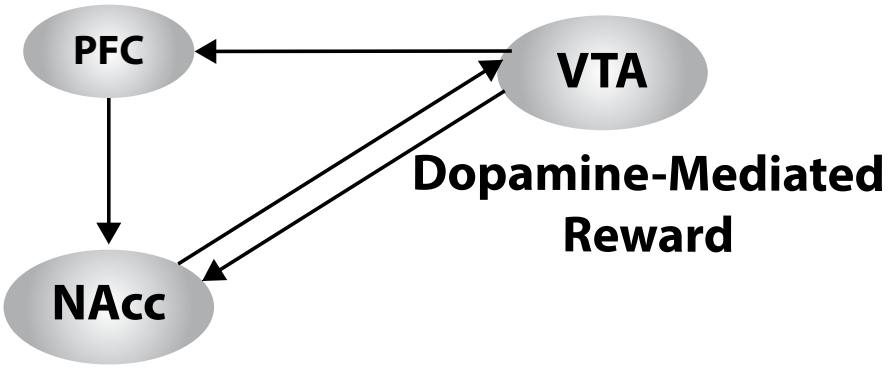


**Figure 1.1 Streamlined View of Key Mesolimbic Structures**

Highlighted here are the two classic structures of mesolimbic reward pathway. The ventral tegmental area initiates dopaminergic signaling to the nucleus accumbens but also receives GABAergic projections from the nucleus accumbens essential to regulating dopaminergic signaling (Ikemoto et al 1997). Though not a part of the mesolimbic pathway proper, the PFC is illustrated due to its participation in pathway modulation.

prefrontal cortex (PFC) which imposes cognitive influence upon actions and drives and is often referred to as the seat of self-control (Goto and Grace, 2008; Faure et al, 2010; Fuchs et al, 2004; Fuchs et al, 2008, Lemos et al, 2012).

As the nucleus of motivation and aversion, the NAcc makes use of information stemming from stress responses to discriminate between feeling a thrill of fear while watching a scary movie and feeling dread or terror when actually walking alone in a dark alley. Direct neural connections from the amygdala (AMYG) give emotional context; from hippocampal projections, declarative memory exerts influence; from PFC projections impulsive actions are moderated. Together, this connectivity lends perspective to the associative environmental cues that are processed in the NAcc. Even indirectly connected brain structures like the paraventricular hypothalamic nucleus (PVH) can exert influence through intervening brain regions and remote glands in the body such as the adrenal cortex can send signals through circulation. An understanding of the mechanisms that underlie normal functioning of the stress and reward systems is needed in order to describe some of the points at which these systems intersect. On that foundation, we can then overlay the actions of METH. Finally, to this we can add aspects of key developmental differences in both stress and reward responses exhibited during the period of adolescence that may suffer dysregulation and alter future behavior toward addictive substances. Let us begin with stress as our cornerstone.

### 1.1.1 Stress Defined as Distress

First, stress is defined in its simplest terms as any challenge to a system's homeostatic balance. However, stressors may either elicit a state of eustress (beneficial challenge) such as going out for a pleasant jog, or distress which constitutes a threat to our well-being either emotionally or physically (like preparing for a dissertation defense). Distress is what we typically think of when using the term "stress" and this common definition will be applied here as well.

The neurological outcomes of stress are determined through complex physical and emotional processing that recruit distinct neuronal populations from defined brain regions predicated, in part, by incoming information. Physical stress such as hemorrhage carries information from baroreceptors to the nucleus of the solitary tract prior to stimulating neurons in the hypothalamic seat of stress,

the PVH, without recruiting cortical structures. Pain, which represents a combined physical and emotional stress, stimulates sensory nerves in the periphery which send information to a host of cortical structures that enable interpretation of the stimulus, ascribe emotional texture, stimulate physiological responses that attempt to ameliorate the pain, and generate motor responses in addition to recruiting neurons in the hypothalamus. Emotional stress such as being confined to a small space without the possibility of escape or being exposed to predator odor is perhaps a less "messy" and more relatable stress. It, too, is characterized by a unique neuron-recruitment profile that involves emotional processing, declarative and associative memory, motivation states, and (sometimes) locomotor responses. Regardless of the stress, however, the core neuroendocrine responses evoked under the differing conditions of stress and even eustress are shared.

### 1.1.2 Stress Activates the Sympatho-Adrenal-Medullary Axis and Generates Neurotransmitters Epinephrine and Norepinephrine

Stress first activates the sympatho-adrenal-medullary or SAM axis (this is also the cascade known as the "fight or flight" response). The SAM axis prepares the body for immediate response to a stressor, using input from the sympathetic nervous system to release epinephrine (Epi) and norepinephrine (NE) from nerve terminals and the medulla of the adrenal glands (Ulrich-Lai and Herman, 2009; Johnson et al, 1992) (Figure 1.2). These neuroendocrine mediators stimulate vasoconstriction in the viscera and increase blood resources to heart and skeletal muscle, vasodilate the bronchioles for increased blood oxygenation, and increase heart rate.

Concurrent with peripheral secretion of Epi and NE, in the brain there is secretion of NE by neurons in the locus coeruleus (LC). This structure is a nexus of connectivity that receives projections from medial prefrontal cortex (mPFC), lateral hypothalamus (LH), and sends projections to neurons in the amygdala (AMYG) and extended AMYG (including bed nucleus of the stria terminalis or BNST), thalamus , hypothalamus and ventral tegmental area (VTA) (Valentino et al, 1992; Radley et al, 2008; Kravets et al, 2015; Wallace et al, 1989; Lopez et al, 1999; Buffalari and Grace, 2009; Pacack et al; 1995). Together, NE released from SAM activation and from the LC stimulates the second branch of the stress response, the hypothalamic-pituitary-adrenal (HPA) axis.

### 1.1.3 The HPA Axis Is the Second Branch of Stress Response and Produces Three Major Products

Whereas adrenaline can be viewed as the neuroendocrine end product in SAM axis activation, the end product for the HPA axis is cortisol in humans or corticosterone in rats (collectively referred to here as CORT). The HPA axis starts with the secretion of corticotropin-releasing factor (CRF) from distinct subpopulations of neurons in the PVH depending on the nature of the stress applied. CRF then enters the hypophyseal portal system which carries the signal to the anterior pituitary and stimulates release of adrenocorticotropic hormone (ACTH) (Kawata et al, 1983; Ulrich-Lai and Herman, 2009; Smith and Vale, 2006). Finally, the adrenal cortex, under the influence of ACTH produces CORT which serves to not only influence emotional processing, memory formation, and immune and metabolic functions, but also to negatively regulate the HPA axis at the level of CRF and ACTH production (Figure 1.2) (Ulrich-Lai and Herman, 2009; Smith and Vale, 2006). Negative feedback for the HPA axis occurs at several levels. CRF can actually inhibit the production of additional CRF. ACTH inhibits ACTH and CRF production, and CORT acts to suppress further secretion of additional CORT, ACTH and CRF. Furthermore, CORT has two mechanisms of negative feedback: fast and slow. Fast feedback is a non-genomic mechanism that shows assayable effect within 10 minutes of stress stimulus. Slow feedback requires 30 minutes after stress onset (Maser-Gluth et al, 1984; Popoli et al, 2012). In short, while the focus of SAM pathway activation is immediate survival, HPA axis activation serves to support recovery and homeostatic return through subsequent immune and metabolic adjustments. Both branches of the stress response are also vital in memory and learning, and both systems are subject to dysregulation be it from repeated or prolonged activation of the stress response or from inappropriate processing of the conditions and qualia of the presented stress or memory of the stress.

### 1.1.4 Anatomical Connections between PVH and NAcc Are not Direct

Examining direct neuronal projections among structures provides an anatomical perspective of the general flow of information in the stress response as it relates to reward. Looking at the lines of structural connectivity (Figure 1.3), we find that direct links between the “stress nucleus” (PVH),

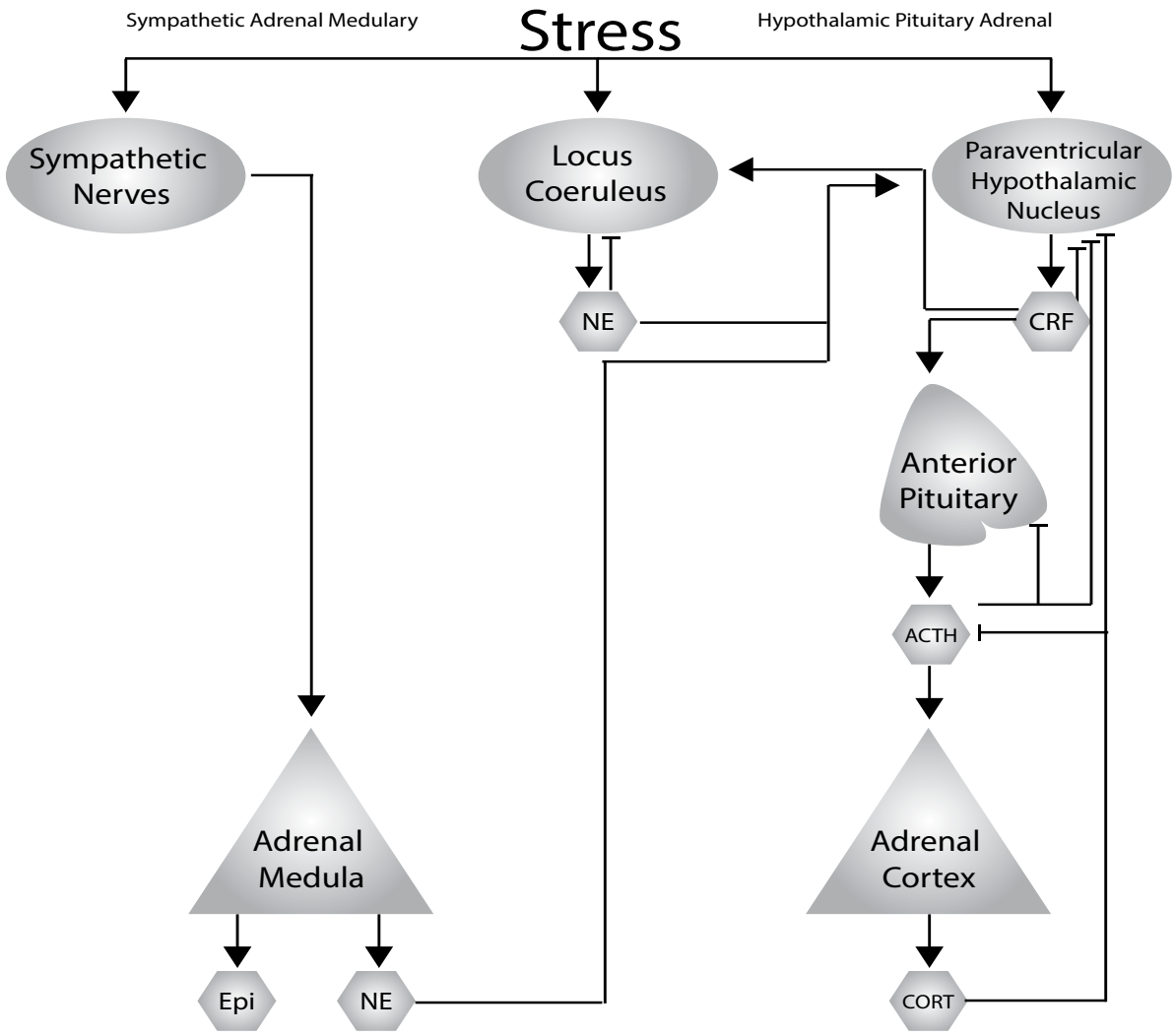


**Figure 1.2 SAM Pathway and the HPA Axis in Parallel**

Experience of a stressor initiates a cascade of events to allow immediate response and enable recovery. Arrows indicate activation increased production of signal. Note that while CRF induces NE secretion in the LC, NE serves to negatively regulate the system. CORT provides negative feedback to the HPA axis. Flat lines indicate suppression. Signaling molecules are rendered in hexagons.

and the "reward/motivation" nucleus (NAcc) have yet to be documented in the literature. However, within the mesolimbic system, the VTA does receive CRF projections from the PVH providing a second-degree relationship between PVH and NAcc in addition to a first-pass in modulating final DAergic release in the NAcc and PFC (Wang et al, 2005; Swanson et al, 1983; Roderos et al, 2007).

There are numerous additional structures that pass information to and from the PVH and the NAcc based on several experiential aspects, including: emotion (AMYG), executive function (PFC), and feeding or metabolic status (LH) (Figure 1.3).

CRF receptors (CRFRs) are also distributed throughout the brain including in the NAcc, indicating that these cells are capable of being activated by CRF secreted from the PVH. One caveat is that CRF is not solely secreted by cells in the PVH under conditions of stress. Several other

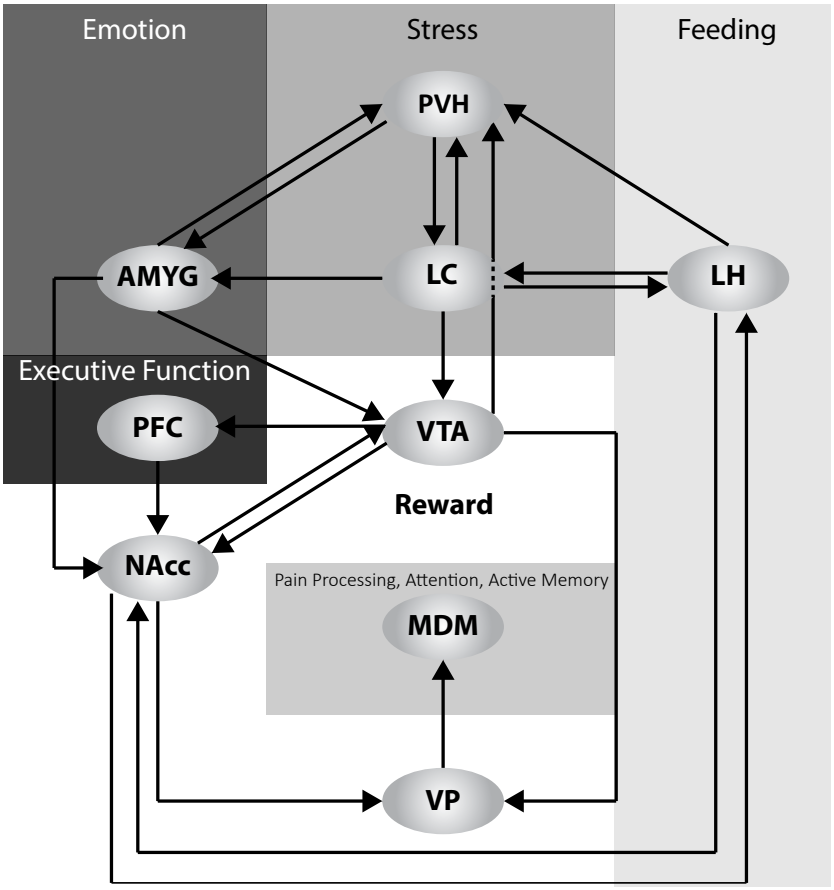


**Figure 1.3 Structural Connectivity of Stress and Reward**

This illustration is non-comprehensive in connectivity among the myriad structures linking the stress and reward pathways of the brain. Hippocampal connections have been omitted. The nigrostriatal pathway is not addressed in this image. Mediodorsal thalamic nucleus (MDM) and lateral hypothalamus (LH) are not addressed in this text but are included given the role MDM plays in experiential attention and that LH contributes in appetitive drives, both of which are emerging as an increasingly significant factors in the broader addiction story. Structure afferents and efferents were obtained through use of BAMS (Bota et al, 2003; http:\\brancusi.usc.edu).

structures that are in communication with the NAcc (AMYG, BNST, and hippocampus) have also been shown to produce CRF mRNA (Palkovits, 2000; Givalois et al, 2000; Funk et al, 2006). Thus it is difficult to definitively draw direct links between stress and reward structures without conducting challenging assays to directly assign the source of CRF acting in the NAcc as one specific structure. It is, however, possible to at least demonstrate that the PVH and NAcc are similarly activated under conditions of a specified stress paradigm, denoting the potential contribution of PVH-sourced CRF (please see the Results section for further review of neuronal recruitment in these structures in response to our stress paradigm).

## 1.2 Examining Molecular Mechanisms Involved in Motivation and Stress

Placing molecular events into anatomical context: DA neurons in the VTA release DA in the NAcc following an action potential. However, it is good to be acquainted with the multiple specific processes involved in DAergic neurotransmission (or at least the highlights thereof) in order to fully appreciate the manner in which stress can impact the functioning of the system. The main stages of the DAergic cycle are: DA synthesis, vesicular packaging, vesicular release, receptor binding, and DA clearance (Figure 1.4). Synthesis of DA involves conversion of tyrosine into L-DOPA by tyrosine hydroxylase (TH) (for review, see Daubner et al, 2011). This enzyme is particularly significant as it is the rate-limiting enzyme in the conversion process. Expression of TH is governed in part by the dopamine autoreceptor D2 (Best et al, 2009; Daubner et al, 2011; Ford, 2014). After synthesis, DA (in addition to other monoamine neurotransmitters) is taken into vesicles via vesicular monoamine transporter 2 (VMAT2) and retained until action potentials spur vesicular binding and release of DA into the synaptic cleft. Released DA then can either bind to post-synaptic DA receptors (D1 being the best characterized of these in addiction literature), bind pre-synaptic receptors (the D2 autoreceptor), be degraded in the synaptic cleft, or be taken back into the pre-synaptic neuron via dopamine transporter (DAT) and metabolized. While reward is mediated through D1 and D1-type receptors, the function of D2 on DA synthesis identifies this receptor in a critical regulatory role.

### 1.2.1 The Stress Hormone CORT Has Specific Means of Influencing DA Neurotransmission

CORT has well-characterized receptor-mediated effects both pre- and post-synaptically.

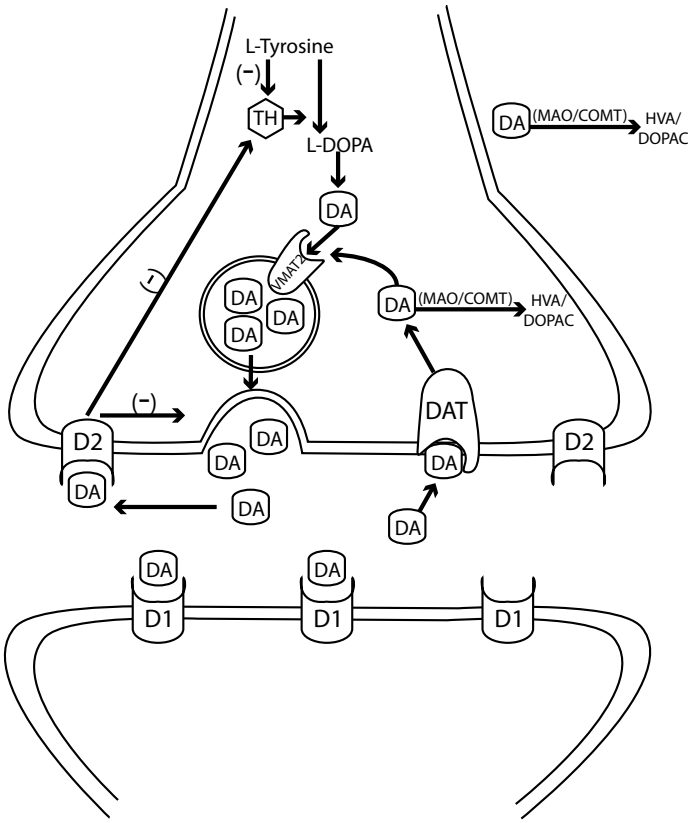


**Figure 1.4 The Dopaminergic Cycle**

Dopaminergic signaling is controlled, in part, through negative feedback mechanisms governed by D2 binding DA. Additional mechanisms of clearance are intracellular and extracellular breakdown of the monoamine into homovanillic acid (HVA) or 3,4-dihydroxyphenyl acetic acid (DOPAC) or 3-methoxytyramine (not shown). This is mediated through activities of monoamine oxidases (MAOs) or chatecol-O-methyltransferase (COMT).

Pre-synaptically, CORT binds glucocorticoid receptor (GR) and enhances DA release via glutamate release (Figure 1.6). Post-synaptically, reward-related effects occur through CORT/GR interactions with transcription factors such as Fos to induce systemic changes. Reward related effects can also occur through secondary signal cascades that promote Ca2+ uptake and alter the pH of the environment with the net result of shifting arachidonic acid metabolism to produce the endocannabinoid 2-arachidonoylglycerol (2-AG) (Hill, 2010) (for reviews see Popoli et al, 2013 and Lopez et al 2008). Furthermore, in the NAcc, the presence of CORT may actually hamper DA clearance through disrupting the function of organic cation transporter 3 (OCT3) (Graf et al, 2013).

Taken together, CORT not only acts to facilitate stress coping and recovery and inhibit the HPA axis, but also contributes to reward circuitry function by facilitating neuronal excitation, promoting production of endocannabinoids, and enhancing DAergic signaling in the synaptic cleft.

### 1.2.2 The Influence of CRF on Addictive Behaviors Is Based on Receptor, Location and Ligand

CRF and signaling events mediated through CRFRs also play a large role in stress-associated and cue-related drug-seeking behaviors (Figures 1.5 and 1.6). Administration of CRF to multiple locations in the brain including NAcc, AMYG, VTA, or BNST have been shown to facilitate drug reinstatement for cocaine, methamphetamine, heroin, or alcohol. Furthermore, blockade of CRFRs have been shown to attenuate stress-induced drug reinstatement (Lu et al, 2001; Lu et al 2000; Le et al, 2000; Shaham et al, 1997; Erb et al, 2001; Erb et al, 1999). However, the receptors governing attenuation of reinstatement appear to differ based on the region of the brain examined. For example, blockade of CRFR1 in the BNST is effective in diminishing reinstatement of drug-seeking, whereas in the VTA this is achieved only by blocking CRFR2 and not CRFR1 (Erb et al, 2001; Meloni et al, 2006; Wang et al 2006; Wang et al 2007; Lu et al, 2001; Lu et al 2000; Shaham et al, 1998; Williams et al, 2014).

To further complicate matters, the urocortins (UCNs) are additional CRFR ligands that, like CRF, are upregulated by stress. CRFRs and their ligands are differentially expressed throughout the brain, and exhibit variable affinities. CRFR1 is relatively ubiquitous, binds UCN1 and has a higher affinity for CRF than does CRFR2 (Hauger et al, 2009). CRFR2 is far more specific in localization, having been documented in NAcc, BNST, AMYG, LS, and the dorsal raphe nucleus (Lemos et al, 2012; Van Pett et al, 2000; Chalmers et al, 1995), and displays higher affinity for UCN2,3 but will also bind UCN1 and CRF. Furthermore, the activation of these receptors appears to govern different functions in the stress response.

In CRFR1 knockout mice, anxiety measures associated with alcohol withdrawal are significantly reduced (Timpl et al, 1998). Coupled with the observation of decreased ACTH production in these same mice, binding of CRFR1 can be considered anxiogenic. Conversely, CRFR2 knockouts present a more rapid elevation in ACTH and CORT levels post-restraint stress and a marked

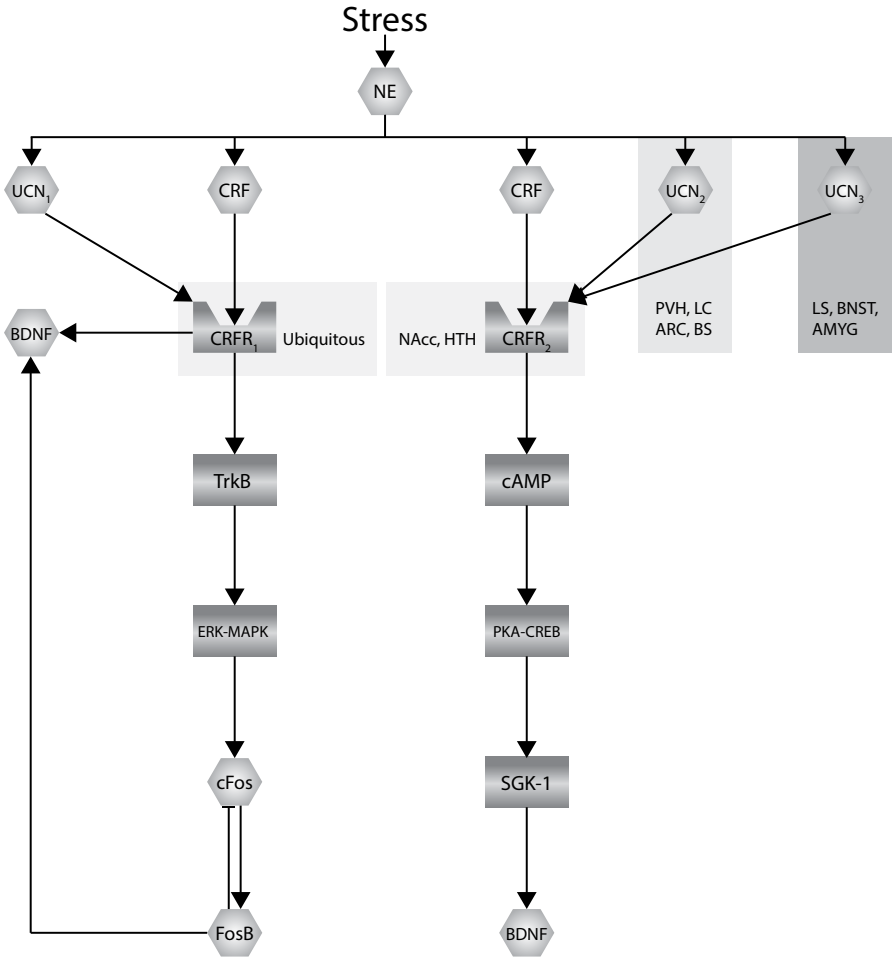


**Figure 1.5 Stress-evoked Non-CORT Molecular Cascades**

Urocortins (UCN) and CRF both trigger a cascade of events throughout sites in the brain and body. $CRFR_2$ in the NAcc and HTH has higher affinity for $UCN_2$ and $UCN_3$ than for CRF (Hauger et al, 2009). These UCNs are also stress-inducible and are specifically distributed among multiple structures that contribute to connections between reward and stress. Arcuate nucleus (ARC), brain stem (BS), hypothalamus (HTH) lateral septum (LS). Downstream products such as cFos are particularly relevant in the context of neuron activation assays where it acts as a classic and well-characterized marker in immunohistochemistry. FosB (and ΔFosB) overexpression has been demonstrated to promote reward-seeking or addictive behaviors (Nestler, 2011). BDNF overexpression, particularly in VTA, can induce a shift in GABA-ergic signaling from inhibitory to excitatory and induce addictive behaviors (Vargas-Perez et al, 2009).

difference in behavioral measures of anxiety in the elevated plus maze, suggesting that CRFR2 has an anxiolytic function (Bale et al, 2000). As an aside, Neufeld-Cohen et al (2010) published a study demonstrating that the UCNs as a whole are essential in appropriately moderating anxiety after stressors have passed, and may represent yet another point of vulnerability in the dysregulation of

stress processing which may contribute to inappropriate responses to rewarding stimuli. To further illustrate the complexity of the relationship of the CRF system to reward, the CRFR2 agonist UCN3 administered in the central amygdala (CeA) of ethanol-dependent rats was shown to decrease ethanol self-administration, but paradoxically increases self-administration in non-dependent rats (Funk and Koob, 2007).

Iemolo et al (2013), in a complex examination of regular versus palatable food diets, administered a selective inhibitor of CRFR1 in three regions of the amygdalar complex. When administered the CeA, palatable food consumption was enhanced, and anxiety behaviors were mitigated in animals experiencing subsequent palatable food withdrawal. Infusions into the basolateral amygdala increased normal food intake but abolished palatable food withdrawal hypophagia in a dose-dependent fashion and failed to diminish withdrawal-induced anxiety. No differences were observed for animals administered antagonist in the BNST. Collectively, dose/strength of signal and location in the brain can profoundly alter the cross-influence and expression of stress-related and motivation-related behaviors.

Finally, in mice, Lemos and colleagues (2012) documented conditioned place preference (CPP) for the chamber associated with CRF infusions into the NAcc, suggesting that this provided a pleasant or rewarding stimulus. Furthermore, CRF increased DA release through the activation of both CRFR1,2. However, when mice were severely stressed through a forced swim paradigm, CRF no longer elicited DA release, and mice avoided the CRF-associated chamber in CPP, suggesting that CRF administration was now perceived as an aversive stimulus. These effects were persistent among animals tested even 90 days after forced swim, demonstrating that at least certain stressful events may induce long-term alterations in reward perception.

### 1.2.3 Assembly of a Unified Picture of Stress at the DA Synapse

Figure 1.6 encapsulates the events of stress and subsequent effects on DA neurotransmission. In summary, CORT and CRF, through GR and CRFR1, respectively, facilitate DA release through alterations in CA2+ influx and increase of glutamatergic activity. CRF (or UCNs) acting on CRFR2 increases GABAergic activity with a net result in decreasing DA release (Freund et al, 2003). Fast

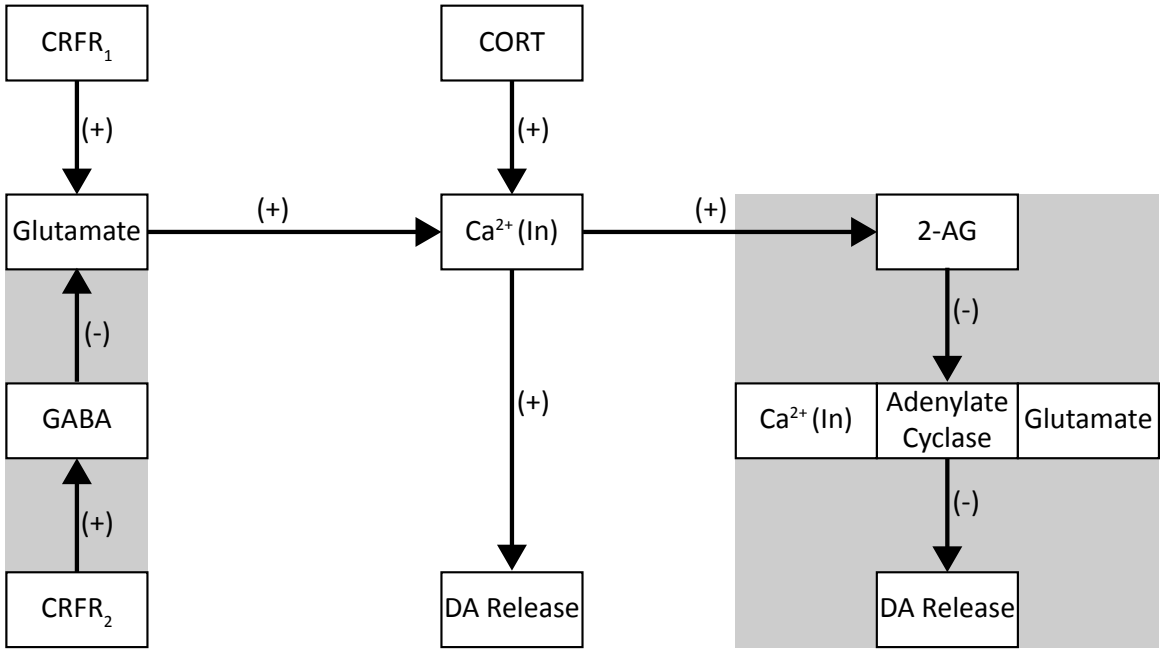


**Figure 1.6 The Intersection of Stress and Dopaminergic Signaling**

Contributions from both early and late products of the HPA axis have opposing functions that both enhance and inhibit DA release. While CORT immediately functions to promote DA signaling, subsequent effects of CORT lead to the suppression multiple pathways of neuron excitation by way of endocannabinoid signaling. Actions of CRF largely depend upon the receptor that binds it with $CRFR_2$ resulting in GABA inhibition and $CRFR_1$ resulting in glutamate excitation. Highlighted in gray are the inhibitory pathways of DA release in the NAcc.

feedback mechanisms for CORT also engage a pathway ultimately reducing DA release. Fine-tuning these responses involves a balance of the signals stemming from unique brain nuclei. In this manner, stress and reward are intimately interconnected.

### 1.3 METH—Molecules, Metabolism, and Mechanisms

The following section seeks to address the aspects in which METH interacts with the dopaminergic system and can introduce dysregulation resulting in escalation and addition. The general overview is that METH's properties as a highly lipophilic weak base enable the drug to easily cross the blood brain barrier (BBB) and to passively cross membranes permitting rapid and potent effects, and to collect in acidic vesicles thereby shifting the internal pH. Taken from another

viewpoint, the lipophilicity allows access while basicity and molecular structure support METH's functional effects.

### 1.3.1 METH Structure and Properties Enable Multiple Interactions with Monoaminergic Machinery

The chemical characteristics of METH allow it to enter cells and vesicles through two modes: 1) high lipophilicity permits passive diffusion across membranes; 2) structural homology with monoamines allows active or facilitated transport via membrane transporters. To gain perspective on the structural similarities of amphetamine-class drugs and the monoamine neurotransmitters, Figure 1.7 is provided.

As a weak base, once METH is concentrated within monoamine vesicles through either passive diffusion or active transport, vesicle pH rises, collapsing the gradient required for monoamine accumulation. In addition to this, vesicular monoamine transporter 2 (VMAT2), is reversed in function, and actively transports DA, serotonin (SER) and NE into the cytosol of the neuron terminals (Wimalasena, 2011). This surge in the cytosolic concentration of monoamines contributes to a shift in the functioning of their reuptake transporters (DAT, SERT, and NET) from a net intake of monoamine signal from the synaptic cleft to a net outflow of neurotransmitters from the presynaptic terminal. Furthermore, there is evidence suggesting that METH and other amphetamines may also physically interact with transporters (particularly with DAT), by partially blocking monoamines from reaching active sites and by inducing a conformational shift in transporters that results in the net export of monoamines (Sulzer et al, 2005). Among the monoamines affected by METH exposure, the greatest effect is seen in DA release, making the dopaminergic system one of particular interest in this field of research (Sulzer et al, 2005).

While structural homology among METH and other amphetamine derivatives does permit physical interactions with transporters, it does not appear to directly interact with post-synaptic monoamine receptors. Thus, it does not appear to act as a signaling analogue. It does, however, indirectly influence the activation of receptors by way of increasing the concentration and duration of monoaminergic signal in the synaptic cleft. In this manner, extended use of METH can alter the

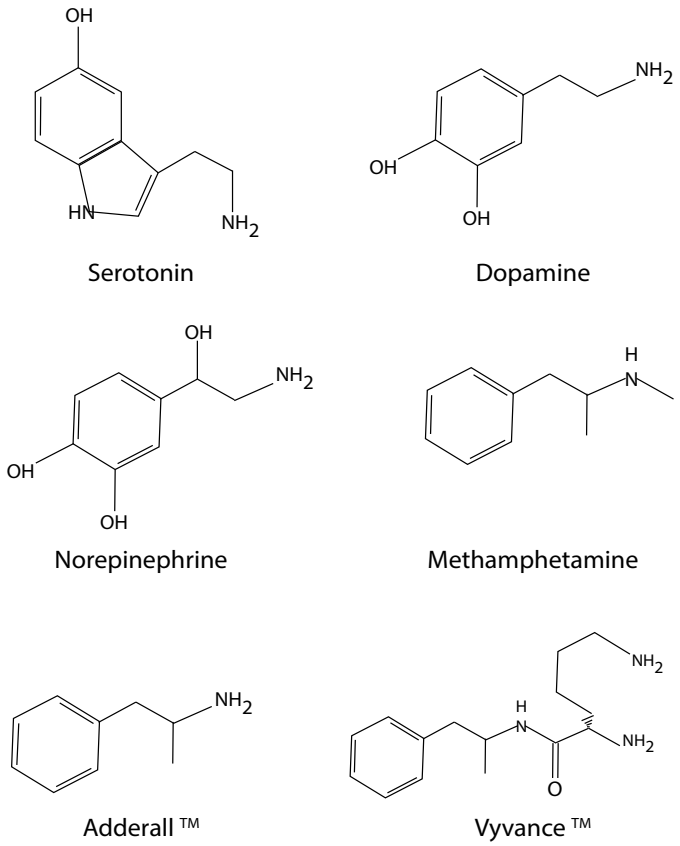


**Figure 1.7 Structural Homologies Among METH, METH-Related Compounds and Monoamines**

expression of monoamine receptors including the autoregulatory dopamine receptor D2.

As it relates to METH addiction, in cases of high-dose consumption, TH is significantly reduced in control samples from striatal tissues including the NAcc, an effect that has been correlated with damage to dopaminergic neurons (Broening et al, 1997; Larsen et al, 2002; Bowyer et al, 2006; Krasnova et al, 2010). However, in the initial stages of METH exposure, TH expression transiently increases. This, coupled with the reversals seen in VMAT2, leads to DA increases in the cytosol of the pre-synaptic neuron which not only provides substrate for DAT to transport into the synaptic cleft, but also creates a condition of oxidative stress in the cell. Eventually, these oxidative conditions are believed, in part, to contribute to formation of inactive DAT complexes seen during the early stages (24 hours) of withdrawal (Hadlock et al, 2010; McFadden et al, 2012). The net

result of long-term drug exposure has been recorded as a downregulation of dopamine receptors and a decrease in DAT that can lead to depressive affect due to dampened dopaminergic signaling thus requiring ever-increasing amounts of drug to attain the same level of euphoria (Volkow et al, 2001; Krasnova et al, 2010; Schwendt et al, 2009; Koob and Le Moal, 1997 and 2008; Wilson, 1996; Wang, 2012). To summarize a list of these effects, Figure 1.8 compares the normal versus METH-induced conditions at the dopaminergic terminals in the NAcc.

### 1.3.2 METH Metabolism Provides Insights into Withdrawal Symptom Appearance and Abatement

Though not pivotal to this study which focuses on pre-drug exposure conditions and subsequent behavior toward freely-accessible drug, the time course of METH metabolism should at least be mentioned, as it is relevant to protocols employed by this study in post-study tissue collection and may inform analysis of drug acquisition behavior. With respect to routes of administration, prescription METH and METH derivatives are taken orally and off-label use of these compounds typically utilizes the oral route, though METH can also be inhaled (smoked) or intravenously injected. The route of administration does influence the duration and concentration of METH in the brain, though clearance mechanisms have been most extensively documented in oral administration routes.

Once internalized, METH enters the bloodstream and is distributed readily to highly blood-perfused sites including the heart and brain. The euphoric effects of METH are reported to last for as long as four hours, and the plasma half-life is extensive (12 hours) relative to that of similar sympathomemetics like cocaine (Chauhan et al, 2014; Cho et al, 2002). It should also be noted that METH exposure activates the SAM and HPA axis thus providing stress influences that may enhance the rewarding state. The dominant route of METH clearance occurs through urination, though the efficiency with which this occurs depends largely on urine pH (Davis,et al, 1971). METH undergoes several steps of conversion, including hydroxylation and demethylation, the latter of which produces the psychoactive drug amphetamine (Figure 1.7) (Li et al, 2009). Complete clearance of METH and metabolites occurs between 48 and 72 hours after ingestion (Caldwell et al, 1972). The

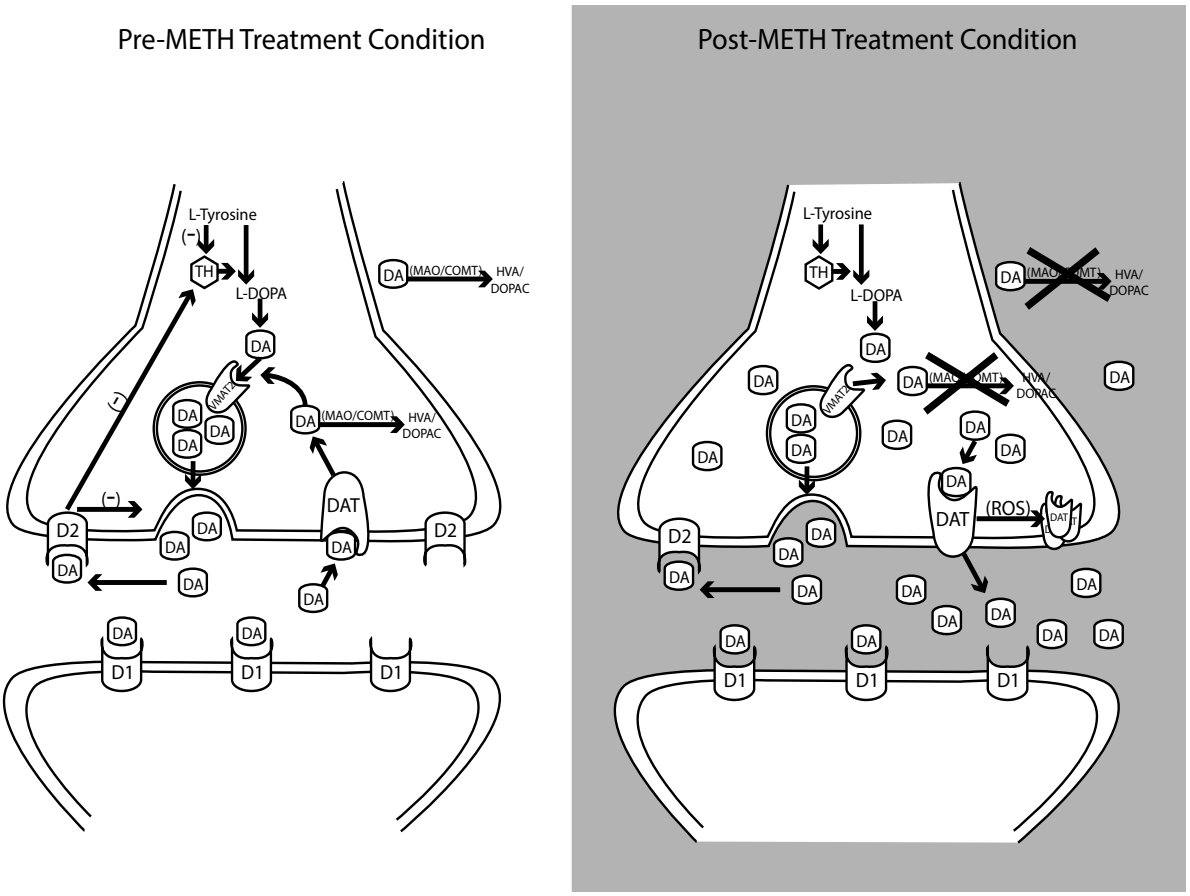


**Figure 1.8 Summarizing the Effects of METH on the Dopamine Cycle: This is your brain. This is your brain on drugs.**

The immediate effects of METH exposure are demonstrated below. TH is transiently upregulated and contributes to a growing DA pool in the cytosol. The function of VMAT2 and DAT are reversed and DA is no longer vesicularized leading to more cytosolic DA which now facilitates formation of reactive oxygen species (ROS). Oxidative stress facilitates the formation of DAT complexes. Intracellular and extracellular MAO activity is hindered, allowing the pool of DA to persist and bind DA receptors until the cells can no longer support this. Long-term abuse leads to retraction of dopamine receptors and reduced DAT that can persist for many months (not illustrated) (Volkow et al, 2001).

span of time between plasma half-life and full clearance also marks the peak period of withdrawal symptoms (craving, anxiety, depression) in abstaining chronic METH users (McGregor et al, 2005). In these withdrawal states, the emotional anxiety and drug craving experienced represents an additional source of stress stimulus which has been implicated in driving drug-seeking behavior and drug relapse (Koob et al, 2008; McGregor et al, 2005; Jang et al, 2013).

### 1.4 Adolescence Represents a Period of Change in Stress and Reward Responses

Armed with a basic understanding of the interplay of stress and reward systems and the means by which METH and closely related compounds disrupt this function, we can now highlight aspects of adolescent development that may suggest stress as an influential factor in later responses to METH. Published research addressing changes in stress response systems and the mesolimbic reward pathway during the adolescent period is a growing body of work, though still less prolific than what is found concerning responses in adults and particularly adult males. However, an excellent review by Burke and Miczek (2014) compiles much of that research and provides, quite elegantly, a thorough treatment of this very topic. Here, I highlight some of the content provided by those authors and offer observations found in additional resources.

First, an effective chart published by Burk and Miczek succinctly illustrates the developmental progression of the stress and dopamine reward systems from PND 10 through adulthood based on literature review. Presented in Figure 1.9 is a modified version of their diagram, highlighting the findings for nucleus accumbens and HPA markers and layering an additional observation. In HPA axis function, the response of ACTH (an intermediate product of HPA axis activation) follows an inverted "u" curve across the duration of adolescence whereas CORT levels gradually drop over the course of early adolescence and stabilize mid-way through the period (Foilb et al, 2011). In higher resolution, the time course work conducted by this group shows that early ACTH responses to stress (30 minutes post-restraint stress onset) are more exaggerated from PND 30-50 whereas circulating and adrenal CORT are sharply elevated at 30 minutes post stress initiation and maintain elevated levels even 90 minutes after stress. This same effect is not seen in PND 40+ animals whose CORT time course closely tracks that of adults (PND 60+).

For dopaminergic activity in the NAcc, there are also distinctions in development, if somewhat less dramatic than what occurs within PFC (data not illustrated). As depicted in our version of the chart, during the early stages of adolescence there is an increase in what is termed in the review as "dopaminergic fibers" found in the NAcc. This measure was originally obtained

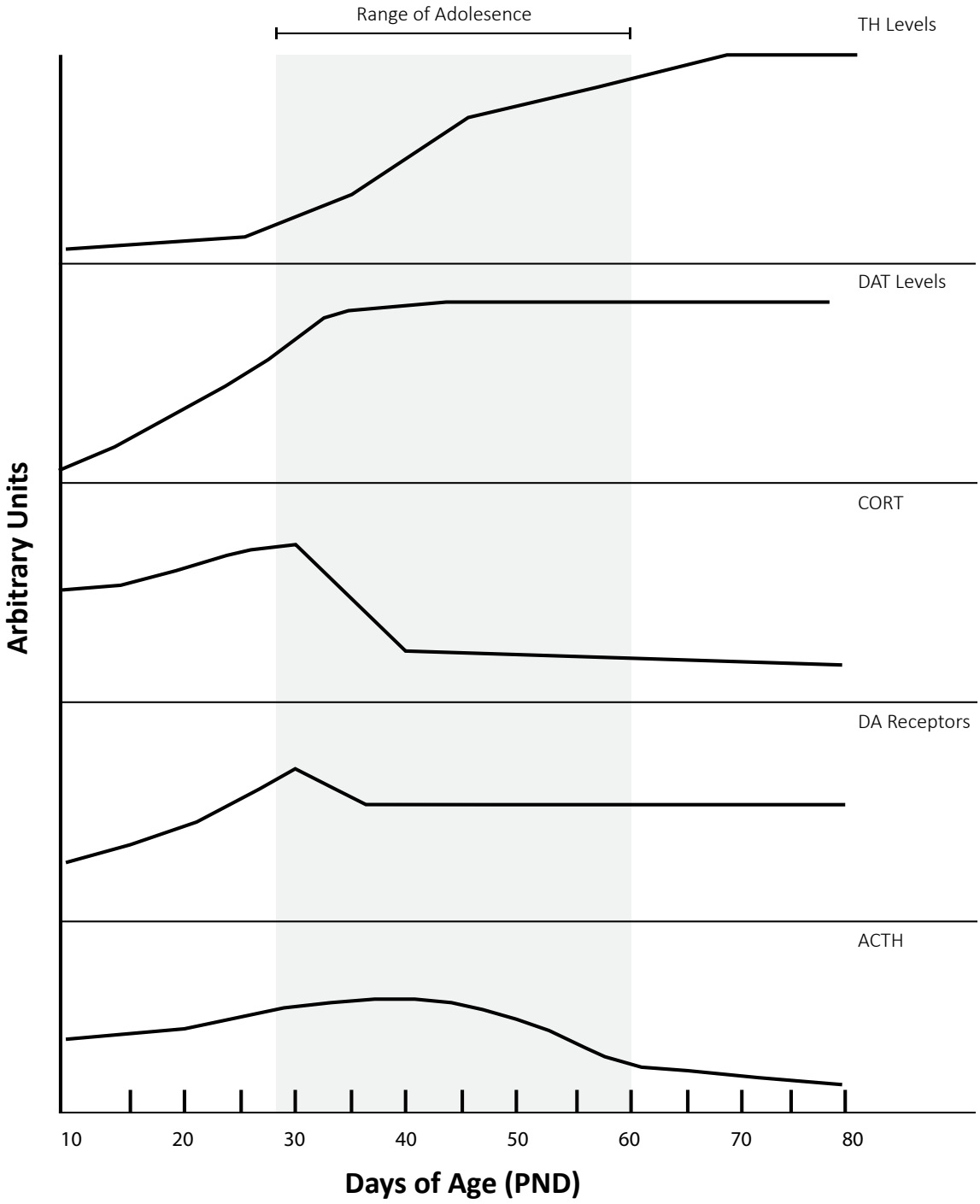


**Figure 1.9 Phases of Stress and Reward Shifts Across the Early Life of the Rat.**

Gray shaded region denotes the broadly-accepted age range for adolescence in rats. Grey lines establish DA-related baseline conditions across the developmental span. Black lines are stress-induced levels observed in stress-related hormones. Vertical axis represents an arbitrary unit of measure.

in autoradiography studies of DAT which showed relatively low levels of DAT label at PND 7 that increased by PND 60 by ~6-fold (Tarazi et al, 1998). Given that DAT activity increases steadily within the NAcc suggests that DA clearance mechanisms are in development in the early stages of adolescence with the potential effect of greater signal delivered upon motivating stimuli (an outcome supported in human studies reported in Ernst et al, 2005 later discussed), and may rely more heavily upon D2-mediated regulation or the limiting of TH available in NAcc terminals. This last speculation is enhanced by examining the work of Naneix et al (2012), and Leslie et al (1991) which contributed to the DA receptor track depicted in Figure 1.9. First, TH expression in the NAcc of early adolescents is significantly lower than adults with a spike observed at PND 45 and continued increase throughout development. The nadir of TH coincides with lower presence of DAT and dopamine receptors (including autoreceptor D2), placing putative natural limits on available DA during a time of impaired DA regulation. This is further supported by accompanying observations of DA elevation at PND 45 when regulatory systems are coming online (Naneix et al, 2012). The initial increase in TH occurs not long after DAT stabilizes at adult levels. Furthermore, while the receptors are not parsed out in the figure, distinctions in receptor presence are important to note. D2 spikes in quantity at PND 35 (as do D5 and D4) perhaps in presage of increasing demands for dopaminergic regulation in the NAcc in contrast to D1 receptors which appear early in development (PND 7) and stabilize by PND 14 at only slightly elevated levels (Leslie et al, 1991) (Naneix et al, 2012).

To examine behavioral paradigms that suggest long-term (persisting into adulthood) consequences stemming from stress exposures, the literature is difficult to correlate. A diversity of stress paradigms are applied at various stages in adolescent development and can yield highly variable results. However, focusing on studies that are compatible in age and at least semi-consistent in model, we can glean a few examples of stress and the impact on feeding, anxiety (evidence of a dysregulated stress response), and response to rewarding stimuli (often sucrose).

In rat models using acute versus chronic restraint and immune challenge, acute and chronic restraint administered during early adolescence had long-lasting behavioral and physiological

effects when animals were subsequently stress-challenged as adults (Traslaviña et al, 2014). CORT levels in adults exposed to an acute stressor were significantly higher than controls, an effect not seen in immune-challenged animals. However, in behavioral measures, both chronic restraint and chronic immune challenge resulted in decreased time spent in the open arms of an elevated plus maze (EPM) test, indicating that both conditions experienced as an adolescent increased a measure of anxiety among adult animals. Chronic restraint used in a comparison against chronic variable stress and chronic isolation stress also produced immediate and long-term changes in body weight, and decreased intake of a palatable substance, suggesting that restraint stress in particular was effective in augmenting reward and motivation responses long-term (Bourke and Neigh, 2011). Additionally, social isolation stress during adolescence was observed to decrease consumption of palatable substances during the adolescent window in a study by Hong et al (2012). Body weight discrepancies observed in chronically restrained adolescents was supported by work from Cruz et al (2012) when compared to chronic variable stress and control animals in addition to decreased locomotor responses in a novel environment.

In studies examining human responses in adolescents versus adults in a gambling paradigm, Ernst and colleagues demonstrated that the nucleus accumbens of teenagers was more active in response to reward withholding and showed less change in the amygdala versus adult counterparts (Ernst et al, 2005). This demonstrates a certain amount of emotional resilience in response to negative outcomes, and more robust motivated behavior despite the occurrence of failure among adolescents. This demonstrates distinct differences in reward and emotion processing that may also help refine the nature of adversity that spawns adverse addiction outcomes.

Thus, as we look at the intersection of drug and reward maturation, the blanket assumption is that any acute or chronic stress applied during periods of adolescent development will influence later responses to stress and reward. The growing body of literature suggests that not only the type of stress applied, but the duration and the age at which that stress is administered is an integral determinant in future responses to addictive substances and rewarding stimuli. Of the systems reviewed, restraint stress appears to offer consistent persistence of influences on measures of

reward and anxiety and offers a system that is not only well characterized in adult animal models but one that is a relatively "clean" emotional stress. Examining the developmental aspects of stress and reward, PND 36-54 offers a unique window of relatively well developed DA signaling that overlaps with transitions of stress feedback mechanisms from juvenile to near-adult levels, making this an intriguing age to investigate.

**1.5 Summary of the Literature Highlights a Need to Assess Stress as a Risk Factor of Addiction**

- Stress can produce variable outcomes on the functioning of reward circuitry including the efficacy with which DA is released in the mesolimbic dopamine reward pathway.
- Components of stress are associated with drug relapse in recovering addicts or extinguished animals.
- Variability seen in behavioral measures (including behavior toward drug) and in physiological responses to stress is dictated by the type of stressor applied, the duration or repetition of exposure, and the age at which it is applied.
- Depending upon the paradigm, stress administered during adolescence can alter stress and reward responses both behaviorally and molecularly well into adulthood, potentially as a function of disruption in developmental events.
- Restraint stress is a well-characterized paradigm that offers a strong emotional stress capable of inducing persistent effects (including from adolescence to adulthood) without introducing aspects of physical stress.
- METH and related compounds can be prescribed during adolescence or adulthood for treatment of certain conditions, necessitating a means by which patients at risk for addiction can be identified during physician visits.
- Key molecular components of the dopamine reward pathway can be affected by METH exposure.
- METH exposure and METH withdrawal activate the SAM and HPA axis but little is known about the influence stress exposures prior to METH exposure exerts on behavior toward that drug.

As stated in the Preface, the purpose of this body of research is to establish more defined

measures of stress criteria required to establish METH addiction vulnerability with the goal of contributing to patient assessment prior to prescribing METH-like medications. The aims of this research, therefore, are to determine:

1) If chronic exposure to emotionally stressful events prior to drug exposure increases vulnerability for drug use escalation; and

2) If ontogeny exerts influence over the impact of stress pre-exposure on drug abuse vulnerability.

Given the contributions of stress to the function of the reward system ***we hypothesize that chronic stress will result in escalation regardless of the age of administration and that these changes will be reflected in alterations to the expression of dopaminergic markers TH, D2, and DAT***.

# 2. MATERIALS AND METHODS

## 2.1 Animal Protocols

### 2.1.1 General Care

Adult (PND 70-74 at entry into study) and adolescent (PND 34-36 at entry into study) male Wistar rats were used in this study. To reduce the risk of negative social interactions, and to ensure consistency across manipulations, animals were singly housed beginning 3-4 days prior to study entry. In the case of animals requiring transfers from Bioscience to the Psychology facilities, animals were transferred and singly housed 7 days prior to study entry. Vivarium house lights were maintained on a regular 12 hour light/dark cycle in both facilities, and the room controlled for temperature and humidity. Food and water were provided ad libidum throughout the study with the exception of the daily 6 hour period during which animals were in the VSA protocol (see below).

### 2.1.2 Animal Restraint Stress and Behavioral Scoring

All restraints were carried out 1.5-2.5 hours after onset of the light cycle, a time consistent with low endogenous CORT levels. Restraint or exposure sessions were conducted in the animals' home cages in the housing room using vented acrylic restrainers with adjustable closure slides. Animal length and girth were assessed to ensure the restrainer selected for use did not impinge upon any region of the body in order to reduce the possibility of pressure or pain stimuli from contributing to the stress experienced. Animals were either restrained for 30 minutes per day for 14 days (repeated stress group- R), exposed to the restrainer for 13 days for 30 minutes per day then restrained on the 14th day for 30 minutes (acute stress group- A), or exposed to the restrainer for 14 days for 30 minutes per day (control stress group- C) (see Figure 2.1 and 2.2 for experimental overview and time line, and Table 2.1 for animal allocations). All restrainers were removed from the home cages at the end of the stress period to control for novel object presentation, and cleaned daily to remove animal scent, fecal residue and urine.

During restraint sessions in the final adult male cohort, animals were observed for 10

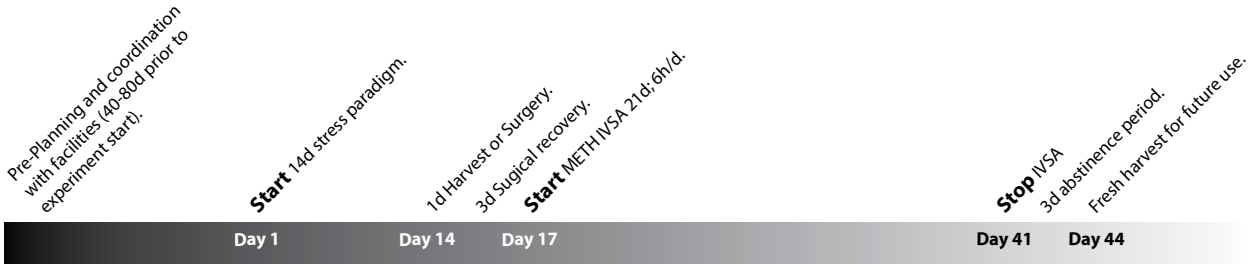


**Figure 2.1 Study Timeline for IVSA**

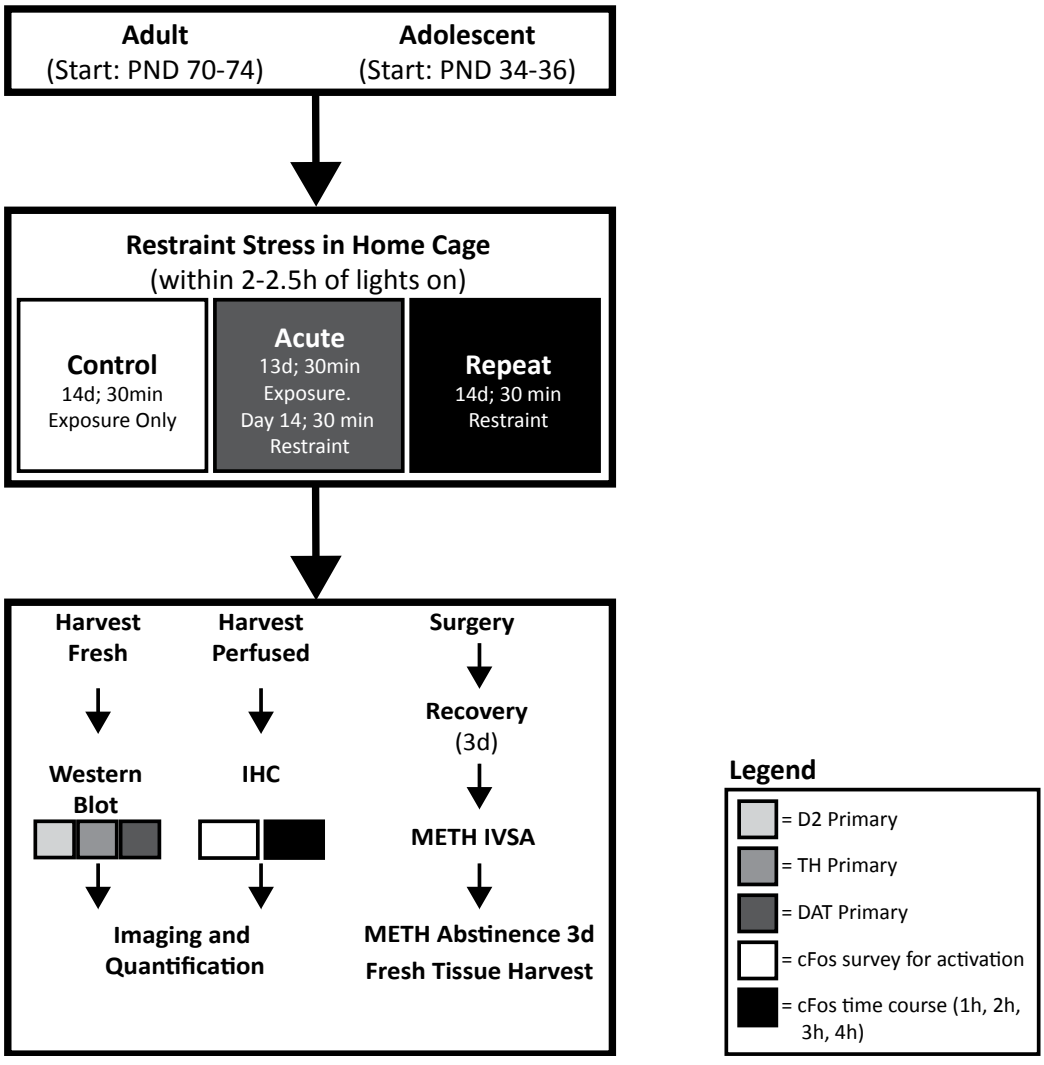


**Figure 2.2 Animal Allocation and Design**

**Table 2.1 Animal Allocations for Studies**

| | | Control Adult | Control Adolescent | Acute Adult | Acute Adolescent | Repeat Adult | Repeat Adolescent |
|---|---|---|---|---|---|---|---|
| **IHC** | *PVH* | 6 | 6 | 5 | 6 | 5 | 6 |
| | *NAcc* | 6 | 6 | 5 | 6 | 5 | 6 |
| **WB** | *TH* | 4 | 5 | 7 | 6 | 6 | 8 |
| | *D2* | 4 | 5 | 7 | 6 | 6 | 8 |
| | *DAT* | 6 | 4 | 5 | 5 | 5 | 7 |
| **IVSA*** | | 7 | 5 | 5 | 4 | 6 | 4 |

***Three control adult animals were placed into saline groups for activity comparisons in IVSA.***

seconds every five minutes for locomotor activity during restraint. Activity scores of 1 were assigned to animals observed chewing, struggling, or attempting to turn around in the restrainer during the window of observation. Animals that were quiescent during observation were assigned a score of zero. Scores were then tallied for each day to give a daily activity score and summed across the duration of the 14 days to obtain a total activity score.

### 2.1.3 Catheter Construction and Patency Regimen

Catheter construction was done in-house using an 11 mm 22 gauge catheter guide with 5 mm upper reveal. The lower section of the guide was bent into a "J" curve prior to tubing fitting and the ends were smoothed and checked for flow using isopropyl alcohol. The main catheter tubing (0.33 mm I.D. silastic) was softened in Citrisolv solvent prior to fitting over the lower portion of the catheter guide with an additional length silastic tubing (0.64 mm I.D. ) acting as a protective sheath to the line at the inlet juncture (Figure 2.3). Catheter guides and tubing were then affixed to a 3.5-4 cm diameter circular mesh backing (Sefar Propytex 500 µm) using dental acrylic set in an aluminum block mold. Once set, the catheters were removed from the mold and a small bead of

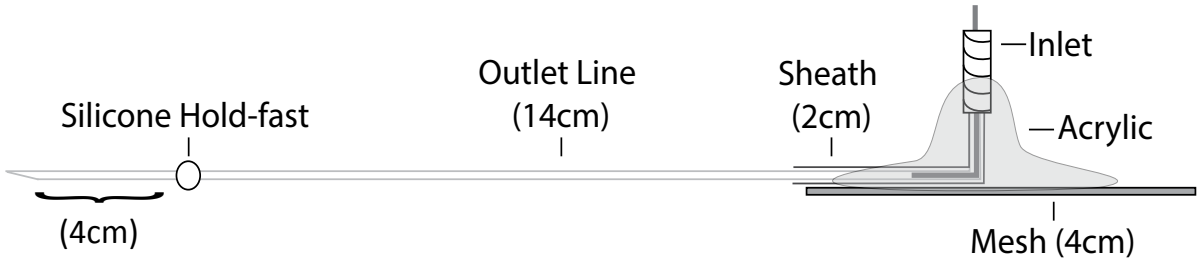


**Figure 2.3 Catheter Construction Diagram**

clear silicone adhesive was applied as a hold-fast 4 cm from the end of the outlet tubing (Figure 2.3). After allowing overnight curing, the catheters were tested for patency with ethanol and autoclaved prior to implantation. Immediately before implantation, patency was assessed one final time with sterile saline. Post-implantation, catheter patency was checked through administration of Brevital (methohexital sodium) and animal muscle tone observed (Table 2.1). Daily flushes both before and after IVSA sessions with 0.2 ml Timentin in heparinized saline were done to maintain catheters. Animals that failed patency checks or lost patency during the study were excluded from statistical analysis.

**Table 2.2 Study Drugs, Administration Routes, and Dose**

| Compound | Provider | Purpose | Concentration | Dose | Delivery |
|---|---|---|---|---|---|
| Ketamine | Bionichepharma | Anesthetic/analgesic | | 50 mg/kg | I.P. |
| Xylazine | Lloyd Laboratories | Anesthetic/muscle relaxant | | 5 mg/kg | I.P. |
| Acepromazine | Vedco | Anesthetic | | 1 mg/kg | I.P. |
| Flunixin meglumine | Bimeda | Anti-inflammatory/analgesic | | 2.5 mg/kg | S.C. |
| Timintin | | Antibiotic | 100 mg/ml | 200 µl | I.V. |
| Heparin | | Anticoagulant | 1000 USP/ml | 200 µl | I.V. |
| Brevital | | Paralytic/patency check | 10 mg/ml | 200 µl | I.V. |
| Sodium Pentobarbitol | Sigma | Anesthetic/euthanasia | | 100 mg/kg | I.P. |
| Isofluorane | Vedco | Anesthetic maintenance | 1.5-2.0% in $O_2$ | | Inhaled |
| Methamphetamine | Sigma | Addictive psychostimulant | 50 µg/kg/inf | 200 µl infusions self-administered | I.V. |

### 2.1.4 Surgery

Anesthetic induction was carried out using intraperitoneal injection (1 ml/kg) of a cocktail of ketamine, xylazine, and acepromazine (Rompun mix) (see Table 2.1). Upon induction, animals were depilated and the surgical field prepared with alcohol and betadine to reduce infectious particle load. A deep level of anesthetia was maintained with isofluorane (1.5-2%) in oxygen for the duration of the surgery. Incisions were made starting below the scapula and extending 5-6

cm (dependent upon animal size) parallel to the spine of the animal. A subcutaneous pocket was created using blunt dissection to accommodate the catheter support mesh. Blunt dissection was extended up over the shoulder and to the ventral portion of the neck to create a path for the catheter line.

Upon locating the jugular pulse, an incision 1-1.5 cm was made $90^0$ to the line of the jugular vein. At this stage, the catheter inlet was placed in the subcutaneous pocket and the outlet line run subcutaneously over the right shoulder. The jugular vein was then isolated and a small incision made in the vein to accommodate the catheter outlet line, now trimmed at a 20-$30^0$ angle to a length appropriate for the run from jugular to heart. Once the catheter was in place, patency was assessed via blood draw and sutured using non-absorbable suture line to tie below the silicone hold-fast securing the line inside the vein, and again above the silicone hold-fast to the vein to reduce movement of the catheter line. To ensure constriction of the catheter was not introduced by sutures, patency was again checked after each suture. Cyanoacrylate glue was then used as a final measure of securing the sutures and sealing the vein. Wound clips (Stoelting, Wood Dale) were used to close the incisions at the neck and along the back, with sutures used to secure the area immediately adjacent to the inlet protrusion.

Wounds were treated with a standard OTC topical antibiotic with lidocaine (Neosporin), and Flunixin analgesic administered (Table 2.1) before removing the animal from isofluorane. Saline was administered subcutaneously for hydration as needed (~3 ml). Heparin with Timentin antibiotic (Table 2.1) was used to flush the catheter line prior to capping off with sealed tubing, and animals were allowed to recover in home cages supplied with surgical bedding. Additionally, during the first 45 minutes of recovery, half of the cage floor was warmed with heating pads on a moderate setting, providing a region of warmth for recovery, and a cool region for the animal to move to when warming was no longer desired. Animal observations and pain management were carried out over the next three days as guided by protocol and Veterinary Services recommendations.

**2.1.5 Intravenous Self-Administration (IVSA)**

Following three days of post-surgical recovery, catheter patency was assessed and animals

were assigned to operant chambers. Food training was not used prior to introducing animals to the chambers to avoid possible confounds that may arise from food and stress interplay, and to minimize the time in between stress treatment and access to drug. The catheters were flushed with heparinized saline containing antibiotic and drug concentrations adjusted per animal based on the weights of the previous day. Tygon tubing (I.D. 0.020) encased in a coiled steel protective line fitted with a swage to allow catheter inlet securement was connected to a micro-infusion pump (Harvard Apparatus, Holliston) set to dispense 50 µl/s of solution upon press of the active lever. Drug solutions in 20 ml syringes were placed in the pumps and pumps were used to flush out ethanol solution (used to reduce risk of microbial contamination of the lines) and ensure no voids were present prior to the animal being connected. Operant chambers (Med Associates, St. Albans) were completely enclosed in white-noise providing, light-restricted cabinets, allowing control of chamber lights (kept on for the duration of the study to synchronize with the animals' schedule).

The IVSA program was set to deploy an active (front) lever and an inactive (back) lever simultaneously. Active levers were set to activate a 20 second cue light and dispense 200 µl of drug immediately upon press followed by a 20 second time out period where further activity on the active lever would not be rewarded with drug. This was done to minimize risk of overdose, limiting METH intake to a maximum of 3 self-administrations per minute (150 µg/kg/minute or 54 mg/kg/session maximum intake). A session summary is provided in Table 2.2. An inactive lever press provided neither drug nor cue light upon activation. All sessions were started 60-90 minutes after vivarium lights switched on.

Lever press data was collected and parsed out in 10 minute bins for the 6 hour duration of drug availability. At the end of each session, the levers retracted and the house lights were switched off. Animals were removed from their within 3 minutes of session end, catheters flushed with heparinized saline and antibiotic, and end caps and protective inlet covers were replaced. Animals were weighed to establish the concentration of drug required the following day, and examined for general condition before being returned to the home cage. The chambers were then cleaned, examined for required maintenance and all lines were flushed with ethanol in preparation

for the following sessions. Animals that failed to meet a minimum threshold of performance were excluded from data analysis.

**Table 2.3 IVSA Settings Summary**

| Session Length | Dispense Time | Dispense Vol | Time Out | House Light | Stimulus Light | Stimulus Light Time | #Active Levers | # Inactive Levers |
|---|---|---|---|---|---|---|---|---|
| 360 min | 4 sec | 200 µl | 20 sec | On | Active Lever Only | 20 sec | 1 | 1 |

## 2.2 Tissue Harvest

Brain tissues were collected from C, A, and R animals to determine the effects of stress on molecular markers associated with drug intake and reward. Two hours after onset of the stress or exposure period on their final treatment day, some animals were lethally anesthetized with sodium pentobarbital (see Table 2.1 for dosage). Immediately following decapitation, brains were isolated, coronally blocked into four sections including one section encompassing the rostral-to-caudal extent of nucleus accumbens (Figure 2.4 and Figure 2.5), and rapidly frozen on dry ice for storage at -80$^0$C. Separate groups of animals underwent transcardial perfusion; these rats were

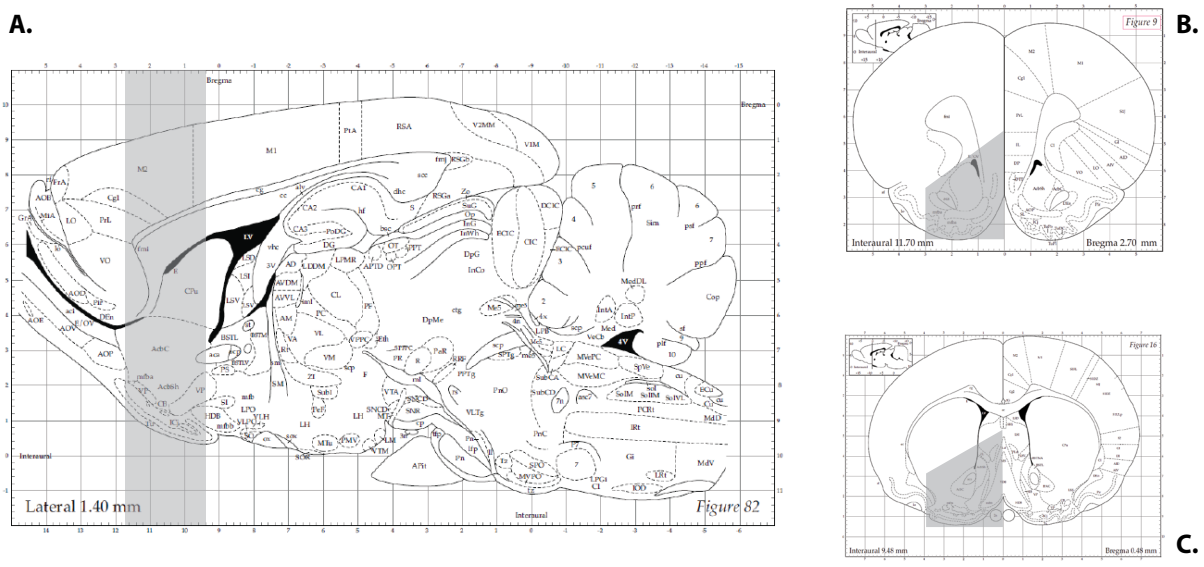


**Figure 2.4 Section Demarcation for Nucleus Accumbens Enrichment for Western Blot**

Panel A. Sagital view is shaded to denote the region of the nucleus accumbens. Panel B. Rostral-most extent of the coronal section. Panel C. Caudal-most extent of the coronal section. Shaded regions denote incision lines used to enrich for nucleus accumbens. Atlas images from Paxinos and Watson, 1998.

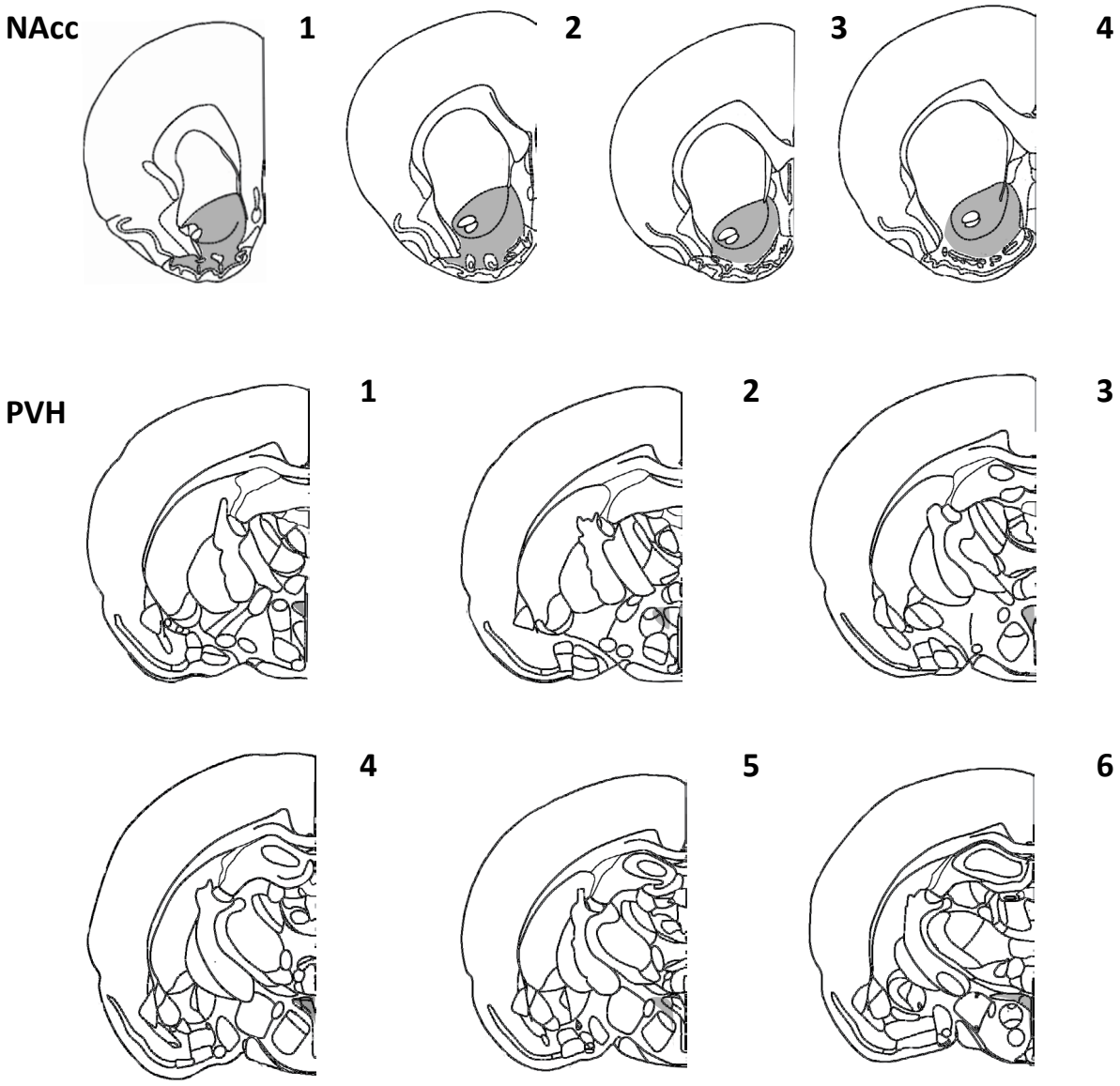


**Figure 2.5 Atlas Sections for NAcc and PVH**

Illustrated above are the four sections of NAcc selected for Fos immunopositive neuron counts as well as the six sections of PVH. Gray shading denotes the structural boundaries used in counting. All samples were sectioned at 30 μm. Images adapted from *The Rat Brain in Stereotaxic Coordinates, Fourth Ed*. Paxinos and Watson, 1988.

lethally anesthetized with sodium pentobarbitol two hours after the onset of their final treatment and perfused with ~100 mls normal saline (0.85%) at $4^0$C. This was followed by perfusion with ~500 mls $4^0$C, 4% paraformaldehyde at pH 9.4, buffered with sodium tetraborate. Brains were then

isolated and postfixed for 5 hours at 4$^0$C to continue passive exposure to the fixative. Brains were then allowed to sink in potassium and phosphate buffered saline (KPBS) with 20% sucrose (18-24 hours, 4$^0$C) whereupon brains were blocked at the cerebellum and coronally either flash frozen in hexane and stored at-80$^0$C until sectioning, or immediately sectioned at 30 µm on a freezing stage tabletop microtome (Leica) in five series of five wells in 24-well plates with ~ 300 µl cryoprotective solution (0.74% sodium phosphate monobasic, 2.73% sodium phosphate dibasic, 30% ethylene glycol, 20% glycerol) and stored at-20$^0$C until used in immunohistochemistry.

**2.3 Immunohistochemistry (IHC)**

Classic studies in neuronal activation use immunoreactivity with the immediate early gene product Fos. This marker is well characterized, and demonstrates consistent performance in multiple neuronal populations including neurons of the PVH under a number of stress conditions (excluding hypertonic stress) (Seasholtz et al, 1988; Imaki et al, 1992; Hoffman et al, 1991; Kajaer et al, 1994; Chan et al, 1993; Covenas et al, 1993; Kononen et al, 1992). This marker is further suited to our purposes given evidence that Fos immunoreactivity co-labels well with CRF-expressing neurons in the PVH (Chat et al, 1993; Seasholtz et al, 1988; Imaki et al, 1992). Thus, to investigate states of activation within reward-associated structures of the brain, we surveyed across six sections of the PVH and across four sections of the nucleus accumbens shell and core for Fos-immunoreactive neurons (see Figure 2.3). Briefly, coronal sections containing these structures were rinsed twice in KPBS for 10 minutes, treated with 0.3% hydrogen peroxide for 10 minutes followed by two 10 minute rinses in KPBS and treatment in 1% sodium borohydride in KPBS. This was followed by KPBS rinses (on average, 6 X 10 minute rinses) and placement of tissue into a solution of blocking buffer (2% normal goat serum (NGS) (Vector) and 0.3% Triton x 100 in KPBS) and rabbit-anti-Fos antibody at a dilution of 1:25,000 (see Table 2.3 for antibody information). After 16-18 hours of incubation in primary antibody solution at 4$^0$C on an orbital table (50 rpm), tissues were removed and rinsed twice for 10 minutes in KPBS prior to incubation in biotinylated secondary antibody (goat-anti-rabbit gamma globulin) at a dilution of 1:200 in blocking buffer followed by 1 hour of room temperature incubation on an orbital table. The solution was again washed twice for ten

minutes in KPBS followed by use of a solution of avidin DH and biotinylated horseradish peroxidase H solutions contained in the standard Vectastain Elite ABC kit (Vector), using 1 drop of each reagent per 10 mls of KPBS. This was then allowed to complex with the secondary-labeled tissue followed by treatment in sodium acetate and subsequent nickel-enhanced diaminobenzadine (DAB) reaction in the presence of β-D-glucose, glucose oxidase and ammonium chloride (Hancock, 1984). After 4-6 minutes of reaction, two 10 minute rinses in sodium acetate were followed by rinses in KBPS and tissue sections were mounted on gelatin-sublimated slides. After drying, mounted tissue was then sequentially dehydrated in an ethanol series and clarified in xylene prior to application of DPX mounting solution and coverslip.

**2.4 Western Blot Analysis (WB)**

For Western blots, unilateral sections of ventral striatum containing the nucleus accumbens with an approximate wet weight of 4 mg were isolated as depicted in Figure 2.4. Briefly, using the brain atlas and stereotaxic coordinates of The Rat Brain in Stereotaxic Coordinates Fourth Ed. (Paxinos and Watson, 1998) for reference, coronal sections were made from freshly dissected brains using a coronal 1mm matrix to block a 2 mm section encompassing the majority of the nucleus accumbens in the rostral-to-caudal extent with the goal of capturing the Bregma coordinate region from ~+0.70- +2.20. These slices were further refined by making a midline incision followed by an angled incision roughly perpendicular to the midline (Figure 2.4). Due to the high content of dopaminergic markers (most notably DAT) in the dorsal striatum, removal of caudate putamen was prioritized by this approach. However, the distribution of additional small ventral striatal structures throughout the nucleus accumbens shell and core did not allow for consistent removal of these structures. Thus our preparations included the following structures: anterior olfactory nucleus posterior part, cell bridges of the ventral striatum, dorsal endopiriform nucleus, islands of Calleja, nucleus of the horizontal limb of the diagonal band, lateral septal nucleus, median forebrain bundle intermediate part, medial septal nucleus, piriform cortex, semilunar nucleus, septohippocampal nucleus, olfactory tubercle, lateral stripe of the striatum, nucleus of the vertical limb of the diagonal band, ventral pallidum. Isolated sections were placed in 500 µl of lysis

buffer (TPER, Thermo Scientific) and 5µl of HALT protease inhibitor cocktail (Thermo Scientific), and homogenized on ice using glass homogenizers until the tissue was completely homogenized. Homogenates were then spun down at 5,000 rpm for five minutes, and supernatants collected for analysis or storage at-80$^0$C.

Protein concentration was determined using the recommended protocol in the Pierce BCA Assay Kit (Thermo Scientific, Rockford) for microplates. Protein samples were then adjusted to ensure samples run contained 25 µg of protein in 20 µl volumes. All samples were prepared in denaturing conditions using 4 µl sample buffer: 20% glycerol, 10% sodium dodecyl sulfate, 0.01% bromphenol blue, in 1M Tris buffer. Protein samples for use in D2 and TH labeling were heated for 7 minutes at 70$^0$C; however due to complex formation at high temperatures, DAT preparations were not heated prior to loading in pre-cast 10-20% gradient gels (Bio-Rad, Hercules). Stacking current was 80 V for 20 minutes followed by 90 minutes at 100 V or until the tracking dye reached ~ 4 mm from the bottom of the gel. Gels were then transferred onto 2 µm polyvinyl difluoride membrane soaked in 100% methanol prior to stacking the transfer layers. Membranes were then removed and rinsed 3 times for 5 minutes each on orbital shaker in TBS (see Appendix I) and blocked in a milk solution for two hours prior to introduction of primary antibodies (see Table 2.3). Primary antibodies were allowed to bind over 20 hours at 4$^0$C with the exception of DAT which was allowed 48 hours or longer to bind. Membranes were rinsed in TBS 3 times for 5 minutes then block solution containing anti-actin was allowed to bind at room temperature for 2 hours followed by an additional 3 rinses in TBS prior to the introduction of horseradish peroxidase-conjugated antibodies in blocking solution (see Table 2.3). Secondary antibodies were allowed to bind for 2 hours at room temperature (TH, and D2 primaries) or for 6 hours at room temperature (DAT). The membranes were then rinsed a final 5 times in TBS and chemiluminescent reagent was added and allowed to react for two minutes prior to film exposures.

**Table 2.4 Antibodies Used in Study**

| Type | Antigen | System | Manufacturer | Cat # | Conc. | Process | Conjugation |
|---|---|---|---|---|---|---|---|
| 1$^0$ | cFos | Rabbit | Calbiochem | PC38 | 1:25,000 | IHC | |
| 1$^0$ | D2 | Rabbit | Milipore | AB5084P | 1:2,000 | WB | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $1^0$ | DAT | Rabbit | Santa Cruz | SC-14002 | 1:500 | WB | |
| $1^0$ | TH | Mouse | Milipore | MAB318 | 1:2000 | WB | |
| $1^0$ | Actin | Rabbit | Thermo Scientific | PNA2066 | 1:2000 | WB | |
| $2^0$ | Mouse | Goat | Southern Biotech | | 1:2000 | WB | HRP |
| $2^0$ | Rabbit | Goat | Southern Biotech | | 1:2000 | WB | HRP |
| $2^0$ | Rabbit | Goat | Vector | | 1:200 | IHC | Biotin |

## 2.5 Imaging, Cell Counts, and Densitometry

### 2.5.1 Immunohistochemistry

Slides were analyzed through imaged using the 10X objective on a Zeiss Axioskop 40 Boundaries of structures were determined via reference landmarks in The Rat Atlas (Paxinos and Watson, 1998) cross-referenced with nissl-stained series for the animals, and counts of Fos-immunopositive neurons were conducted. Nucleus accumbens regions were surveyed from Bregma level 1.20 mm to 1.70 mm and bilateral counts made. Paraventricular nucleus of the hypothalamus regions were surveyd from Bregma level-1.30 mm to-2.12 mm (refer to figures 2.5 and 2.6).

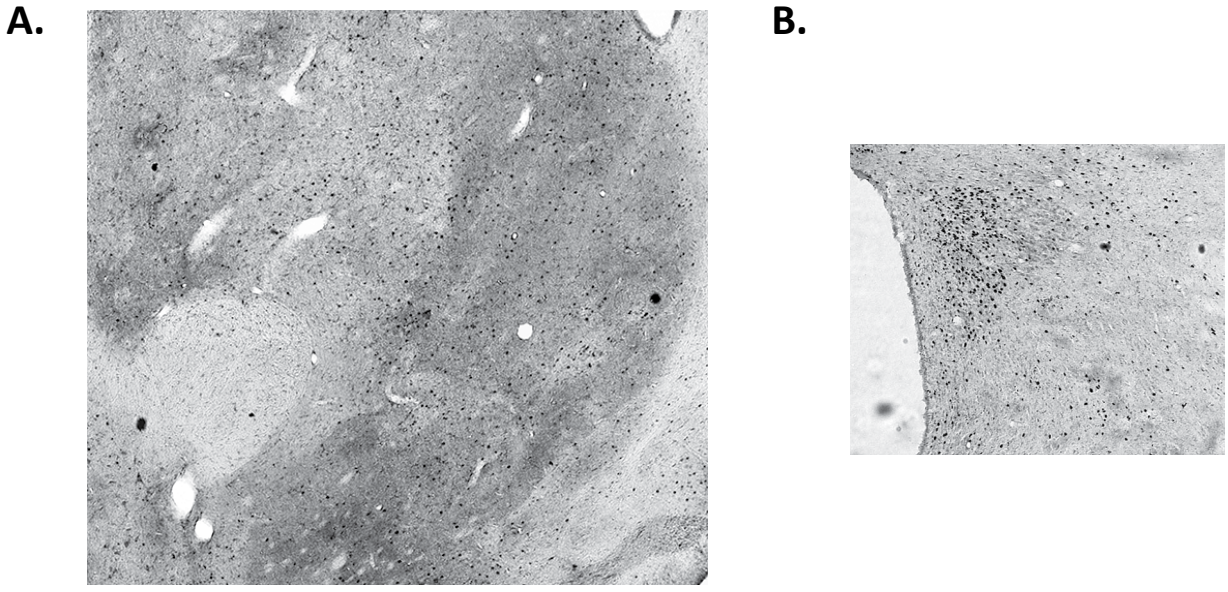


**Figure 2.6 Fos Immunopositive Sample Regions of Survey for NAcc and PVH**

Representative section of Fos-immunolabeled tissue from A) NAcc (Bregma ~1.70 mm), and B) PVH (Bregma ~-1.70 mm) imaged at 5X magnification for purposes of illustration.

### 2.5.2 Densitometry

Exposed Western blot films were scanned using a Kodak ESP 3250 model scanning bed at a resolution of 1000 ppi. Resulting images were then analyzed after converting images to black and white, inverting, and selecting band regions for histogram analysis in Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose)

### 2.6 Statistical Analysis

For neuron counts and Western blot data, one-way ANOVA was used. Pairwise comparisons were conducted between ages for the respective stress conditions, and within age groups between Controls and Acutes, between Acutes and Repeats, and between Repeats and Controls. To examine differences observed among stress conditions within one age, active lever presses (attempts to self-administer METH) were analyzed using a two-way repeated measures ANOVA with stress as a between group factor and days IVSA as a within-subjects factor. Pairwise comparisons were made between Controls and Acutes, between Acutes and Repeats, and between Repeats and Controls in all cases. Behavioral comparisons of age at time of stress were also made between corresponding stress treatments using two-way ANOVA with age as a between subjects factor and days IVSA as the within subjects factor. Tukey's post hoc analysis was used in all measures.

# 3. RESULTS AND DISCUSSION

## 3.1 Immunohistochemical Survey of Fos-Immunopositive Neurons

### 3. 1 .1 Use of Fos Immunopositive Label as a Marker of Neuronal Activation: Rationale and Limitations

Using Fos immunoreactivity in the PVH as a means of assessing neuronal activation in response to stress is well-established. It is not a direct measure of CRF secretion nor do Fos-immunopositive (Fos+) neurons exclusively co-express CRF. However, when stimulated by stress, Fos+ neurons do reliably co-label CRF+ neurons regardless of the stress paradigm used with the exception of high-salt challenge (Herrera and Robertson, 1996; Kovacs, 1998 [review]; Li et al, 1996; Chan et al, 1993; Wamsteeker et al, 2013). Thus, for a study that is not focused on the neuroanatomy and characterization of specific neuronal populations, using Fos+ neurons as a partial proxy for CRF+ neuronal recruitment circumvents the difficulties intrinsic to dealing with currently-available anti-CRF antibodies which are plagued by high background and low sensitivity. While this logic is more challenging to extend to the use of Fos+ in measuring neuronal activation in the NAcc with specific respect to CRF or CRFR neurons, stress and subsequent glutamate elevations (refer to Figure 1.5, Figure 1.6, see Figure 3.1) have been documented to elevate Fos in response to both pleasant and noxious stimuli (Schwarzschild et al, 1997; Pecina et al, 2006; Faure et al, 2010). Thus Fos+ is a marker suitable for measuring neuronal recruitment under conditions of stress and reward, but less biased in representing specific subpopulations of CRF/CRFR co-localization in the NAcc.

This being said, the neurological responses to stress in non-adult models are less well-characterized. While, this survey offers the field novel information which may complement the serological studies done to date on circulating CORT and ACTH for adolescent rodents (refer to Figure 1.9), it does not specifically address issues of CRF co-expression, nor does it specifically seek to characterize the neurons with respect to any other secondary marker, and care should be taken in any conjectures regarding the adolescent data sets.

In general, the relationship between expression of Fos+, downstream signaling and

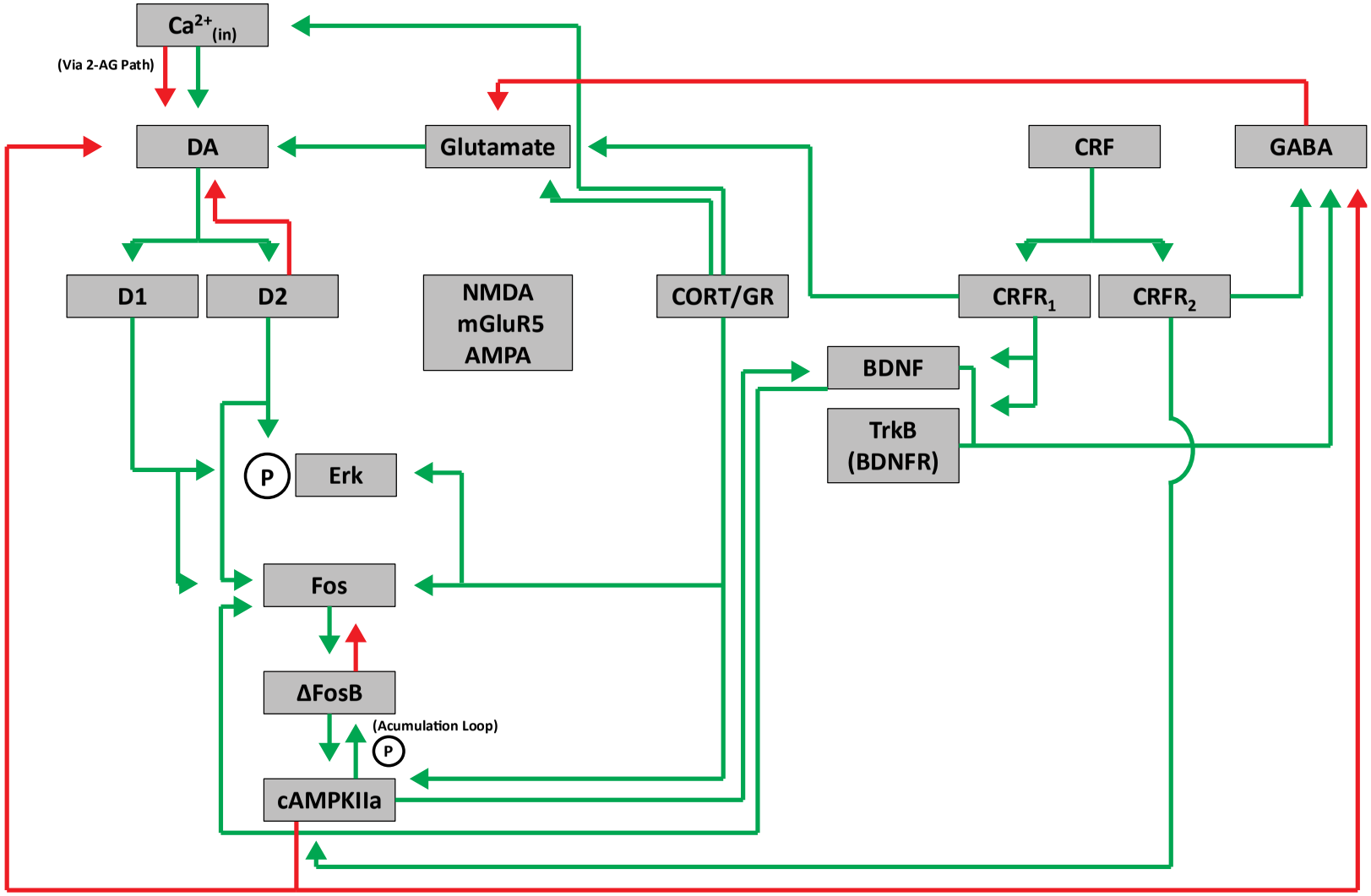


**Figure 3. 1 Cross-Over of Signal Regulation in Stress and Reward Responses in the Nucleus Accumbens**

Green Arrows indicate elevation or activation, Red arrows indicate downregulation or inhibition. Circled P indicates phosphorylation.

transcription events, and the actions of CORT and CRF in eliciting these cascades forms an interesting overlap with GABAergic and glutamatergic signaling, dopamine production and efflux, calcium influx, dopamine receptor binding efficiencies, generation of opioid receptor ligands, intracellular oxidative states, monoamine transporter function, and ΔFosB accumulation and subsequent suppression of Fos production (Parnaudeau et al, 2014; Robison et al, 2013; Besheer et al, 2014; Sun et al, 2008; Bertran-Gonzalez et al, 2008; Mombereau et al, 2007; Schoffelmeer et al, 1995; Djouma et al, 2006). A small portion of this is mapped out for NAcc in Figure 3.1, and even within this restricted window of interactions, it quickly becomes apparent how stress and dopamine systems interrelate in both direct and indirect fashion. Furthermore, given the delicate nature of system negative regulation, small disruptions have the potential to throw the entire system out of balance, especially considering the persistent nature of phosphorylated ΔFosB pools that accumulate when the system is activated repeatedly as it is in both chronic stress and chronic drug use.

### 3.1.2 The NAcc Shell Exhibits Greater Immunoreactivity than the NAcc Core

Analysis of Fos-immunopositive (Fos+) neurons in the NAcc revealed several key differences in the expression of Fos in the shell region compared to the core. The largest differences in response to stress were seen in the shell. This is consistent with the findings from multiple studies that showed preferential activation of the shell versus the core in response to both rewarding and emotionally aversive stimuli (Lemos et al, 2012; Faure et al, 2010; Faure et al, 2008; Fuchs et al, 2008; Goto and Grace, 2008; Ito et al, 2008; Pacchioni et al, 2007; Pecina et al, 2006). In adolescents, there was a nearly-significant main effect of section from which Fos counts were taken [$F(3, 42) = 3.597$; $p = 0.056$]. However, the main effect of stress treatment [$F(1, 14) = 10.604$; $p = 0.002$] and section interaction with treatment [$F(6, 42) = 3.117$; $p = 0.047$] were both significant indicating that not only the type of stress applied shifted the expression of Fos+, but this change was also influenced by which coronal section was being surveyed. Using Tukey's post hoc analysis, differences emerged among adolescent stress responses in the shell overall (Figure 3.2 Panel A).

The anterior-most sections through the NAcc of repeated adolescents (RO) had a significantly lower number of stress-induced Fos+ neurons than repeated adults (RA) for those same sections (Figure 3.2 Panel A). Comparisons of total Fos+ counts between age groups also showed a main effect of age [$F(1,27) = 7.613$; $p = 0.01$] but a marginal treatment X age interaction [$F(2, 34) = 3.084$; $p = 0.057$]. In post hoc analysis of the total Fos+ count for sections surveyed, strong differences were observed in acute adolescents (AO) versus acute adults (AA), and between AO and RO (Figure 3.2 Panel B).

In the NAcc core, Fos+ counts were low overall compared to values attained in the shell. Again, this is consistent with a reduced involvement of this structure in responses to emotional stress. Adolescents showed no main effect of series, but did demonstrate a main effect of treatment [$F(1, 14) = 6.463$; $p = 0.010$] with significant differences in AO versus control adolescents (CO) and RO (Figure 3.2 Panel D). As with adolescents, for adults, only the treatment condition exhibited significant main effect [$F(1,13) = 13.166$; $p = 0.001$]; however a main effect was seen in age [$F(1,26] = 16.006$; $p < 0.001$] and in post hoc analysis, AO and RO both exhibited significantly lower Fos+ counts by section than their adult counterparts (Figure 3.2 Panel C). In contrast to the shell in RA versus control adults (CA), wherein the anterior-most section exhibited significant elevations in Fos+ cells but showed no significant differences when the total was summed from all sections surveyed, the core totals from RA were significantly elevated versus CA values, yet this significance was not maintained when examined by section. Thus, the entire NAcc core that was analyzed appears to uniformly contribute to chronic stress responses, whereas the shell exhibits an anatomical preference in terms of the location of cells engaged in chronic stress responses through these sections. While this data is intriguing, without specific tracing studies or co-localization mapping through these and through more anterior and posterior sections of the structure, little more can be inferred.

### 3.1.3 Interpreting the Data: Stress-Induced Fos+ Neurons of the NAcc Shell and Core

While the section-level analysis provides a limited view of events in the NAcc, this region has been carefully selected to sample areas populated with neurons that respond to both stress

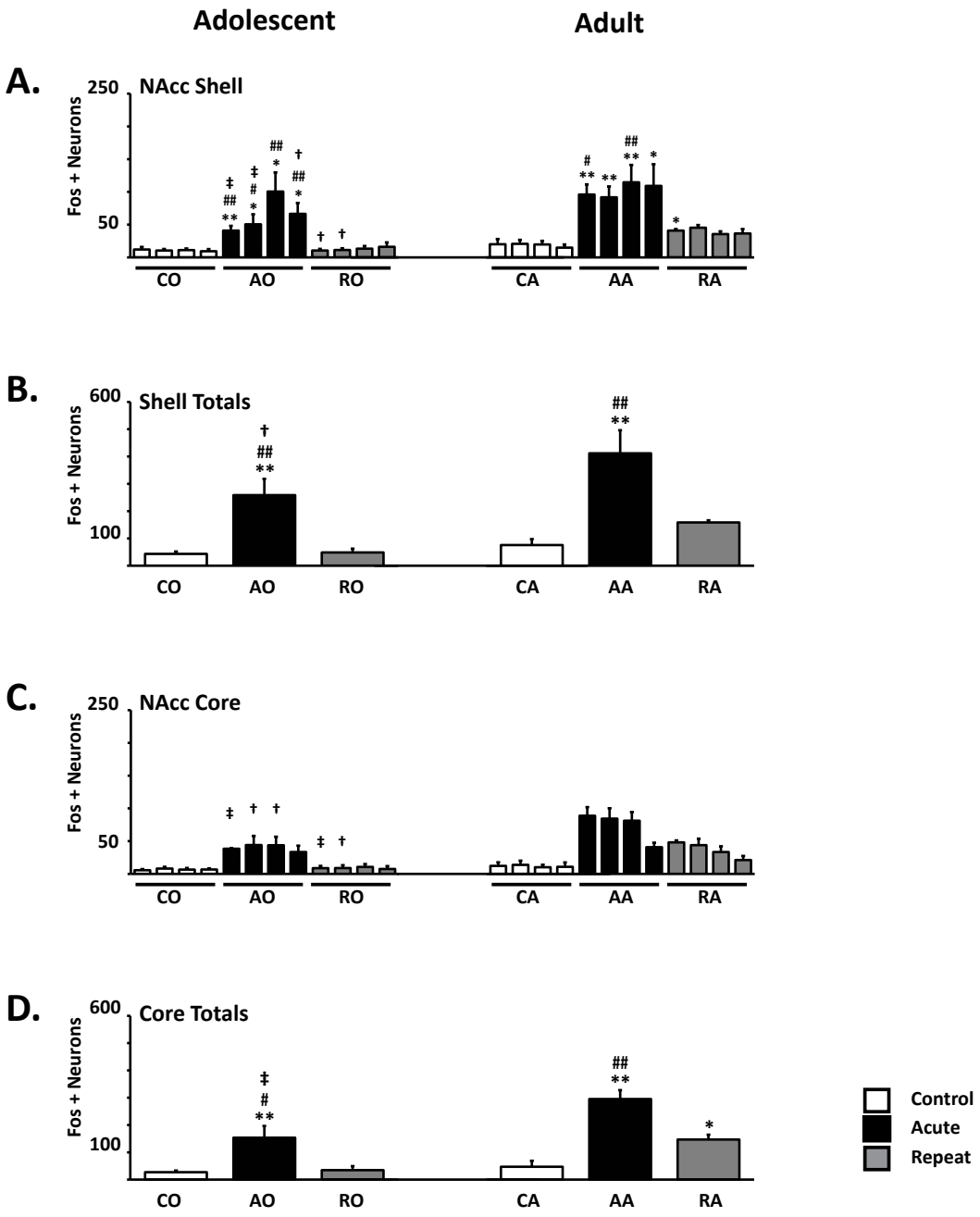


**Figure 3. 2 NAcc Fos Immunopositive Neuron Counts for Adolescent and Adult Males**

Counts per 30 µm section of the NAcc from the rostral-to-caudal extent of the four sections examined are listed from left to right of shell (Panel A) and core (Panel B). All data represents the group mean. Error is expressed in SEM. For total NAcc shell (Panel C) and core (Panel D), two-way ANOVA was conducted with Tukey's post hoc analysis. Significance versus Control: * = $p < 0.05$; ** = $p < 0.005$. Significance versus Repeat: # = $p < 0.05$; ## = $p < 0.005$. Significance versus Adult: † = $p < 0.05$; ‡ = $p = < 0.005$. Control (C) = White. Acute (A) = Black . Repeated (R) = Gray.

cues and hedonic stimuli. DAergic neurons under regulation of GABA and glutamate, and CRFR+ neurons are both represented, and have been shown to correspond to Fos+ elevation arising from stress and reward, in addition to being associated with motivated behaviors ranging from defensive locomotion in response to stressful stimuli to hedonic responses to a pleasant stimulus (sucrose) and increased feeding (Faure et al, 2010; Pecina et al, 2006, Rodaros et al, 2007; Swanson et al, 1983; Wamsteeker et al, 2013).

In adolescent NAcc shells, while AO did have strongly significant increases compared to CO and, to a lesser extent, RO, fewer neurons were recruited versus their adult counterparts despite having similar neuron density. This prompts the question of whether the adolescents are responding less avidly and adversely to acute stress. Interestingly, the anterior-most sections (which are more strongly correlated with motivated behavior in the face of hedonic stimuli) in repeatedly stressed adolescents have far less neuronal activation than do the adults. In prelude to discussion regarding the behavior of adolescents in METH self-administration, the observed failure to escalate in this group may be attributable to changes in the responsiveness of neurons within this region to the rewarding stressor of drug.

The overall Fos+ profiles of the NAcc core still follow the pattern of elevation in response to acute stress and a return to near-control values under chronic stress. However, the differences between the RA and RO and AA and AO are significant. While the core is less associated with the rewarding effects of drug, it is essential in cue-association and motor planning, thus still maintaining a role of importance in addiction (Kelley et al, 1997). However, given that repeated Fos induction results in a gradual increase in phosphorylated ΔFosB which, in turn, attenuates the ability for Fos to be induced (Figure 3.1) stronger expression of Fos may indicate either a disruption in FosB-associated functions, or that different subpopulations of neurons are responding to continued challenge. It should be mentioned here that immunofluorescent surveys were attempted for FosB and Fos co-localization in the initial stages of the study, but due to the perfusion protocol employed, sufficient signal-to-noise could not be attained; however, future studies may benefit from minor protocol adjustments in order to accommodate this survey.

### 3.1.4 The PVH in Adults Displays Greater Response to Acute Stress than Observed in Adolescents

Fos+ expression in response to acute and chronic homotypic (unvaried) stress for both adolescents and adults in the PVH follows a classic pattern: a large response to the acute stress with diminished responses, or habituation, as a consequence of repeated exposures. Thus, the overall curve of the graph for total Fos+ counts in the structure for both adolescents and adults was not unexpected. Interestingly, while main effects were seen in both adolescents [$F(2, 7) = 7.691$; $p = 0.017$] and adults [$F(2, 12) = 19.093$; $p < 0.001$], the responses by section within AAs were far more robust and uniformly distributed than that seen in AOs (Figure 3.3). Furthermore, while there were no main effects of section for adolescents assessed by two-way ANOVA, there was both a main effect of section [$F(5, 60) = 6.221$; $p = 0.008$], and an interaction of treatment and section [$F(10, 60) = 2.311$; $p = 0.023$] observed. By examining Fos+ expression in both NAcc and PVH in this higher resolution format, the differences between adolescent and adult stress-induction of neural activity emerge.

As an aside, it was interesting to note that for both NAcc and PVH the standard error of AO was much higher than of AA, and in fact, while not as dramatic, higher error was observed among all treatment groups in adolescents versus their adult counterparts. From a research perspective, while this could be attributable to random variation in animal responsiveness to stress, it should be emphasized that rat adolescence is a time of rapid change, and exceedingly compressed in its window of time compared to humans. Within a span of days, the neurological underpinnings of stress and motivation are shifting from a juvenile to adult state (refer to Figure 1.9 in the introduction). Thus, when examining the individual animals included in this survey, the lowest Fos+ counts were observed in the youngest animals of the age bracket, but did not meet the criteria for exclusion. Thus, when designing studies using adolescent rats, a ±2 day margin may be too generous, and has the potential to introduce confounds to the study.

### 3.1.5 Interpreting the Data: Stress-Induced Fos+ Neurons of the PVH

The total Fos+ count profiles are not significantly different in adolescents (~PND 50) versus adults (PND 76-82) (Figure3.3) however, there are subtle shifts in Fos+ distribution progressing

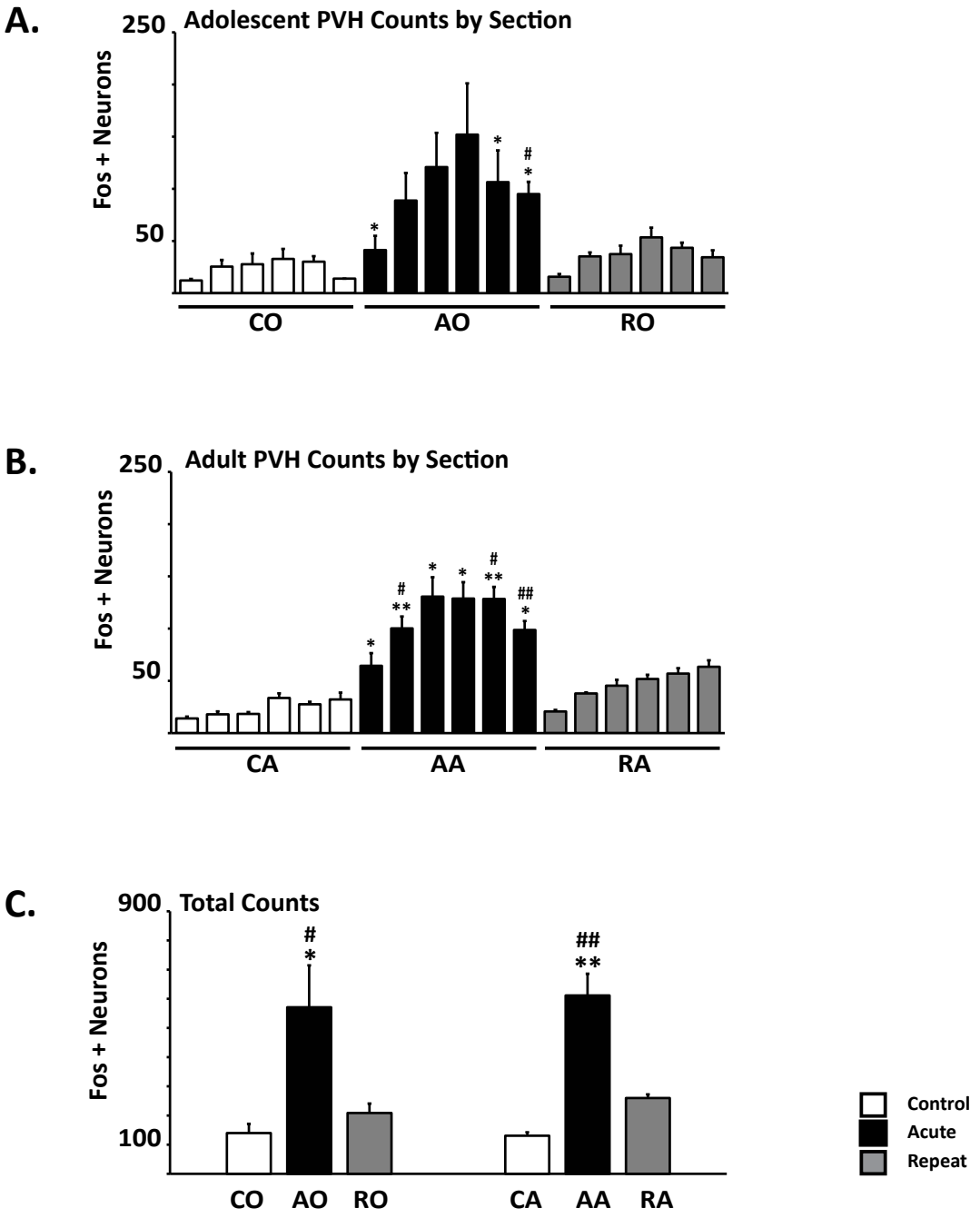


**Figure 3. 3 PVH Fos Immunopositive Neuron Counts for Adolescent and Adult Males**

Counts per 30 µm section of the PVH from the rostral-to-caudal extent are listed from left to right. All data represents the group mean. Error is expressed in SEM. Age at the time of tissue harvest for adolescents (Panel A) was PND 49 ± 1. Age at the time of tissue harvest for adults (Panel B) was PND 86 ± 2. For total PVH data (Panel C), two-way ANOVA was conducted to determine main effects of age, though no significant differences emerged between age groups for a given stress treatment. Significance versus Control:* = $p < 0.05$; ** = $p < 0.005$. Significance versus Repeat: # = $p < 0.05$; ## = $p < 0.005$. Control (C) = White. Acute (A) = Black. Repeated (R) = Gray.

from the anterior to the posterior of the structure. Analysis of coronal sections in acute versus control revealed a less uniformly robust increase in Fos+ neurons in adolescents compared to adults. Furthermore, RO exhibited significant difference from acute only in the caudal-most section whereas significance was distributed throughout the PVH in adults for these groups. This suggests one of two possibilities in adolescents: 1) that neurons involved in the initial response to stress in the anterior portions of the PVH are not highly recruited and warrant further characterization, or that 2) adaptation to chronic predictable emotional stress is not as efficient as in adults. Given the lack of significant difference in Fos+ neurons between controls and repeated restraint groups seen in both ages, and given the lack of pervasive statistical difference from controls among acute adolescents, the first interpretation is more likely. General contributions from the survey of this structure are to establish 1) that rats experienced stress during the restraint treatment, 2) that adolescents did not exhibit radical departure from the pattern of attenuated Fos+ in RO, 3) a baseline pattern against which NAcc comparisons could be made under the assumption that PVH-sourced CRF may be partially involved in stress-associated neuron activation observed.

**3.2 Western Blot Data for TH, D2, and DAT Show No Significant Differences Among Groups**

Stress has been documented in many studies to influence the production and function of dopaminergic componentry including the downregulation of D2, and differential effects on TH in males versus females. Thus, to ascertain if stress altered the expression of key markers in the dopaminergic pathway, terminating in the NAcc, NAcc-enriched preparations of coronal slices were assessed via Western blot conducted under reducing conditions. The results were somewhat surprising given our initial hypothesis that changes in protein expression resultant from stress treatments would be observed based on the findings of Lucas et al (2007) wherein in situ hybridization demonstrated shifts in striatal D2 and DAT. While anticipating some differences arising from electing to evaluate protein expression in our study in lieu of mRNA expression, no significant differences were observed (Figure 3.4). In each group, fold-induction of the protein determined through densitometry, exhibited no main effects of age [$F_{TH}(1,38) = 1.086$; $p = 0.305$, $F_{D2}(1,38) = 2.068$; $p = 0.160$, $F_{DAT}(1,31) = 0.759$; $p = 0.392$] or treatment [$F_{TH}(2,38) = 1.392$; $p =$

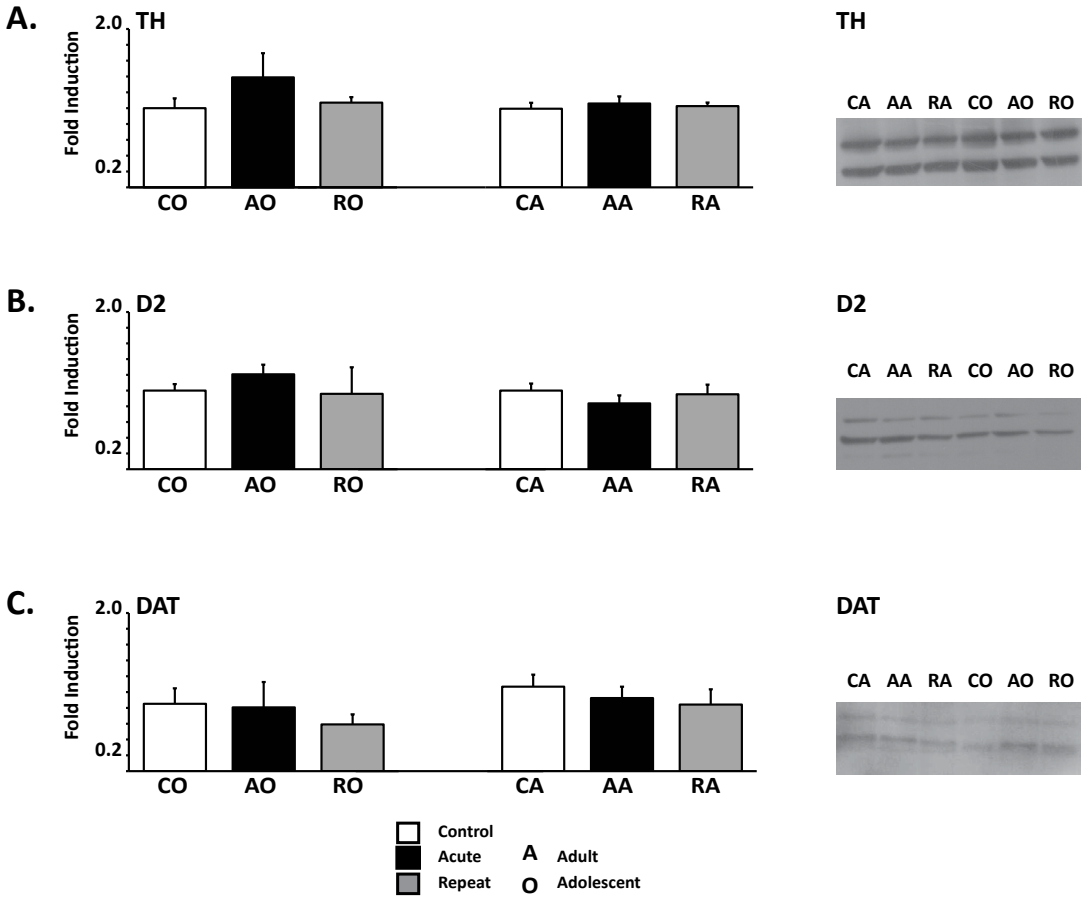


**Figure 3.4 Fold Induction of Protein Markers TH, D2, and DAT in Adolescent and Adult Males**

Above, are the fold-induction values obtained through densitometric analysis of Western blots of NAcc-enriched preparations from the right hemisphere. On the right, sample blots representing each of the conditions are presented. Data is drawn from triplicate replicates for each sample. All samples normalized to actin. Molecular weights; TH ~60 kDA, D2 ~50, DAT ~ 50 kDa (non-glycosylated), Actin~42 kDa. Control = White. Acute = Black. Repeat = Gray.

0.263, $F_{D2}$ (2,38) = 0.21; $p$ = 0.812, $F_{DAT}$ (2,31) = 0.819; $p$ = 0.452]. In pairwise comparisons of age within stress groups, no differences were observed, nor were there differences for stress treatment comparisons within age groups.

While TH exhibits little change beyond rapid transient increases or decreases in healthy neurons, even the transient increases observed in published studies of enzyme expression after stress treatments were not observed (Palkovitz et al, 1975). This may be attributable in part to a poor selection for tissue harvest time in relation to stress termination; however, given the age of the

adolescents at time of harvest, among controls, TH expression would be expected to be lower than control adults. This was not the case. Also, given the role of D2 in blocking the phosphorylation site at Ser 40, it is possible that the *interactions* rather than the expression of these dopaminergic markers are altered under stress conditions (Lindgren et al, 2001). Initial exploration of this question could be conducted through the use of antibodies specific for phosphorylated TH at the Ser 40 residue.

Furthermore, with respect to D2, several studies suggest that stress, rather than changing the level of expression of dopamine receptors, merely alters the binding efficiencies of the receptors (Ito et al, 2000; Henry et al, 1995; Papp et al, 1994; Tomic and Joksimovic, 1991). It is, therefore, not unprecedented that receptor levels remain static yet the overall performance of the dopaminergic reward pathway still exhibits signs of effect. Though, as previously discussed, the role of stress hormones in setting the release thresholds for dopamine transmission suggests that alterations brought on by stress treatments are occurring at earlier levels in the hierarchy of dopamine regulation, and not due to the total expression of these dopaminergic markers.

With respect to DAT, the oxidative state of the neuron under stress can induce complex formation in DAT. Total protein remains unchanged, but protein function may be severely damaged and non-reducing conditions that would allow us to observe the complexed species of DAT were not used in this study. Thus, in terms of experimental design, certain flaws must be acknowledged. While a great deal of information can be gained from Western blots, it is 1) a snapshot and therefore vulnerable to inconsistent results as a function of choosing a time poorly, and 2) a non-discriminating assay. In preparation of total proteins, no synaptosomal or membrane-associated enrichment steps were taken and gels were run under reducing conditions which does not protect DAT complex from breaking back down into component DAT. Thus proteins observed may have been in a cellular compartment or a state of post-translational modification that interferes with function, but still is indistinguishable from functional protein. However, this should not overshadow the fact that 1) serotonergic and noradrenergic systems are also affected by stress, and may contribute to drug vulnerabilities 2) other regions of the brain not surveyed here also contribute to the regulation

of rewarding effects , perception of noxious (stressful) stimuli and motivated behaviors and may exhibit greater sensitivity of response with respect to protein expression 3) stress hormones can significantly alter the innate thresholds required for dopaminergic transmission and these effects do not hinge upon the downstream expression of the markers examined.

### 3.3 Analysis of Behavior for METH Self-Administration

While the molecular data for this study were not conclusive, neuronal activation profiles in the PVH in response to restraint stress did demonstrate similar elevations among acutely stressed animals and decreases among repeatedly stressed animals relative to acute: a function that is linked to neuroplasticity in the face of chronic challenge. This general pattern extends to the NAcc shell, and, to a lesser extent, the core, and is recapitulated in the adolescent groups. However, with a lack of difference in dopaminergic marker expression among the groups, the nature of the neuroplastic events are still elusive. The question remains: does stress impact behavior? Shifting focus from molecular mechanisms and neuroanatomy, we return to this practical question, in keeping with the goal of this research: to determine if stress fundamentally alters response to METH. Following, are the results of 21 sessions of 6 hour drug access. The duration of access was selected based on the findings that, even though METH acquisition is highest in the first 60 minutes of drug access, escalation is only observed in longer (6 hours) versus shorter (1 hour) access windows (Kitamura et al, 2006). The study length (number of IVSA sessions) was determined by observations made during previous studies in our own laboratory of overall animal health when self-administering METH and the ability to sustain catheter patency.

#### 3.3.1 An Overview of Acquisition

To assess measures of escalation, the number of active (METH-dispensing) lever presses is presented in Figure 3.5. Panels A and B show the daily number of lever presses for adolescents and adults for the duration of the study. A distinction, however, must be emphasized between active lever presses and actual drug received. A time-out period after successful drug delivery was in effect which prevented further active lever presses from dispensing METH for the 20 seconds following. Thus, there are discrepancies between the dose of METH acquired versus the actual

attempts made that prevent use of dose (mg/Kg) values or rewarded presses as a direct measure of escalation. With no topics addressed in the post-METH status of these animals, dose is less significant to incorporate in in-depth analysis than behavioral metrics. Dose data is used in analyses to determine its function as a potential predictor of future lever responding in later sections.

In a repeated measures ANOVA, Adolescent-stressed groups, had a main effect of day [$F(20, 220) = 2.116$; $p = 0.005$]; however significant differences from early values in the study were not observed in post hoc analysis. No main effect was present for treatment [$F(2, 11) = 0.801$; $p = 0.797$], nor were interactions of day and treatment observed [$F(20, 40) = 0.852$; $p = = 0.453$]. These findings contrasted with our initial predictions of stress during adolescence providing a risk factor toward addition, though, admittedly in the survey of neurons activated in the PVH and NAcc by stress, the effects of repeated stress appeared to restore activation profiles to that of controls, and the presences of reduced responding among acutes suggests that the stress that we applied, while capable of recruiting neurons, did not stimulate the animals to the same degree. In contrast, adult-stressed groups had a main effect of day [$F(20, 300) = 11.853$; $p < 0.001$] and of treatment [$F(2, 15) = 5.50$; $p = 0.016$], but no interactions between day and treatment conditions. Observation of significant differences from the first day's intake was seen starting at the study midpoint and continued through the final days of the study, indicating escalation (Figure 3.5 Panel B), and the repeatedly stressed groups exhibited multiple days of significantly higher drug intake versus controls, in keeping with our hypothesis. Thus, at first glance there is a disconnect between the failure of stress to elicit changes in the dopaminergic markers assayed and the behavior displayed by repeatedly stressed adults; however, neurons in the NAcc are clearly stimulated under conditions of acute stress, and even remain elevated in the NAcc core after repeated treatments suggesting persistent sensitivity to the stress in this structure. This would suggest that the mechanism of stress-induced drug sensitivity lies not in elevations or reductions of proteins TH, D2, and DAT, but reside in more functional aspects or within the mechanisms governed by stress hormones and their receptors.

As to the differences seen in adolescents versus adults, both in failure of stress to elicit

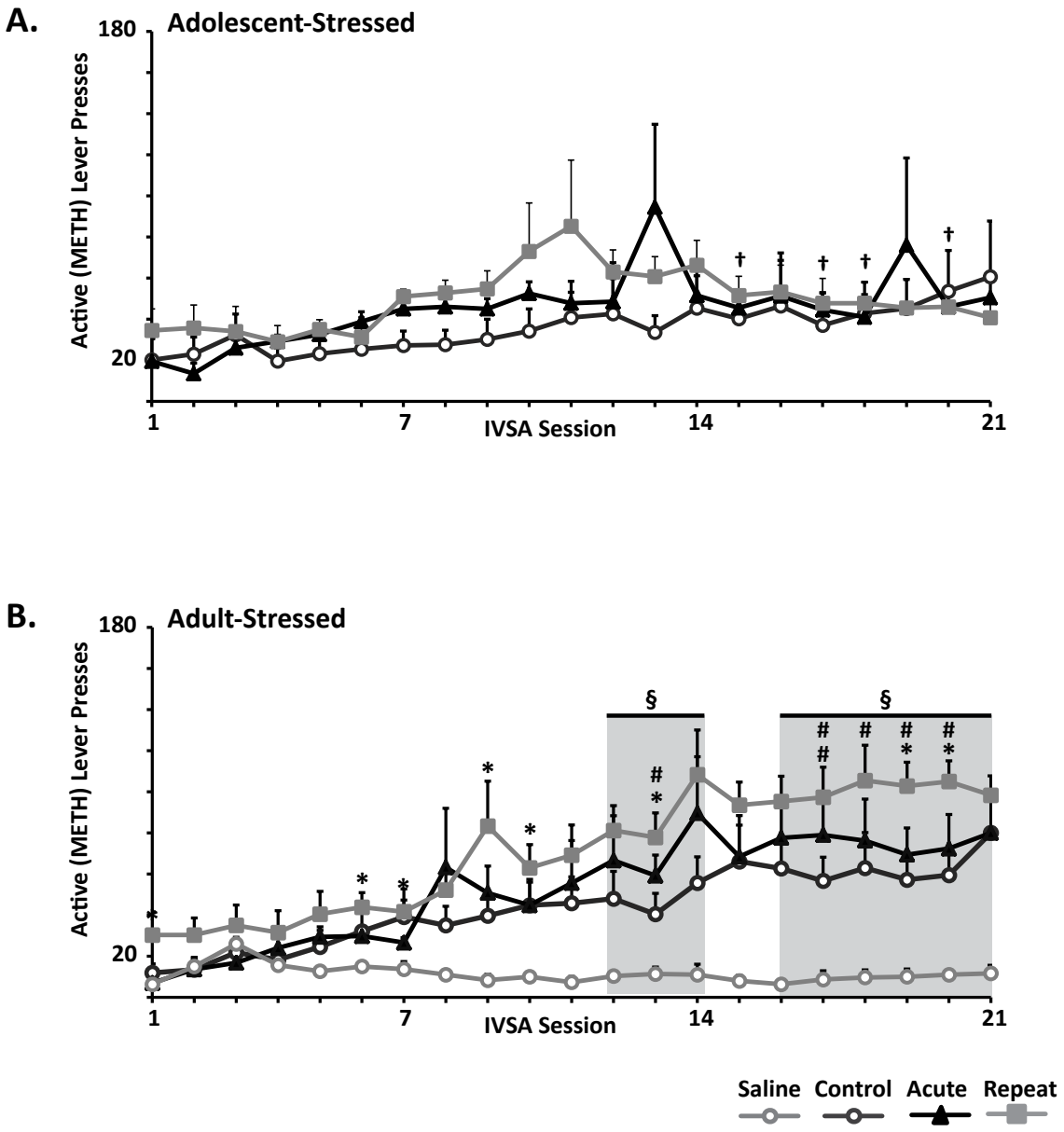


**Figure 3. 5 IVSA Active Lever Data for Adolescent- and Adult-Stressed Rats**

§ = $p < 0.05$ versus the initial IVSA session in a pairwise comparison across all groups. * = $p < 0.05$ versus control values for that session. # = $p < 0.05$ versus initial values. † = $p < 0.05$ versus adult for that session. Gray shading is for the purpose of highlighting the region of drug escalation.

any vulnerabilities to METH addiction, and in the failure for adolescents to escalate intake by the end of study, part of this may be attributable to the developmental differences exhibited by certain components of the stress and dopaminergic componentry during the stress paradigm and subsequent drug exposures (Figures 1.9 and 3.6). By the start of METH IVSA, rats that were stressed as adolescents were recovered from surgery by ~PND 54. While adolescent-stressed rats at this age are nearing the standard age of adulthood (PND 60) there is still ongoing maturation of the dopaminergic system until ~PND 70 (Figure 3.6). The duration of METH IVSA for these groups spans the end of the adolescent period to the early stages of stabilization in the dopamine reward pathway, making the groups neither fully adolescent nor fully adult during the majority of their drug-taking experience, and developmental processes alone may account for the overall low and consistent administrations observed (Figure 3.6). Adolescent-stressed rats are ending METH IVSA before adult-stressed animals, and this small offset in age between adolescent-stressed and adult-stressed animals when starting METH IVSA, warrants caution in drawing any sweeping conclusions

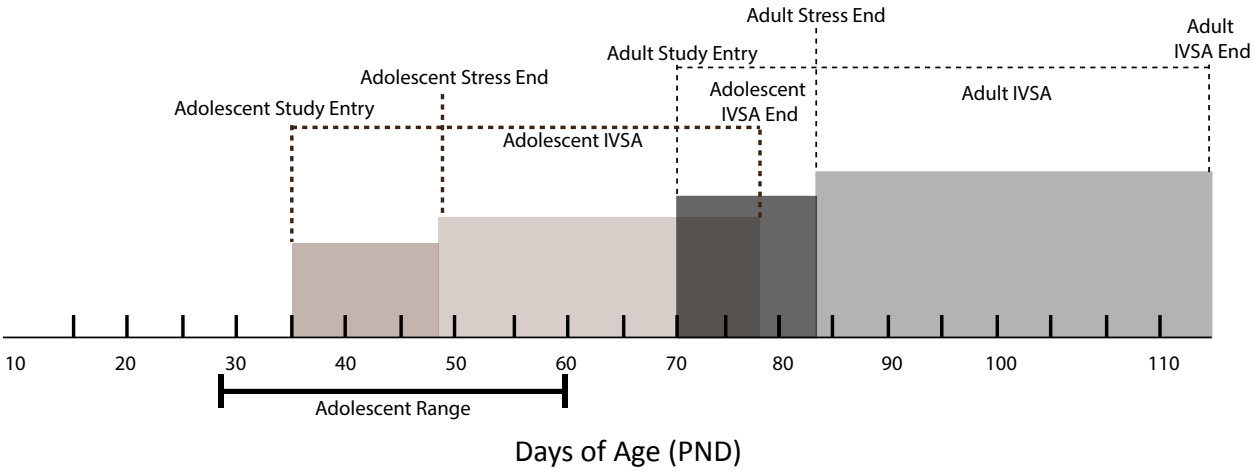


**Figure 3. 6 Study Age Distribution--A Failure to Overlap**

Dark brown covers the region of adolescence during which the stress paradigm occurs. Light brown regions denote the METH IVSA portion of study. Shades of gray are similarly distributed for adults. Region of age overlap occurs ~PND 70-83 but do not share a common protocol during that window.

between the two age groups. One further caution must be taken as the animals in our study were singly housed starting ~6 days prior to study entry. While this was done to control for any adverse interactions among rats that may occur in group housing situations, the fact remains that rats are social animals and isolation represents a stress in and of itself. Adults are more resilient to social stressors but adolescence represents a time of heightened sensitivity to social stressors (Spear, 2000; Nelson et al, 2005; Vidal et al, 2007; Spear, 2012; Bozarth et al, 1989; Moore et al, 2014). As such, while mechanisms may exist which impart a certain protection against addiction in adolescence through early adulthood in our model, it is difficult to tease this out in the face of potentially having introduced a profoundly different stress experience to our adolescents. In this regard, mechanisms that presage anhedonia may be responsible for the flat rate of drug intake, and the general observances during animal handling of reduced responsiveness and righting reflexes displayed by these animals versus their adult counterparts.

### 3.3.2 Refined Analysis of Drug Intake During the Window of Access

While daily totals for active lever activity provide a gross measure of overall escalation, data collection for the 6 hour period takes place on a more refined scale. Collected in bins of 10 minutes, this information offers the opportunity to see the ebb and flow of acquisition attempts across a long period of time allowing us to see periods motivated drug seeking marked by increased lever activity and satiety indicated by periods of low acquisition attempts. This allows us to overlay a time course onto the known measures stress, desire, and satiety as addiction progresses. Visual representation of this data in the form of heat maps (Figure 3.7) provides a means of rapid and intuitive evaluation of an otherwise daunting data set. Figure 3.7 provides an overall assessment of activity across 21 days (rows) and 36 10-minute bins (columns starting at the first bin) for adults and adolescents across all conditions. On the right, data from both the first and last hours in IVSA have been isolated to highlight two critical time points in METH IVSA that yield radically contrasting profiles. One consistency among groups readily stands out. After the first day of IVSA, lightest shades (the highest drug acquisition) occurs within the initial three bins (30 minutes) of drug access which fits with the model of binging following a forced abstinence.

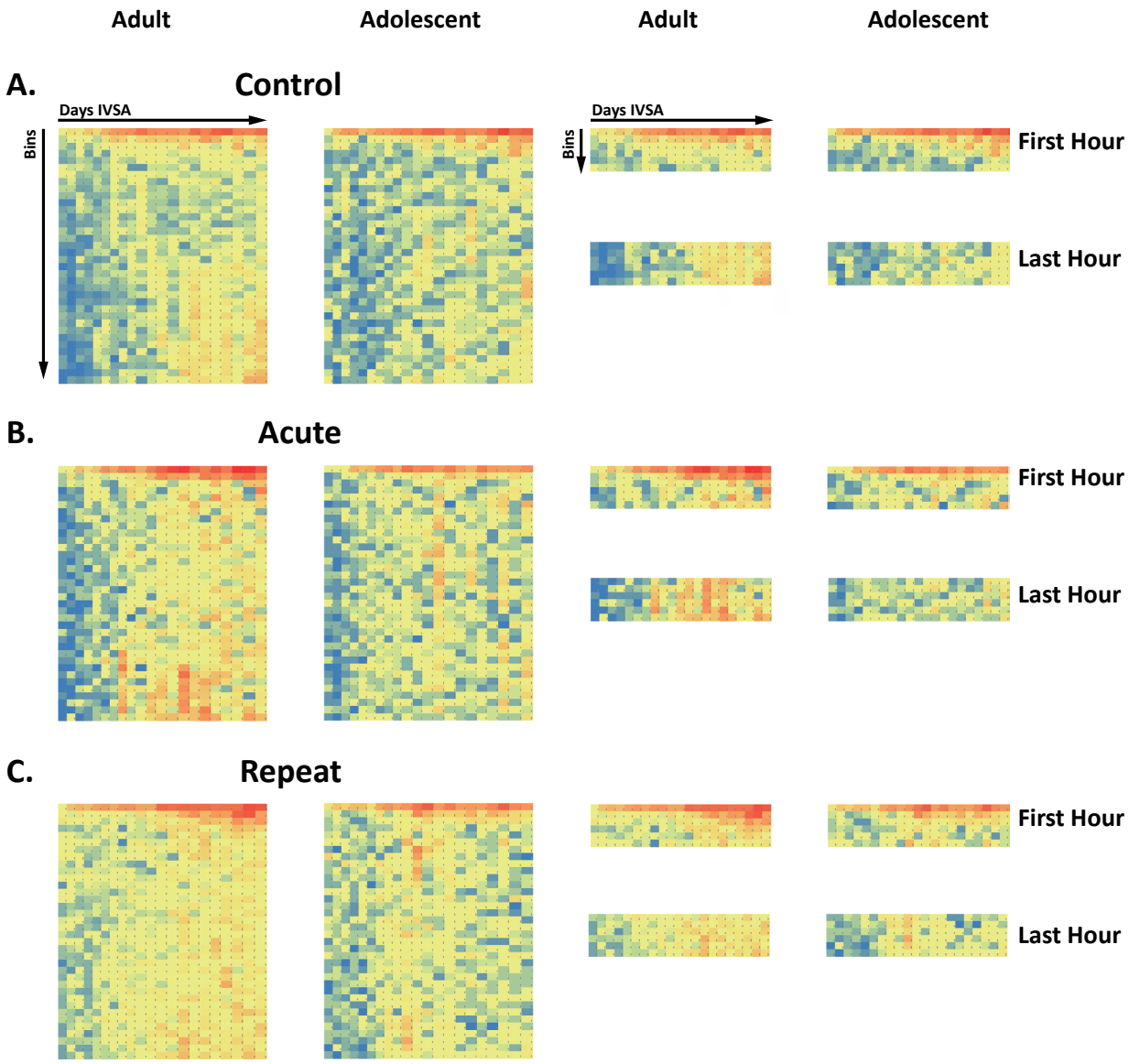


**Figure 3. 7 IVSA Active Lever Heat Maps for Adolescent- and Adult-Stressed Rats**

Above are behavioral heat maps showing active lever presses across 21 days of IVSA (left to right) during 36 10 -minute bins (top to bottom). All IVSA data were pooled across groups and study duration for the purposes of setting conditional formatting thresholds. Minimum threshold (blue) is set to zero and the maximum (red) was determined by the highest number of presses achieved in the data pool. Conditional formatting is set for 50% yellow at the median of the pool.

Recalling the general progression of meth metabolism, the 18 hour abstinence extends beyond the pharmacological half-life of METH in rats (and humans) but stops shy of full drug clearance. Peak appearances of withdrawal symptoms (including drug craving) and behaviors (including measures of anxiety and anhedonia) are marked within the first 24 hours of abstinence. Thus, when looking at the bin data for groups, regardless of the overall activity on the active lever, the animals seek out METH with avidity, indicating that low daily lever presses do not correspond to drug aversion, that METH is providing animals with a rewarding (or at least alleviating) experience, and that, to this extent of the definition, there is a certain level of drive to obtain the drug exhibited in all cases.

The depth to which that band of high activity in the first hour progresses begins to highlight differences among the groups. Within adults, first hour lever press after the first bins progresses from low activity in the first week to progressively higher activity in the second and third week for CA and AA. However, RA displays immediate high activity on the active lever that is maintained with relative consistency throughout the first hour. In viewing the last hour, CA appears to have graded steps across the study duration, gradually increasing activity seen in the final hour to levels approaching that of RA. In AA, the jump from low to high activity in the last hour occurs after the first week of IVSA, and again, RA appears to be an "early adopter" of increased drug intake. Viewing the full distribution of activity, CA has a period following the first hour of relative quiescence with a sudden jump to increased lever pressing near the midpoint of the access window during the final week of IVSA. The time of decreased activity falls within the 2-4 hour window in which the euphoric effects of METH are experienced and aligns with patterns of METH-induced DA outlined by DiChiara and Imperato (1998) wherein a peak of dopamine release is observed within the first half hour after administration followed by a radical decrease ~90 minutes thereafter and continuing to fall to baseline over the next three hours. It would follow that as the rewarding effects fade, self-administration would resume. This line of reasoning would also suggest, based on the progression from low activity in the last hour to progressively higher rates of last-hour acquisition, the maintenance of reward progressively diminishes with repeated METH exposure. This motif is

lost in AA, wherein a sharp demarcation exists between persistence of low and of high acquisition after the first hour. This, coupled with the almost-immediate steady rate of activity seen in RA suggests that stress of this nature may influence the ability to achieve or maintain satiety, thereby driving the escalation seen in addiction.

The adolescents offer more of a challenge to interpret. The first hour of IVSA does show high activity on the active lever consistent with behavior displayed by adults. Given the failure for adolescents to escalate, this behavior addresses concerns over the METH dose. The behavior would suggest that the does selected is neither too high and thus aversive, nor too low and unable to elicit sufficient reward to drive avid early response after 18 hours of forced withdrawal. However, in the subsequent bins, adolescents displayed relatively sporadic self-administration of drug along the bin axis. Interestingly, in the second week of IVSA for RO, there appears to be an increase in active lever responding that then returns to an erratic distribution of activity in the final week. In attempts to address the cryptic appearance of this surge in activity, the age of the animal and their developmental stage should be examined. In Figure 3.8, we see an interesting intersection of events that approximately spans the period of heightened activity. Here ACTH under normal unstressed conditions is found to be in rapid decline during the time corresponding to the start of increased METH acquisition in repeatedly stressed animals. The termination of the activity is enticingly concurrent with the plateau of TH.

Under normal, unstressed, non-social isolation conditions, TH in adolescents should be lower than that of adults: a phenomenon not observed among our adolescents in Western blots. Furthermore chronic stress conditions in adolescents showed lower neuronal recruitment overall in both PVH and NAcc, offering the possibility of altered responsiveness in either stress initiation or in negative regulation of the HPA axis of which ACTH is a part. The potential of our stress to introduce an alteration in reward responding during two surges in neurotrasmitter and neurohormonal development, suggests that there may be a transient period of vulnerability toward METH that chronic stress may facilitate. It has been demonstrated that chronic stress in early life results in long-term deficiencies in ACTH levels which may introduce a transient relief from suppressive

effects of HPA axis products though the mechanisms underling the proposed correlation between TH levels, at PND 70 and the termination of increased drug acquisition are somewhat murky given the limitations of our current study (Juruena, 2014; Lattin et al, 2012; Renard et al, 2007, Aguilera et al, 1996 ). In sum, the chronic stress condition appears to have a temporary window of effect, in increasing drug acquisition, however does not appear to persist into early adulthood. Furthermore, the social isolation stress applied during adolescence may contribute to an overall anhedonic state and protocols in future research should be adjusted accordingly.

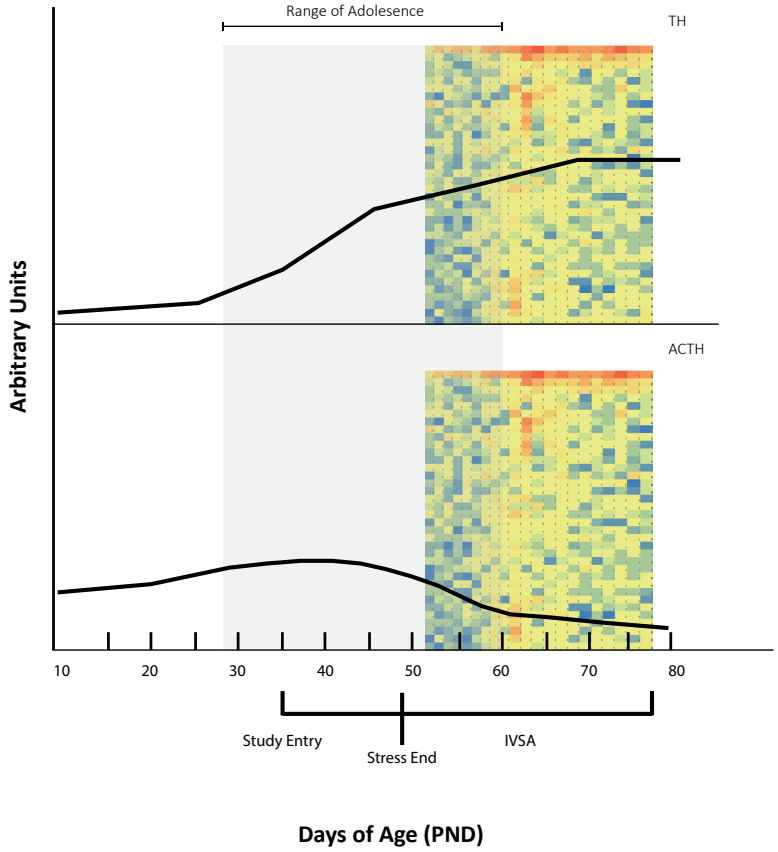


**Figure 3. 8 Overlay of Stress and Dopamine-Reward Development with RO Heat Map Data**

### 3.3.3 The Drug Dose Attained Does not Affect Drug Acquisition on the Following Day

Robust METH IVSA behavior and strong group differences among adults shifted focus toward a more in-depth investigation of potential contributing factors. Molecular and behavioral responses

to METH can be dose-dependent and exhibit both a minimum dose required to elicit reward and escalation and a maximum threshold beyond which aversion is experienced (Cunningham et al, 1992; Masukawa et al, 1992; Richards et al, 1999; Kitamura et al, 2006). A simple linear regression was applied to the actual dose of METH acquired versus the active lever increase or decrease observed on the following day of all individuals within each treatment group (Figure 3.9). The R-squared values for all groups were < 0.2 suggesting little correlation between drug intake and a subsequent increase or decrease in active lever performance.

### 3.3.4 Experiencing Time-Out on the Drug Lever Does not Correlate to Higher Inactive Lever Response

Given the inclusion of a time out period of 20 seconds after a press on the active lever delivers a METH dose, the potential for unrewarded lever presses introduces the possibility of aberrant lever behavior including increased presses on the inactive lever. Averages across group for each bin of data collected during the entire study were used to obtain the values for time out presses (Figure 3.10 Panel A), rewarded presses (Figure 3.10 Panel B) and the inactive lever responses corresponding to data of subsequent bins. In plotting the data for time out activity against subsequent bins for inactive press, there is no significant correlation which would indicate unrewarded presses result in randomized lever selection. Neither does an increase in the amount of METH received (rewarded press) presage an increase or decrease in inactive lever press as a function of increased locomotor responses leading to non-specific (accidental) or non-goal-associated lever responding. However, without secondary measures that specifically test impulsivity or compulsivity, it is difficult to apply specific interpretations to the data.

### 3.3.5 Struggle Observed During Restraint Stress May Predict Subsequent Behavior toward METH

Due to early indications of RA to have increased METH self-administration, a subsequent cohort of RA rats was carefully monitored during the duration of restraint. While scoring methods in the survey were a crude and binary metric (please refer to section 2.1.2 in Materials and Methods) a pattern did emerge from the overall assessment of animal activity during restraint stress that in simple linear regression correlated to the activity of not only the active (Figure 3.11 Panel A), but

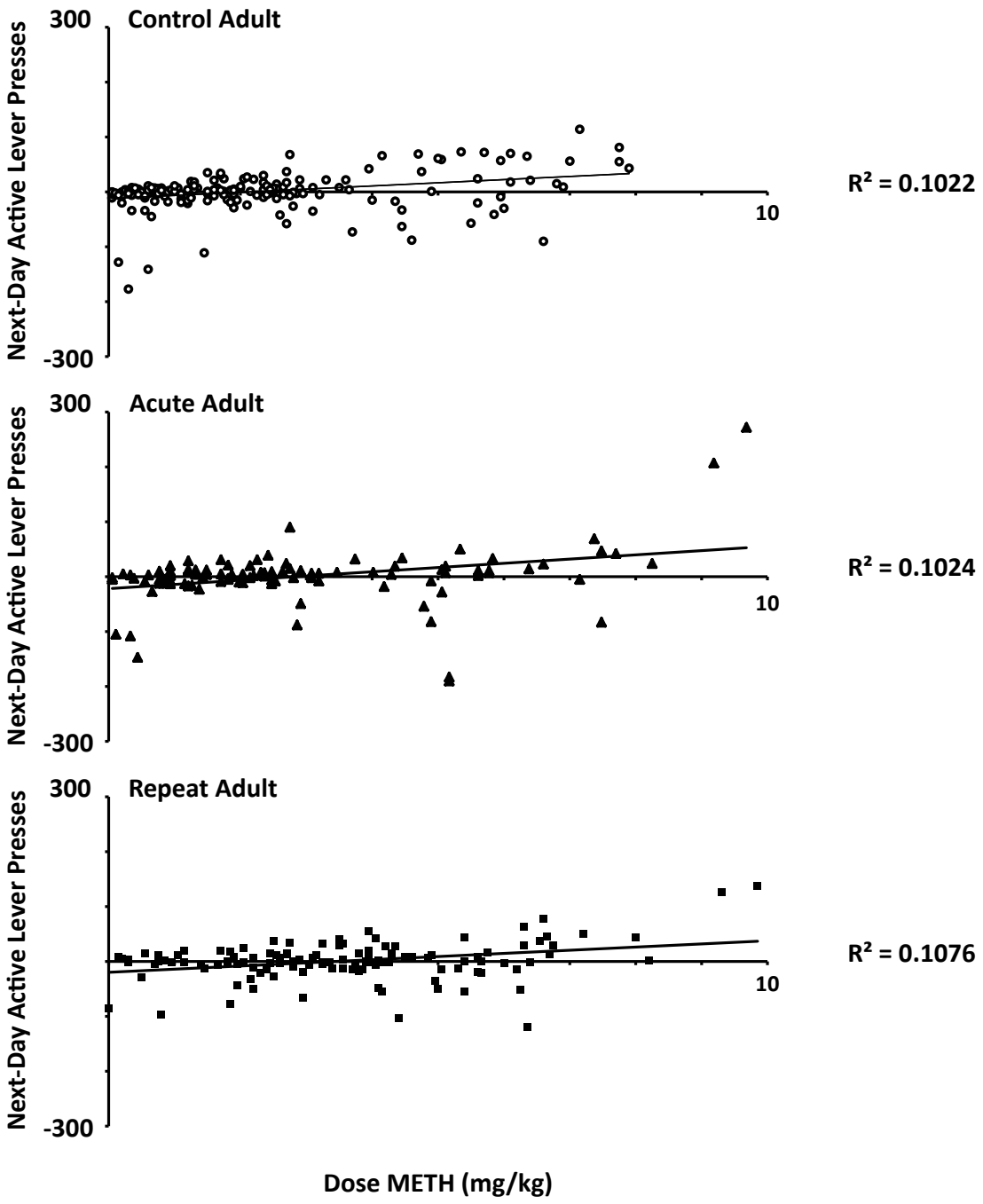


**Figure 3. 9 The Effects of Prior METH Dose on Next-Day Acquisition Attempts**

Data displayed are non-averaged points. Each treatment group's individuals have the daily change in active lever response (increase or decrease) plotted verses the preceding day's dose of METH.

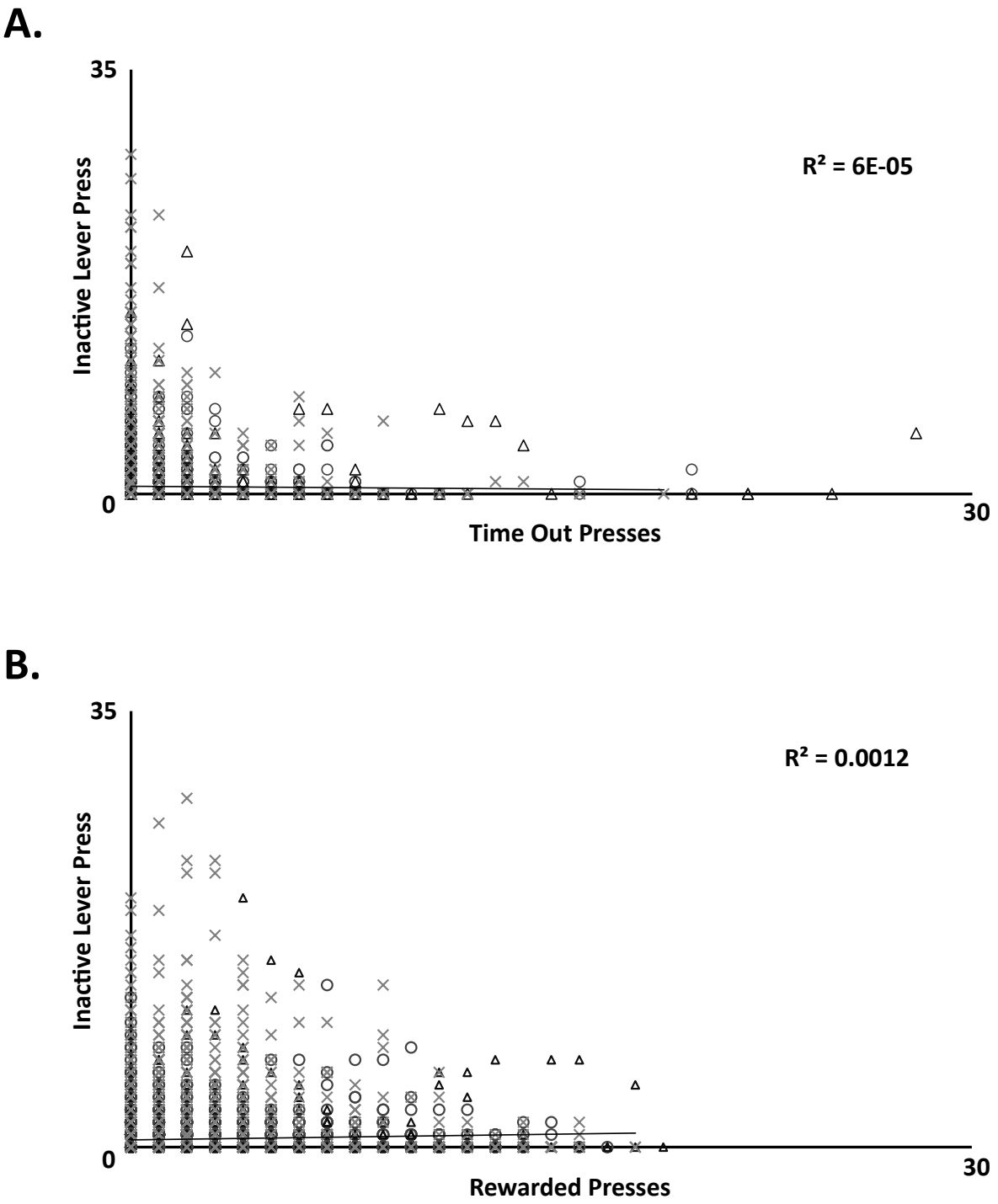


**Figure 3.10 The Effects of Pressing during Time Out and of METH Dose on Next-Bin Inactive Lever Presses**

Group means per 10-minute bin for the study duration are displayed. R-squared values from simple linear regression are listed. No direct correlation between prior bin activity and subsequent inactive lever responses can be drawn. Averages O = Control; Δ = Acute. X = Repeated.

also the inactive (Figure 3.11 Panel B) lever. This preliminary data has the disadvantage of small sample size (n = 6), though from two different litters. The initial group of animals undergoing behavioral observation was slightly larger (n = 9), however only 6 rats met requirements for study inclusion and therefore, the remaining 3 were not included in the analysis. Animal behavior was not associated with a specific litter.

While replication of this experiment is needed before firm conclusions can be drawn, the current data set would suggest that more targeted drug acquisition behaviors indicative of goal-seeking and learning (be it declarative or associative) might be predicted by the level of struggle observed in restraint stress. Whether this indicates that a fundamental state of the individual exists corresponding to both focused METH acquisition and to persistence of struggle, or if the stress and response thereto shape future responses to METH is unknown. Given that low persistence of struggle corresponds in these data to reduced METH acquisition and higher rates of inaccuracy, the functions of anhedonia, cue-associated learning, and goal directed behavior toward addictive substances becomes another question for future investigations of risk factors.

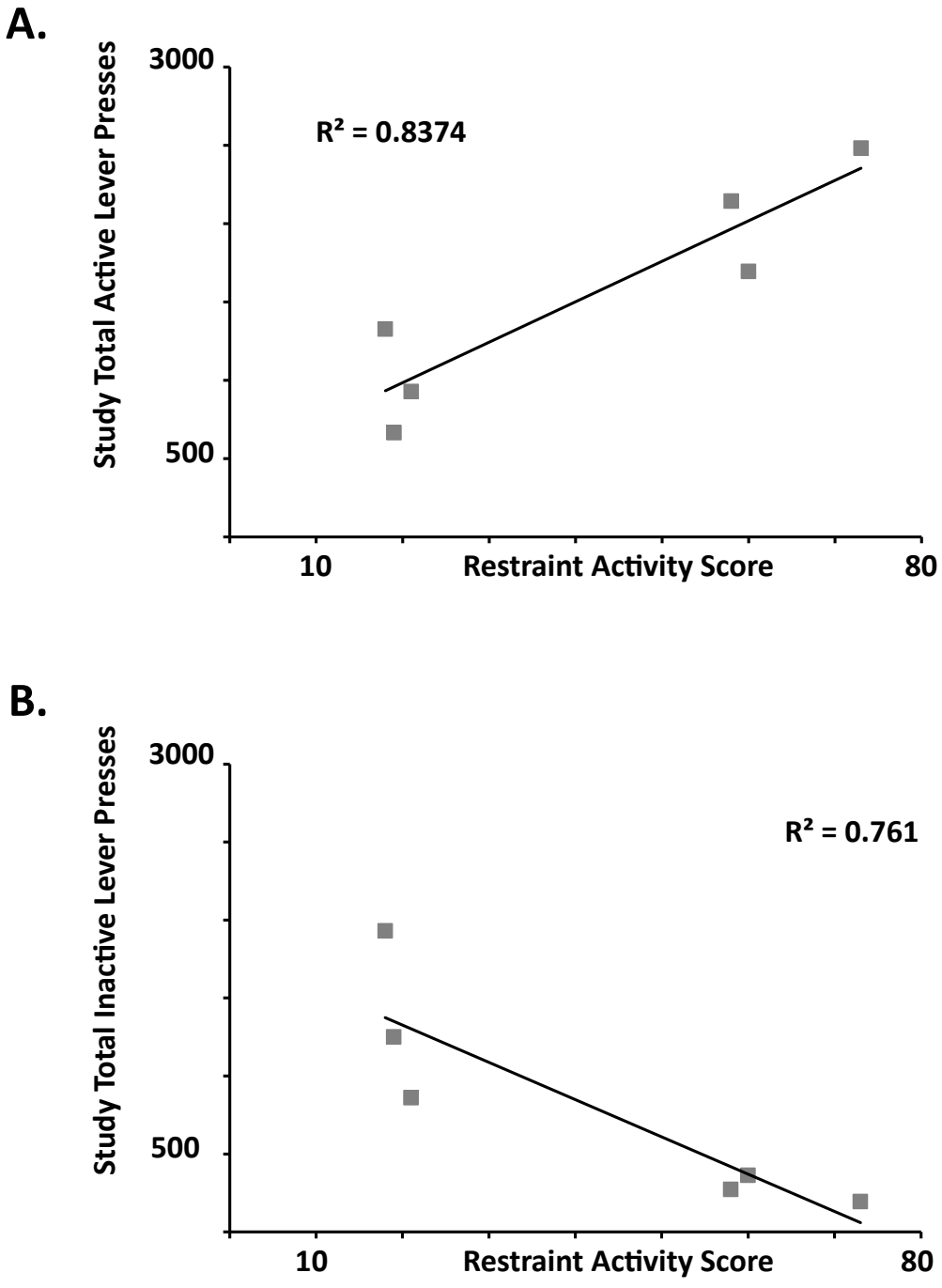


**Figure 3. 11 Activity during Restraint Stress Correlates to METH IVSA Lever Behaviors**

Active (Panel A) and inactive (Panel B) lever presses were summed for the study duration and compared to total activity scores of during the restraint stress treatment. R-squared values from linear regression are provided on the graph.

# 4. CONCLUSIONS AND SUMMARY

## 4.1 Assessing the Big Picture

The purpose of this study was to determine if a chronic or an acute emotional stressor experienced during a particular phase of life is capable of influencing the path of future drug exposures toward a course of addiction. Using a strong emotional stressor (restraint stress) in a rat model, we accomplished the following:

1. We evoked a stress response that could be measured by counting activated neurons and that, with chronic administration manifested neuroplastic changes in stress and reward nuclei of the brain.
2. We observed that the responses to stress in adolescents, while producing similar patterns of neuron activation, does so with significantly less robustness and shows some differences in anatomical preference of recruitment from the rostral to caudal extent surveyed.
3. We confirmed that food training is not necessary to establish robust and directed lever pressing for METH, which gives future researchers the freedom to establish new time lines for experimental manipulations and observations.
4. We established that the total protein expression for the METH-sensitive markers TH, D2, and DAT does not highlight the mechanism behind observed behavioral differences in METH IVSA for stressed groups versus non-stressed controls, or for adults versus adolescents, and we suggest that other systems of signaling or additional metrics of function should be assayed in future studies.
5. We established that the differences observed between adolescents and adults in neuron recruitment are translated to METH self-administration wherein responses are generally lower for adolescents, though not the manner in which these observations are related.
6. We discovered that among chronically stressed adolescents, the pattern of METH acquisition may be reflective of developmental events that are disrupted by stress and may warrant investigation from the perspective of anhedonia and depression.
7. We demonstrated a method by which long-term neurohormonal and neuroanatomical trends

can be mapped onto IVSA data to facilitate the study of the adolescent age group which is still underrepresented in the literature.

8. We observed measures of activity and struggle during restraint and found a correspondence to more targeted METH acquisition suggesting a root mechanism that warrants further investigation with more stringent experimental conditions.
9. Finally, we answered our initial question and found that:

***Adolescent chronic stress does not appear to be a sustained risk factor for addiction in early adulthood, but rather a transient risk factor. Adult chronic stress does appear to be a risk factor in escalating drug use.***

Numerous studies are required to further support many of the findings herein. New studies that probe some of the questions generated by this research, including extending adolescent IVSA to observe long-term behaviors toward drug that more closely parallel behavior observed in adult-stressed models. Furthermore, investigations of the measures of struggle in adults as a means of predicting future behavior toward drug may help develop a behavioral metric for the purpose of pre-screening animals in the lab setting, and for risk assessment within the medical and social work communities.

## 5. REFERENCES


1. Aguilera, G; Kiss, A; Lu, A; Camacho, C. 1996. Regulation of adrenal steroidogenesis during chronic stress. Endocr Res. 22(4):433-43.
2. Bale, TL; Contarino, A; Smith, GW; Chan, R; Gold, LH; Sawchenko, PE; Koob, GF; Vale, WW; Lee, KF. 2000. Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behavior and are hypersensitive to stress. Nat Genet. 24(4):410-4.
3. Bahi A, Dreyer JL. Chronic psychosocial stress causes delayed extinction and exacerbates reinstatement of ethanol-induced conditioned place preference in mice. Psychopharmacology (Berl). 2014 Jan;231(2):367-77.
4. Berridge, KC. Kringelback, MI. 2008. Affective neuroscience of pleasure: Reward in humans and animals. Psychopharmacolology. 199:457-80.
5. Bertran-Gonzalez, J; Bosch, C Maroteaux, M; Matamales, M; Hervé, D; Valjent, E; Girault, JA; 2008. Opposing patterns of signaling activation in dopamine D1 and D2 receptor-expressing striatal neurons in response to cocaine and haloperidol. J Neurosci. 28(22):5671-85.
6. Besheer, J; Fisher, KR; Jaramillo, AA; Frisbee, S; Cannady, R. 2014. Stress hormone exposure reduces mGluR5 expression in the nucleus accumbens: functional implications for interoceptive sensitivity to alcohol. Neuropsychopharmacology. 39(10):2376-86.
7. Best, JA; Nijhout, HF; Reed, MC. 2009. Homeostatic mechanisms in dopamine synthesis and release: a mathematical model. Theo Bio Med Model. 6(21).
8. Bourke CH, Neigh GN. Behavioral effects of chronic adolescent stress are sustained and sexually dimorphic. Horm Behav. 2011 Jun;60(1):112-20.
9. Bota, M; Dong, HW; Swanson, LW. 2003. From gene networks to brain networks. Nat. Neurosci.6:795–9.
10. Bowyer, JF; Robinson, B; Ali, S; Schmued, LC. 2008. Neurotoxic-related changes in tyrosine hydroxylase, microglia, myelin, and the blood-brain barrier in the caudate-putamen from acute methamphetamine exposure. Synapse. 82(3):193-204.

11. Boyd, CJ. 1993. The antecedents of women's crack cocaine abuse: family substance abuse, sexual abuse, depression and illicit drug use. J of Substance Abuse Treat. 10:433-8.

12. Bozarth, MA; Murray, A; Wise, RA. 1989. Influence of housing conditions on the acquisition of intravenous heroin and cocaine self-administration in rats. Pharmacol Biochem Behav. 33(4): 903-7.

13. Brecht, ML; von Maryhauser, C; Anglin, MD. 2000. Predictors of relapse after treatment formethamphetamine use. J Psychoactive Drug 32(2): 211-20.

14. Broening, HW; Pu, C; Vorhees, CV . 1997. Methamphetamine selectively damages dopaminergic innervation to the nucleus accumbens core while sparing the shell. Synapse. 27(2):153-60.

15. Buffalari, DM; See, RE. 2009. Footshock stress potentiates cue-induced cocain-seeking in an animal model of relapse. Physiol Behav. 98: 614-7.

16. Buffalari, DM; Grace, AA. 2009. Chronic cold stress increases excitatory effects of norepinephrine on spontaneous and evoked activity of basolateral amygdala neurons. Int J Neurophsychopharmacol. 12(1): 95-107.

17. Burke, AR; Miczek, KA. 2014. Stress in adolescence and drugs of abuse in rodent models: role of dopamine, CRF, and HPA axis. Psychopharmacology. 231(8): 1557-80.

18. Caldwell, J; Dring, LG; Williams, R.T. 1972. Metabolism of [14C]methamphetamine in man, the guinea pig and the rat. Biochem. J. 129:11-22.

19. Chan, RKW; Brown, ER; Ericsson, A; Kovacs, KJ; Sawchenko, PE. 1993. A comparison of two-immediate-early genes, c-fos and NGFI-B, as markers for functional activation in stress-related neuroendocrine circuitry. J Neurosci. 13(12): 5126-38.

20. Chalmers, DT; Lovenberg, TW; DeSouza, EB. 1995. Localiation of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in the rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci. 15(10): 6340-50.

21. Chauhan, H; Killinger, BA; Miller, CV; Moszczynska, A. 2014. Single and binge methamphetamine administrations have different effects on the levels of dopamine D2 autoreceptor

and dopamine transporter in rat striatum. Int. J. Mol. Sci. 15(4): 5884-5906.

22. Chen, X; Xu, L; Radcliffe, P; Sun, B; Tank, AW.2008. Activation of tyrosine hydroxylase mRNA translation by cAMP in midbrain dopaminergic neurons. Mol Pharmacol. 73(6):1816-28.

23. Cho, AK; Melega, WP. 2002. Patterns of methamphetamine abuse and their consequences. J Addictive Dis. 21:21-34.

24. Cruickshank, CC; Dyer, KR. 2009. A review of the clinical pharmacology of methamphetamine. Addiction. 104:1085-1099.

25. Cruz, FC; Marin, MT; Leào, RM; Planeta, CS. 2012. Behavioral and neuroendocrine effects of the exposure to chronic restraint or variable stress in early adolescent rats. In J Dev Neurosci. 30(1):19-23.

26. Coveñas, R; de Leòn, M; Cintra, A; Bjelke , B; Gustaffson, JA; Fuxe, K. 1993. Coexistence of c-Fos and glucocorticoid receptor immunoreactivities in the CRF immunoreactive neurons of the paraventricular hypothalamic nucleus of the rat after acute immobilization stress. Neurosci Lett. 149(2): 149-52.

27. Cunningham, CL; Noble, D; 1992 Methamphetamine-induced conditioned place preference or aversion depending on dose and presence of drug. Ann NY Acad Sci. 28;654:431-3.

28. Daubner, SC; Le, T; Wang, S. 2011. Tyrosine Hydroxylase and regulation of dopamine synthesis. Arch Biochem Biophys. 508(1):1-12.

29. Davis, J; Kopin, I; Lemberger, L; Axelrod, J. 1971. Effects of urinary pH on amphetamine metabolism. Ann Ny Acad of Sci. 493-501.

30. DiChiara, G; Imperato, A. 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A. 85(14):5274-8.

31. Djouma, E; Card, K; Lodge, DJ; Lawrence, A. 2006. The CRF 1 receptor antagonist, antalarmin, reverses isolation-induced up-regulation of dopamine D2 receptors in the amygdala and nucleus accumbens of fawn-hooded rats. Eur J Neurosci. 23(12):3319-27.

32. Dube, SR; Felitti, VJ; Dong, M; Chapman, DP; Giles, WH; Anda, RF. 2003. Childhood abuse,

neglect, and household dysfunction and the risk of illicit drug use: the adverse childhood experiences study. Pediatrics. 111:564-72.

33. Erb, S and Stweart, J. 1999. A role for the bed nucleus of the stria terminalis, but not the amygdala in the effectsof corticotropin-releasing factor on stress-induced reinstatement of cocaine seeking. J of Neurosci. 19: RC35.

34. Erb, S; Salamaso, N; Rodaros, D., Stewart, J. 2001. A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. Psychopharmacol. 158:360-5.

35. Ernst, M; Nelson, EE; Jazbec, S; McClure, EB; Monk, CS; Leibenluft, E; Blair, J; Pine, DS. 2005. Amygdala and nucleus accumbens in responses to receipt and omission of gains in adults and adolescents. Neuroimage. 25(4): 1279-91.

36. Ernst, M; Fudge, JL. 2009. A developmental neurobiological model of motivated behavior: anatomical connectivity and ontogeny of the triadic nodes. Neurosci Biobehav. Rev. 33(3):367-82.

37. Faure, A; Reynolds, SM; Richard, JM; Berridge, KC. 2008. Mesolimbic dopamine in desire and dread: enabling motivation to be generated by localized glutamate disruptions in nucleus accumbens. J Neurosci. 28(28); 7184-92.

38. Faure, A; Richard, JM; Berridge, KC. 2010. Desire and dread from the nucleus accumbens: cortical glutamate and subcortical GABA differentially generate motivation and hedonic impact in the rat. PLoS One 5(6): e11223.

39. Fleckenstein, AE; Volz, TJ; Hanson, GR. 2009. Psychostimulant-induced alterations in vesicular monoamine transporter-2 function: neurotoxic and therapeutic implications. Neuropharmacology 56(Suppl 1):133–138.

40. Foilb, AR; Lui, P; Romeo, RD. 2011. The transformation of hormonal stress responses throughout puberty and adolescence. J Endocrinol. 210(3): 391-8.

41. Ford, CP. 2014. The role of D2-autoreceptors in regulating dopamine neuron activity and transmission. Neurosci. 282:13-22.

42. Freund, TF; Katona, I; Piomelli, D. 2003. Role of endogenous cannabinoids in synaptic signaling. Phys Rev. 83(3): 1017-66.

43. Fuchs, RA; Evans, KA; Parker, MC; See, RE. 2004. Differential involvement of the core and shell subregions of the nucleus accumbens in conditioned cue-induced reinstatement of cocaine seeking in rats. Psychopharm. 176:459-65.

44. Fuchs, RA; Ramirez, DR; Bell, GH. 2008. Nucleus accumbens shell and core involvement in drug context-induced reinstatement of cocaine seeking in rats. Psychopharmacology. 200(4): 545-56.

45. Funk, CK and Koob, GF. 2007. A CRF2 agonist administered into the central nucleus of the amygdala decreases ethanol self-administration in ethanol-dependent rats. Brain Res. 1155:172-8.

46. Funk, D; Li, Z; Le, AD. 2006. Effects of environmental and pharmacological stressors on c-fos and the corticotropin-releasing factor mRNA in the rat brain: relationship to the reinstatement of alcohol seeking. Neurosci. 138(1): 235-43.

47. Givalois, L; Arancibia, S; Tapia-Arancibia, L. 2000. Concomitant changes in CRH mRNA levels in rat hippocampus and hypothalamus following immobilization stress. Mol Brain Res. 75(1): 166-71.

48. Goto, Y and Grace, AA. 2008. Limbic and cortical information processing in the nucleus accumbens. Trends Neurosci. 11:552-8.

49. Graf, EN; Wheeler, RA, Baker, DA; Ebben, AL; Hill, JE; McReynolds, JR; Robble, MA; Vranjkovic, O; Wheeler, DS; Mantsch, JR; Gasser, PJ. 2013. Corticosterone acts in the nucleus accumbens to enhance dopamine signaling and potentiate reinstatement of cocaine seeking. J Neurosci. 33(29): 11800-10.

50. Groman, SM; Lee, B; Seu, E; James, AS; Feller, K; Mandelkern, MA; London, ED; Jentch, D. 2012. Dysregulation of D2-mediated dopamine transmission in monkeys after chronic escalating methamphetamine exposure. J Neurosci. 32(17):5843-5852.

51. Hadlock, G.C., Chu, P.W., Walters, E.T. Hanson, G.R., Fleckenstein A.E. 2010. Methamphet-

amine-induced dopamine transporter complex formation and dopaminergic deficits: the role of D2 receptor activation. J. of Pharmacol. and Exp. Ther. 335: 207-212.

52. Hauger, RL; Risbrough, V; Oakley, RH; Iliveres-Reyes, JA, Dautzenberg, FM. 2009. Role of CRF recptor signaling in stress vulnerability, anxiety, and depression. Ann NY Acad Sci. 11

53. Henry, C; Guegant, G; Cador, M; Arnauld, E; Arsaut, J; Le Moal, M; Demotes-Mainard, J. 1995. Prenatal stress in rats facilitates amphetamine-induced sensitization and induces long-lasting changes in dopamine receptors in the nucleus accumbens. Brain Res. 685(1-2):179-86.79:120-43.

54. Herrera, DG and Robertson, HA. 1996. Activation of c-fos in the brain. Prog in Neurobiol. 50: 83- 107.

55. Hill, MN; Patel, S., Campolongo, P; Tasker, JG; Wotjak, CT; Bains, JS. 2010. Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. J Neurosci. 30(45): 14980-6.

56. Hoffman, GE; McDonald, T; Shedwick, R; Nathanielsz, PW. 1991. Activation of cFos in ovine fetal corticotropin-releasing hormone neurons at the time of parturition. Endocrinology. 129(6): 3227-33.

57. Hong, S; Flashner, B; Chiu, M; ver Hoeve, E; Luz, S; Bhatnagar, S. 2012. Social isolation in adolescence alters behaviors in the forced swim and sucrose preference tests in female but not in male rats. Physiol Behav. 105(2):269-75.

58. Iemolo, A; Blasio, A; St Cyr, SA; Jiang, F; Rice, KC; Sabino, V; Cottone, P. 2013. CRF-CRF1 receptor system in the central and basolateral nuclei of the amygdala differentially mediates excessive eating of palatable food. Neuropsychopharm. 38(12):2456-66.

59. Ikemoto, S; Kohl, RR; McBride, WJ. 1997. GABA(A) receptor blockade in the anterior ventral tegmental area increases extracellular levels of dopamine in the nucleus accumbens of rats. J Neurochem. 69(1): 137-43.

60. Imaki, T; Shibasaki, T; Hotta, M; Demura, H. 1992. Early induction of c-fos precedes increased expression of corticotropin-releasing factor messenger ribonucleic acid in the

paraventricular nucleus after immobilization stress. Endocrinology. 131(1): 240-6.

61. Imaki, T; Ziao-Quan, W; Shibasaki, T; Yamada, K; Harada, S; Chikada, N; Narusue, M; Demura, H. 1995. Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. J Clin Invest. 96:231-8.

62. Ito, R; Robbins, TW; Pennartz, CM; Everitt, BJ. 2008. Functional interaction between the hippocampus and nucleus accumbens shell is necessary for the acquisition of appetitive spatial context conditioning. J of Neuro. 28(27): 6950-9.

63. Jang, C.G., Whitfield, T., Schulteis, G., Koob, G.F., Wee, S. 2013. A dysphoric-like state during early withdrawal from extended access to methamphetamine self-administration in rats. Psychopharmacology 225, 753-63.

64. Johnson, EO; Kamilaris, TC; Chrousos, GP; Gold, PW. 1992. Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. Neurosci Biobehav Rev. 16(2): 115-30.

65. Juruena, MF .2014. Early-life stress and HPA axis trigger recurrent adulthood depression. Epilepsy Behav. 38:148-59.

66. Kajaer, A; Larsen, PJ; Knigge, U., Moller, M; Warberg, J. 1994. Histamine stimulates c-fos expression in hypothalamic vasopressin-, oxytocin-, and corticotropin-releasing hormone-containing neurons. Endocrinology. 134(1): 482-91.

67. Kawata, M; Hashimoto, K; Takahara, J; Sano, Y. 1983. Immunohistochemical identification of neurons containing corticotropin-releasing factor in the rat hypothalamus. Cell Tissue Res. 230(2): 239-46.

68. Kelley AE, Smith-Roe SL, Holahan MR. Response-reinforcement learning is dependent on N-methyl-D-aspartate receptor activation in the nucleus accumbens core. Proc Natl Acad Sci U S A. 1997 Oct 28;94(22):12174-9.

69. King, D; Zigmond; MJ; Finlay, JM. 1997. Effects of dopamine depletion in the medial prefrontal cortex on the stress-induced increase in extracellular dopamine in the nucleus accumbens core and shell. Neuroscience. 77(6): 141-53.

70. Kitamura O, Wee S, Specio SE, Koob GF, Pulvirenti L. Escalation of methamphetamine self-administration in rats: a dose-effect function. Psychopharmacology (Berl). 2006 May;186(1):48-53.

71. Kononen, J; Honkaniemi, J; Alho, H; Koistinaho, J; Iadarola, M; Pelto-Huikko, M. 1992. Fos-like immunoreactivity in the rat hypothalamic-pituitary axis after immobilization stress. E Koo, JW; Lobo, MK; Chaudhury, D; Labonté, B; Friedman, A; Heller, E; Peña, CJ; Han, MH; Nestler, EJ. 2014. Loss of BDNF signaling in D1R-expressing NAc neurons enhances morphine reward by reducing GABA inhibition. Neuropsychopharmacology. 2014 Oct;39(11):2646-53.ndocrinology. 130(5): 3041-7.

72. Koob, GF; Le Moal, M. 1997. Drug abuse: hedonic homeostatic dysregulation. Science. 278: 52-8.

73. Koob, GF; Le Moal, M. 2008. Neurobiological mechanisms for opponent motivational processes in addiction. Phil Trans R Soc B. 363: 3113-23.

74. Kosheleff, AR; Rodriguez, D; O'Dell, SJ; Marshall, JF; Izquierdo, A. 2012. Comparison of single-dose and extended methamphetamine administration of reversal learning in rats. Pscyopharmacology, 224: 459-467.

75. Kovacs, KJ. 1998. C-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem. Int. 33: 287-97.

76. Krasnova, IL; Justinova, Z; Ladenheim, B; Jayanthi, S., McCoy, MT; Barnes, C; Warner, JE; Goldberg, SR; Cadet, JL. 2010. Methamphetamine self-administration is associated with persistent biochemical alterations in striatal and cortical dopaminergic terminals in the rat. PLoS One. 5(1): e8790.

77. Kravets, JL; Reyes, BA; Unterwald, EM; Van Bockstaele, EJ. 2015. Direct targeting of peptidergic amygdalar neurons by noradrenergic afferents: linking stress-integrative circuitry. Brain Struct Funct. 220(1): 541-58.

78. Laćan, G; Hadamitzky, M; Kuczenski, R; Melega, WP. 2013. Alterations in the striatal dopamine system during intravenous methamphetamine exposure: Effects of contingent and

noncontingent administration. Synapse 67, 476-488.

79. Larsen, KE; Fon, EA; Hasting, TG; Edwards, RH, Sulzer, D. 2002. Methamphetamine-induced degeneration of dopaminergic neurons involves autophagy and upregulation of dopamine synthesis. J Neurosci. 22(20): 8951-60.

80. Larson, RW; Moneta, G; Richards, MH; Wilson, S. 2002. Continuity, stability and change in daily emotional experience across adolescence. Child Dev.. 73(4): 1151-65.

81. Lattin CR1, Bauer CM, de Bruijn R, Michael Romero L. Hypothalamus-pituitary-adrenal axis activity and the subsequent response to chronic stress differ depending upon life history stage. Gen Comp Endocrinol. 2012 Sep 15;178(3):494-501.

82. Le, AD; Harding, S; Juzytsch, W; Watchus, J; Shalev, U; Shaham, Y. 2000. The role of corticotrophin-releasing factor in stress-induced relapse to alcohol-seeking behavior in rats. Psychopharm. 150:317-24.

83. Lemos, JC; Wanat, MJ; Smith, JS; Ryes, BAS; Hollon, NG; BanCockstaele, EJ; Chavkin, C; Phillips, PEM. 2012. Sever stress switches CRF action in the nucleus accumbens form appetitive to aversive. Nature. 490: 402-6.

84. Leslie, CA; Robertson, MW; Cutler, AJ; Bennett, JP., Jr. 1991. Postnatal development of D1 dopamine receptors in the medial prefrontal cortex, striatum and nucleus accumbens of normal and neonatal 6-hydroxydopamine treated rats: a quantitative autoradiographic analysis. Brain Res Dev Brain Res. 62:109–14.

85. Li, HY; Ericson, A; Sawchenko, PE. 1996. Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms. Proc Natl Acad Sci USA. 93: 2359-64.

86. Li, L; Everhart, T; Jacob, P; Jones, R, Mendelson, J. 2009. Stereoselectivity in the human metabolism of methamphetamine. British J Clin Pharmacol. 69: 187-92.

87. Lopez, JF; Akil, H; Watson, SJ. 1999. Neural circuits mediating stress. Biol Psychiatry. 46(11): 1461-71.

88. Lindgren, N; Xu, ZQ; Herrera-Marscitz, M; Haycock, J; Hokfelt, T; Fisone, G. 2001. Dopamine

D(2) receptors regulate tyrosine hydroxylase activity and phosphorylation at Ser40 in rat striatum. Eur J Neurosci. 13(4): 773-80.

89. Lopez, ML; Franco, A; Tasker, JG. 2008. Glucocorticoids shift arachidonic acid metabolism toward endocannabinoid synthesis: a non-genomic anti-inflammatory switch. European J Pharmacol. 583: 322-39.

90. Lucas, LR; Wang, CJ; McCall, TJ; McEwen, BS. 2007. Effects of immobilization stress on neurochemical markers in the motivational system of the male rat. Brain Res. 1155: 108-115.

91. Marco, EM; Adriani, W; Canese, R; Podo, F; Viveros, MP; Laviola, G. 2007. Enhancement of endocannabinoid signaling during adolescence: modulation of impulsivity and long-term consequences on metabolic brain parameters in eraly maternally deprived rats. Pharmacol Biochem Behav. 86(2);332-45.

92. Maser-Gluth, C; Toygar, A; Vecsei, P. 1984. Time course of plasma corticosterone, 18-hydroxycorticosterone and aldosterone concentrations following CRF administration in the rat. A phase of corticosterone inhibition. Life Sci 35(8): 879-84.

93. Masukawa, Y; Suzuki, T; Misawa, M. 1992. Differential modification of the rewarding effects of methamphetamine and cocaine by opioids and antihistamines. Psychopharmacol. 111(2): 139-43.

94. McFadden, LM; Hadlock, GC; Allen, SC; Vieira-Brock, PL; Stout, KA; Ellis, JD; Hoonakker, A.J; Andrenvak, D.M; Nielsen, S.M; Wilkins, D.G; Hanson, G.R; Fleckenstein, A.E. 2012. Methamphetamine self-administration causes persistent striatal dopaminergic alterations and mitigates the deficits caused by subsequent methamphetamine exposures. J. Pharmacol. Exp. Ther. 340:295-303.

95. McGregor, C; Srisurapanont, M; Jittiwutikarn, J; Laobhripatr, TW; White, JM. 2004. The nature, time course and severity of methamphetamine withdrawal. Addiction. 100:1320-9.

96. Meloni, EG; Gerety, LP; Knoll, AT; Cohen, BM; Carlezon, WA. 2006. Behavioral and anatomical interactions between dopamine and corticotropin-releasing factor in the rat. J Neurosci. 26(14)3855-63.

97. Mombereau, C; Lhuillier, L; Kaupmann, K; Cryan, JF. 2007. GABAB receptor-positive modulation-induced blockade of the rewarding properties of nicotine is associated with a reduction in nucleus accumbens DeltaFosB accumulation. J Pharmacol Exp Ther. 321(1):172-7.

98. Moore, NL; Gauchan, S; Geovese, RF. 2014. Adolescent traumatic stress experience results in less robust conditioned fear and post-extinction fear cue responses in adult rats. Pharmacol Biochem Behav. 120:17-24.

99. Morita K, Morishima M, Sakai K, Kawaguchi Y. Dopaminergic control of motivation and reinforcement learning: a closed-circuit account for reward-oriented behavior.J Neurosci. 2013 May 15;33(20):8866-90.

100. Naneix, F; Marchand, AR; Di Scala, G; Pape, JR; Coutureau, E. 2012. Parallel maturation of goal-directed behavior and dopaminergic systems during adolescence. J Neurosci. 32:16223–32.

101. Nelson, EE; Leibenluft, E; Mclure, E; Pine, DS. 2005. The social reorientation of adolescence: a neuroscience perspective on the process and its relation to psychopatholgy. Psychol Med 35: 163-74.

102. Nestler, EJ. 2013). Cellular basis of memory for addiction. Dialogues Clin. Neurosci. 15 (4): 431–443.

103. Neufeld-Cohen, A; Tsoory, MM; Evans, AK; Getselter, D; Gil, S; Lowry, CA; Vale, WW; Chen, A. 2010. A triple urocortins knockout mouse model reveals and essential role for urocortins in stress recovery. PNAS. 107(44): 19020-5.

104. Office of National Drug Control Policy (ONDCP). The Economic Costs of Drug Abuse in the United States: 2000-2010. ONDCP Publication #207303. Washington, DC: 2014.

105. Pacak, K; Palkovits, M; Kopin, IJ; Goldstein, DS. 1995. Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: in vivo microdialysis studies. Front Neuroendocrinol. 16(2): 89-150.

106. Pacchioni, AM; Cador, M; Bregonzion, C; Cancela, LM. 2007. A glutamate-dopamine interaction in the persistent enhanced response to amphetamine in nucleus accumbens core

but not shell following a single restraint stress. Neuropsychopharm. 32: 682-692.

107. Palkovits, M. 2000. Stress-induced expression of co-localized neuropeptides in hypothalamic and amygdaloid neurons. Euro. J Pharm. 405: 161-6.

108. Palkovits, M; Kobayashi, RM; Kizer, JS; Jacobowitz, DM; Kopin, IJ. 1975. Effects of stress on catecholamines and tyrosine hydroxylase activity of individual hypothalamic nuclei. Neuroendocrinology. 18(2):144-53.

109. Papp, M; Klimek; V, Willner, P. 1994. Parallel changes in dopamine D2 receptor binding in limbic forebrain associated with chronic mild stress-induced anhedonia and its reversal by imipramine. Psychopharmacology . 115(4):441-6.

110. Parnaudeau, S; Dongelmans, ML; Turiault, M; Ambroggi, F; Delbes, AS; Cansell C; Luquet S; Piazza, PV; Tronche, F, Barik, J. 2014. Glucocorticoid receptor gene inactivation in dopamine-innervated areas selectively decreases behavioral responses to amphetamine. Front Behav Neurosci. 12;8:35.

111. Paxinos, G. and Watson, C. 1998. The Rat Brain in Stereotaxic Coordinates, Fourth Ed. Academic Press. Orlando.

112. Pecina, S; Schulkin, J, Berridge, KC. 2006. Nucleus accumbens corticotropin releasing factor increases cue-triggered motivation for sucrose reward: paradoxical positive incentive effects in stress? BMC Biology. 4(8).

113. Pich, EM; Lorang, M; Yeganeh, M; de Fonseca, FR; Raber, J; Koob, GF; Weiss, F. 1995. Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. J Neurosci. 15(8) 5439-47.

114. Pohl, J; Olmstead, MC; Wynne-Edwards, KE; Harkness, K; Menard, JL. 2007. Repeated exposure to stress across the childhood-adolescent period alters rats' anxiety- and depression-like behaviors in adulthood: The importance of stressor type and gender. Behav. Neurosci. 121(3): 462-74.

115. Popoli, M; Yan, Z; McEwen, B; Sanacora, G. 2012. The stressed synapse: the impact of

stress and glucocorticoids on glutamate transmission. Nature Rev Neurosci. 13: 22-37.

116. Preston KL, Epstein DH. Stress in the daily lives of cocaine and heroin users: relationship to mood, craving, relapse triggers, and cocaine use. Psychopharmacology (Berl). 2011 Nov;218(1):29-37.

117. Radley, JJ; Williams, B; Sawchenko, PE. 2008. Noradrenergic innervation of the dorsal medial prefrontal cortex modulates hypothalamo-pituitary-adrenal responses to acute emotional stress. J Neurosci. 28(22): 5806-16.

118. Raudensky, J and Yamamoto, BK. 2007. Effects of chronic unpredictable stress on monoamine transporter immunoreactivity and methamphetamine-induced dopamine release in the nucleus accumbens shell. Synapse. 61:353-5.

119. Renard, GM; Rivarola, MA; Suárez, MM. 2007. Sexual dimorphism in rats: effects of early maternal separation and variablechronic stress on pituitary-adrenal axis and behavior. Int J Dev Neurosci. 25(6):373-9.

120. Richards, JB; Sabol, KE; de Wit, H., (1999) Effects of methamphetamine on the adjusting amount procedure, a model of impulsive behavior in rats. Psychopharmacol. 146:432-9.

121. Robison, AJ; Vialou, V; Mazei-Robison, M; Feng, J; Kourrich, S; Collins, M; Wee, S; Koob, G; Turecki, G; Neve, R; Thomas, M; Nestler, EJ. 2013. Behavioral and structural responses to chronic cocaine require a feedforward loop involving ΔFosB and calcium/calmodulin-dependent protein kinase II in the nucleus accumbens shell. J Neurosci. 33(10):4295-307.

122. Roderos, D; Caruana, DA; Amir, S; Stewart, J. 2007. Corticotropin-releasing factor projetions from the limbic forebrain and paraventricular nucleus of the hypothalamus to the region of the ventral tegmental area. Neuroscience. 150(1): 8-13.

123. Romeo, RD; Bellani, R; Karatsoreos, IN; Chhua, N; Vernov, M; Conrad, CD; McEwen, BS. 2006. Stress history and pubertal development interact to shape hypothalamic pituitary-adrenal axis plasticity. Endocrinology. 147(4): 1664-74.

124. Schoffelmeer, AN; De Vries, TJ; Vanderschuren, LJ; Tjon ,GH; Nestby, P; Wardeh, G; Mulder, AH. 1995. Glucocorticoid receptor activation potentiates the morphine-induced adaptive

increase in dopamine D-1 receptor efficacy in gamma-aminobutyric acid neurons of rat striatum/nucleus accumbens. J Pharmacol Exp Ther.274(3):1154-60.

125. Schwarzschild, MA; Cole, L; Hyman, SE. 1997. Glutamate, but not dopamine, stimulates stress-activated protein kinase and AP-1-mediate transcription in striatal neurons. J Neurosci. 17(10): 3455-3466.

126. Schwendt, M. Rocha, A., See, R.E., Pacchioni, A.M., McGinty, J.F., Kalivas, P.W., 2009. Extended methamphetamine self-administration in rats results in a selective reduction of dopamine transporter accompanied by marked monoaminergic depletion. J. Pharmacol. Exp. Ther. 331, 555-562.

127. Seasholtz, AF; Thompson, RC; Douglass, JO. 1988. Identification of a cyclic adenosine monophosphate-responsive element in the rat corticotropin-releasing hormone gene. Mol Endocrinol. 2(12): 1311-9.

128. Shaham, Y; Erb, S; Leung, S; Buczek, Y; Stewart, J. 1998. CP-154,526, a selective, non-peptide antagonist of the corticotropin-releasing factor 1 receptor attenuates stess-induced relapse to drug seeking in cocaine-and heroin-trained rats. Psychopharmacol. 137:184-90.

129. Shaham, Y; Funk, D; Erb, S; Brown, TJ; Walker, C; Steward, J. 1997. Corticotropin-releasing factor, but not corticosterone, is involved in stress-induced relapse to heroin-seeking in rats. J Neurosci. 17:0-4.

130. Shahani SK, Lingamaneni R, Hemmings HC Jr. 2002. General anesthetic actions on norepinephrine, dopamine, and gamma-aminobutyric acid transporters in stably transfected cells. Anesth Analg. 95(4):893-9.

131. Shepard JD, Bossert JM, Liu SY, Shaham Y. The anxiogenic drug yohimbine reinstates methamphetamine seeking in a rat model of drug relapse. Biol Psychiatry. 2004 Jun 1;55(11):1082-9.

132. Smith, SM; Vale, WW. 2006. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogue Clin Neurosci. 8(4): 383-95.

133. Spear, LP. 2012. Rewards, aversions and adolescence: emerging convergences across labo-

ratory animal and human data. Dev Cogn Neurosci. 1(4): 390-403.

134. Spear, LP. 2000. The adolescent brain and age-related behavioral manifestation. Neurosci Biobehav. Rev. 24(4):417-63.

135. Sulzer, D; Sonders MS; Poulsen, NW; Galli, A. 2005. Mechanisms of neurotransmitter release by amphetamines: a review. Prog Neurobiol. 75: 406–33.

136. Swanson, LW; Sawchenko, J; Vale, WW. 1983. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the brain: an immunohistochemical study. Neuroendocrin. 36: 165-86.

137. Sun; WL, Zhou, L; Hazim, R; Quinones-Jenab, V. Jenab, S. 2008. Effects of dopamine and NMDA receptors on cocaine-induced Fos expression in the striatum of Fischer rats. Brain Res. 1243:1-9.

138. Tarazi, FI; Tomasini, EC; Baldessarini, RJ. 1998 Postnatal development of dopamine and serotonin transporters in rat caudate-putamen and nucleus accumbens septi. Neurosci Lett. 254:21–4.

139. Timpl, P; Spanagel, R., Sillaber, I; Kresse, A; Reul, JM; Stalla, GH; Blanquet, V; Steckler, T; Holsboer, F; Wurst, W. 1998. Impared stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet. 19(2): 162-6.

140. Tomić, M; Joksimović, J. 1991. Glucocorticoid status affects the response of rat striatal dopamine D2 receptors to hyperthermia and turpentine treatment. Endocr Regul. 25(4):225-30.

141. Torregrossa, MM; Xie, M; Taylor, JR. 2012. Chronic corticosterone exposure during adolescence reduces impulsive action but increases impulsive choice and sensitivity to yohimbine in male Sprague-Dawley rats. Neuropscyhopharm. 37(7):1656-70.

142. Traslaviña, GAA; de Oliviera, FL; Franci, CR. 2014. Early adolescent stress alters behavior and the HPA axis response in male and female adult rats: the relevance of the nature and duration of the stressor. Phys Behav. 133:178-89.

143. Ulrich-Lai, Y and Herman, JP. 2009. Neural regulation of endocrine and autonomic stress

responses. Nature Rev Neurosci. 10: 397-409.

144. U .S. Drug Enforcement Administration (USDEA), Office of Diversion Control . (2012) . National Forensic Laboratory Information System: Midyear Report 2011. Springfield, VA: U .S. Drug Enforcement Administration .

145. Valentino, RJ; Page, M; Van Bockstaele, E; Aston-Jones, G. 1992. Corticotropin-releasing factor innervation of the locus coeruleus region: distribution of fibers and sources of input. Neurocience. 48(3): 689-705.

146. Van Pett, KV; Viau, V; Bittencourt, JC; Chan, RKW; Li, HY; Arias, C; Prins, GS; Perrin, M; Vlae, W; Sawchenko, PE. 2000. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J of Comp Neurol. 428(2): 191-212.

147. Vargas-Perez, H; Kee, RT-A; Walton, CH; Hansen, DM; Razavi, R; Clarke, L; Bufalino, MR; Allison, DW; Steffensen, SC; van der Kooy, D. 2009. Ventral Tegmental Area BDNF Induces an Opiate-Dependent–Like Reward State in Naïve Rats. Science.324(5935):1732-1734.

148. Vidal, J; Bie, JD; Branneman, RA; Wallinga, AE; Koolhaas, JM; Buwalda, B. 2007. Social stress during adolescence in Wistar rats induces social anxiety in adulthood without affecting brain monoaminergic content and activity. Physiol Behav. 92(5):824-30.

149. Volkow, N.D., Chang, L., Wang, G., Fowler, J.S., Franceschi, D., Sedler, M., Gatley, S.J., Miller, E., Hitzemann, R., Ding, Y., Logan, J., 2001. Loss of dopamine transporters in methamphetamine abusers recovers with protracted abstinence. J. Neuorsci. 21, 9414- 9418.

150. Wahlstrom, D; White, T; Luciana, M. 2010. Neurobehavioral evidence for changes in dopamine system activity during adolescence. Neurosci Biobehav Rev. 34(5):631-48.

151. Wallace, DM; Magnuson, DJ; Gray, TS. 1989. The amygdalo-brainstem pathway: selective innervation fodopaminergic, noradrenergic and adrenergic cells in the rat. Neurosci Lett. 97(3): 252-8.

152. Wamsteeker, JI; Fuzesi, T; Watts, AG; Bains, JS. 2013. Characterization of corticotrpin-releasing hormone in the paraventricular nucleus of the hypothalamus of Crh-IRESCre Mutant mice. PLos One. 8(5): e64943.

153. Wang, B; Shaham, Y; Zitzman, D; Azari, S; Wise, RA; You, ZB. 2995. Cocain experience establishes control of midbrain glutamate and dopamine by corticotropin releasing factor: a role in stress-induced relapse to drug seeking. J. Neurosci. 25(22)5389-96.

154. Wang, B; You, AB; Rice, KC; Wise, RA. 2007. Stress-induced relapse to cocaine seeking: roles for the CRF(2) receptor and CRF-binding protein in the ventral tegmental area of the rat. Psychopharmacology. 193: 283-94.

155. Wang, G.J., Smith, L., Volkow, N.D., Telang, F., Logan, J., Tomasi, D., Wong, C.T., Hoffman, W., Jayne, M., Alia-Klein, N., Thanos, P., Fowler, J.S., 2012. Decreased dopamine activity predicts relapse in methamphetamine abusers. Mol. Psychiatry 9, 918-925.

156. Wilens, TE; Adler, LA; Adams, J. 2008. Misuse and diversion of stimulants prescribed for ADHD: a systematic review of the literature. J Am Acad Child Adolesc Psychiatry. 47(1):21–31.

157. Williams, CL; Buchta, WC; Riegel, AC. 2014. CRF-R2 and the heterosynaptic regulation of VTA glutamate during reinstatement of cocaine seeking. J Neurosci. 34(31)”10402-14.

158. Wimalasena, K. (2011). Vesicular monoamine transporters: structure-function, pharmacology, and medicinal chemistry. Med Res Rev 31(4): 483-519.

159. Wright, LD; Hèbert, KE; Perrot-Sinal, TS. 2008. Periadolescent stress exposure exerts long-term effects on adult stress responding and expression of prefrontal dopamine receptors in male and female rats. Psychoneuroendocrinology.33(2):130-42.

# APPENDIX I

## STANDARD SOLUTIONS LIST

### I. HISTOLOGY AND GENERAL PURPOSE

Antifreeze

- 1L MilliQ water
- 1.58g Sodium Phosphate (monobasic)
- 5.46g Sodium Phosphate (dibasic)
- Mix well
- Add 600ml Ethylene glycol
- 400ml Glycerol
- Mix well
- Store at 40C

DAB

- 5 mg/ml DAB (2.5g)
- 500ml MilliQ water
- Mix well
- Aliquot into 1ml tubes
- Store in-200C

KPBS (0.02 M)

- 1L MilliQ water
- 19g Potassium Phosphate (dibasic)
- 2.25g Potassium phosphate (monobasic)
- 45g NaCl

- Mix well on stir plate
- pH to 7.4-7.6
- Pour into carboy
- Bring volume to 5L
- Store at 40C

KPBS +Sucrose 20% (100ml)

- 20g Sucrose
- 100ml KPBS

KPBS +0.3% Triton (1l)

- 997ml KPBS
- 3ul Triton X 100
- Mix well

Na Acetate

- 1L MilliQ water
- 13.63 g Na Acetate
- pH to 6.0 with 10% glacial acetic acid
- Store at 40C

NAS (Nicke Ammonium Sulfate)

- -500ml 5% NAS-
- 25g NAS
- 500ML 0.1M Sodium Acetate Buffer
- pH 6.0
- Store at 40C

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Normal Fixative- 4% PFA in Borate Buffer (pH 9.5)

- 1L MilliQ water
- Heat and stir well until it reaches 60-650C
- 4g NaOH pellets/L (for 4L volume, use 16g)
- 40g PFA/L-> (for 4L use 160g)
- 38.14 Sodium Tetraborate (borax)/L (for 4L use 15205g)
- Store at 40C, Filter if needed, pH to 9.5 with 10% glacial acetic acid

Normal Saline (0.85%)

- 8.5g NaCl
- 1L MilliQ water
- Mix well and store at 40C

PBS

- 1L MilliQ water
- 0.256g Sodium Phosphate (monobasic)

o Sodium phosphate (dibasic) anhydrate

- 8.75g NaCL
- pH to 7.6

Thionin (for Nissl Stain)

- 1.44g NaOH
- 12ml glacial acetic acid
- 988mL MilliQ water
- Heat mixture to near 600C
- Heat to near 1000C

- While stirring, slowly add thionin (2.5g for a 0.25% solution)
- Boil for 45 minutes (keep covered)
- Filter and store in amber bottle
- Filter before each use.

## II. PROTEIN WORK

Blocking Buffer (500ml)

- 500ml TBST (10X)
- 5g BSA OR 5g Powdered Milk (Great Value, Bentonville)
- 0.025g NaAzide
- Mix well; store at room temp.

10 X Running (Laemmli) buffer (1L)

- 30.3g Tris Base
- 144.02g Clycine
- 900ml Milli Q water
- 10g SDS
- Mix well, adjust volume to 1L. pH to 8.3
- Store at room temp

1X Running (Laemmli) Buffer (1L)

- 100 ml 10X Running buffer
- 900ml Milli Q water
- Mix well, store at room temp. Can be reused.

Stripping Buffer (1L)

- 20g SDS
- 7.6g Tris base
- 1L MilliQ water
- 7.0 ml Beta-mercaptoethanol
- Mix well, pH to 6.7
- When ready to use, take 20 ml aliquot of buffer for stripping.

SDS PAGE Sample Buffer (10ml)

- 2.4 ml 1M Tris (pH 6.8)
- 0.8g SDS
- 4ml Glycerol
- 0.01% Bromophenol Blue (final – 0.02%)
- 2.8ml MilliQ water
- Stir to dissolve; store at room temp in dark.
- Add Beta-Mercaptoethanol before use (1ml) for reducing conditions

- 10X TBS
- 12.11g Tris Base
- 87.66g NaCl
- 1L MilliQwater
- Mix well, store at room temp

10X TBST

- 12.11g Tris Base
- 87.66g NaCl
- 5ml tween-20
- 1g Na Azide

- 1L MilliQ water
- Mix well, pH to 7 .6; store at room temp.

10X Transfer buffer (1L)

- 900 ml Milli Q water
- 30.3g Tris Base
- 144.1g Glycine
- Adjust volume to 1L
- Store at room temp.

1X Transfer Buffer (1L)

- 100ml 10X transfer buffer
- 900ml MilliQ water
- Mix well, store at room temp.  Can be re-used.

# VITA

After obtaining a B.S. in Biology and graduating ***summa cum laude*** from the University of Texas at El Paso in the winter of 1999, I started graduate research with Dr. Todd Primm in *Mycobacterial spp*. Stationary phase survival genetics, and moved toward proteomic research using tandem mass spectrometry to analyze encystation proteins of the intestinal parasite *Giardia lamblia.* Continually dismayed by the looks of disgust at parties when answering the, "So, what do you do?" Question with, "I work with a diarrheal parasite," I took a hiatus to explore life as a freelance graphic artist, stained glass craftswoman, and Argentine tango dancer with my husband and three cats.

In May of 2010, I wanted to finish what I had started back in 2000 and fell in love with the world of neuroscience and neuroendocrine pathology. As a member of the VIDA project, an interdisciplinary mentorship grant in topics of drug abuse, I was privileged to be able to escape research myopia and enjoy being in a field that was immediately relevant, helpful and relatable.

My greatest joys in graduate school were my teaching opportunities. I love engaging with students and finding out how best to explain new concepts and have a strong interest in building up multi-modal curricula. As a neuroscientist I feel it is my job to study the brain so I can learn how to teach the brain. The more pathways you engage and the more cues you lay on to the concepts you present, the stronger the memory becomes!

**Teaching and Mentoring Experience**

- *SMART MINDS Graduate Mentor 2012, 2013, 2014*
- *Assistant Instructor 2004-2005*
- *Supplemental Instructor 1998*

**Research Experience**

- *Graduate Researcher 2010-Present*
- *Graduate Researcher 2003-2005*
- *Research Assistant 2002-2003*

**Posters and Publications**

- Impact of homotypic stress exposure on methamphetamine self-administration in rats, C.E. D'Arcy, J.N. Hamdan, L.E. O'Dell, M. Miranda-Arango, K.L. Gosselink. Society for Neuroscience 2014.
- Repeated stress increases the rate of methamphetamine escalation in adult male rats, C. D'Arcy, J.N. Hamdan, L.E. O'Dell, K.L. Gosselink. Sun Conference 2014.
- Associated differences in the effects of stress on reward-associated regions of the rat brain. C.E. D'Arcy and K.L. Gosselink. Sun Conference 2012.
- Neurological responses to repeated retraint stress are altered in hypertensive rats. C.E. D'Arcy and K.L. Gosselink. Society for Neuroscience 2011.
- [Illustrations] D. Schulze-Makuch, L.N. Irwin (2004).Life in the Universe. New York, NW: Springer.

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