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Inoculation Stress Hypothesis of Environmental Enrichment

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Abstract

One hallmark of psychiatric conditions is the vast continuum of individual differences in susceptibility vs. resilience resulting from the interaction of genetic and environmental factors. The environmental enrichment paradigm is an animal model that is useful for studying a range of psychiatric conditions, including protective phenotypes in addiction and depression models. The major question is how environmental enrichment, a non-drug and non-surgical manipulation, can produce such robust individual differences in such a wide range of behaviors. This paper draws from a variety of published sources to outline a coherent hypothesis of inoculation stress as a factor producing the protective enrichment phenotypes. The basic tenet suggests that chronic mild stress from living in a complex environment and interacting non-aggressively with conspecifics can inoculate enriched rats against subsequent stressors and/or drugs of abuse. This paper reviews the enrichment phenotypes, mulls the fundamental nature of environmental enrichment vs. isolation, discusses the most appropriate control for environmental enrichment, and challenges the idea that cortisol/corticosterone equals stress. The intent of the inoculation stress hypothesis of environmental enrichment is to provide a scaffold with which to build testable hypotheses for the elucidation of the molecular mechanisms underlying these protective phenotypes and thus provide new therapeutic targets to treat psychiatric/neurological conditions.

Keywords

Environmental enrichment; inoculation stress; resilience; drug addiction; corticosterone

1.1 A history of environmental enrichment research

The “nature vs. nurture” debate began in earnest during the Victorian period, championed by Sir Francis Galton, who was inspired by the works of his cousin Charles Darwin. At issue was whether a person’s expressed traits are a product of heritability (i.e. nature) or by his/her

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own experiences (nurture). Galton, bolstered by Darwin's theories on heritability came down firmly on the side of "nature". The opposing "nurture" side of the debate was best defined centuries before by John Locke's borrowed term "tabula rasa" (i.e. blank slate). The "nurture" side of the argument was further strengthened in the early 1900s by John Watson's theories on behaviorism.

As science evolved (particularly the advent of genetics), the "nature vs. nurture" debate evolved into a "genes vs. environment" debate, respectively. The battle raged on as scientists on both sides of the argument produced irrefutable evidence for their view. Eventually, scientists realized that both arguments were correct—that a person's expressed phenotype was due to an interaction of genes with environment. Thus, the Gene/Environment Interaction Theory was born. In a basic sense, the environment controls (to some degree) how genes are expressed. Thus, gene transcription is where the proverbial "rubber hits the road" and seems to play a significant role in the protective phenotypes produced by environmental enrichment (Green et al., 2010; Lobo et al., 2013; Zhang et al., 2014), which are described below in the beneficial effects of environmental enrichment.

The beginning of modern environmental enrichment research is mostly attributed to Rosenzweig, Renner, Bennett, Diamond and colleagues. This group used the environmental enrichment paradigm to show convincingly that the adult brain still exhibits plasticity and that, just like muscles, brains get stronger with greater use. Rats reared in an enriched condition (EC) have a thicker cortex, more dendritic arborization and greater cognitive abilities than rats reared in an isolated condition (IC) (Diamond et al., 1964; Renner and Rosenzweig, 1987; Rosenzweig and Bennett, 1996). Following these early experiments, many others have used environmental enrichment and found it to be a useful animal model in a variety of fields, particularly because it is a non-drug and non-surgical manipulation.

In parallel with Rosenzweig and colleagues, Harry Harlow was finalizing the ideas for his seminal work on the importance of maternal and social enrichment in rhesus monkeys (Harlow, 1958). Harlow designed inanimate wire and cloth "surrogate" mothers to show that maternal contact is enriching to baby macaques beyond merely providing food. Although Harlow's early work was oriented to the positive effects of maternal enrichment (i.e. affection), his later work shifted perspective to focus on the isolation aspect (i.e. lack of enrichment) rather than the enrichment itself (Harlow and Suomi, 1971).

2.1 What is environmental enrichment?

Environmental enrichment is complex and there are numerous ways to provide enrichment. There is a lack of consistency in protocols for enrichment between different laboratories, but the most common procedure in rats involves rearing the subjects in a large cage with novel objects and social contact with conspecifics for at least 30 days beginning immediately after weaning. The objects are replaced and rearranged daily to maximize novelty. This arrangement provides three key facets of enrichment: novelty, social contact and exercise. It has been shown in rats that all three aspects are rewarding (Bardo and Bevins, 2000; Belke, 2000; Bevins and Bardo, 1999) and all three release dopamine in the nucleus accumbens (Greenwood et al., 2011; Louilot et al., 1986; Rebec et al., 1997). Thus, it can be said that

environmental enrichment is a compound manipulation that provides a daily workout for the dopamine system. Indeed, when the novel objects are replaced each day, the rats display a burst of exploratory activity lasting approximately thirty minutes that is beyond anything seen with locomotor stimulants like cocaine or amphetamine. Additionally, there is a second burst of exploratory/play behavior that occurs at the onset of the dark cycle, the beginning of the rats' normal period of high activity.

Although environmental conditions have a dramatic impact on the behavior of animals, these differing protocols for enriching rats often produce conflicting results. Parameters such as age of the animal, degree of enrichment, duration of enrichment, species and sex can each affect the results of an experiment. The lack of consistency in protocols likely stems from a lack of consensus regarding the definition of what indeed constitutes "environmental enrichment". Some might define enrichment based on environmental complexity–that a more complex environment is more enriching; however, environmental complexity alone is not the whole story. Environmental enrichment, by most definitions, should exert a positive influence on the organism, setting enrichment apart from overtly stressful events that have a negative impact on the organism. Thus, enrichment must provide an overall benefit to the organism. Further confusion in the field also arises from the fact that some researchers compare EC rats only to pair-housed social condition (SC) rats or compare only IC with SC rats (see below for discussion of the appropriate control for enrichment). However, without discounting or dismissing the views of others studying environmental enrichment using different protocols, this paper outlines a theory that the mild daily stresses of the enriched lifestyle are adaptive and inoculate rats to produce protective preclinical phenotypes for addiction and depression.

2.2 What are the beneficial effects of environmental enrichment?

As mentioned above, environmental enrichment contains three basic components: novelty, exercise and social contact. Animals are group-housed in a large cage equipped with children's plastic toys, which are replaced and rearranged every day. In order to study the "preventive" effect of environmental enrichment, rats are usually raised in the enriched condition before exposure to drugs (in the case of addiction research) or stress (in the case of depression research). Environmental enrichment attenuates the reinforcing effects of addictive drugs and produces an antidepressant-like effect (Brenes et al., 2008; Brenes Saenz et al., 2006; Green et al., 2010; Laviola et al., 2008). In addition, environmental enrichment can be studied as a "treatment" model, in which rats are assigned to either an isolated or enriched condition *after* they are exposed to drugs or stress, which has also been shown to produce adaptive consequences (Grimm et al., 2008; Solinas et al., 2008; Thiel et al., 2010). Below is an overview of the beneficial effects of environmental enrichment. To maintain focus, this hypothesis paper is predominantly centered on rodent research, although environmental enrichment has been studied in other species with success (Harlow, 1958; Harlow and Suomi, 1971; Nader et al., 2012; Solinas et al., 2010).

2.3 The protective addiction phenotype

Rats reared in an enriched condition exhibit lower basal locomotor activity than rats in the isolated condition, making interpretation of drug-stimulated locomotor data challenging. Despite this, the available evidence is fairly clear that EC rats show greater locomotor sensitivity to psychostimulants such as amphetamine and cocaine (Bardo et al., 1999; Bowling and Bardo, 1994a; Bowling et al., 1993; Smith et al., 2009) while at the same time showing reduced sensitization to repeated exposure (Bardo et al., 1995; Green et al., 2003; Smith et al., 1997). In the conditioned place preference (CPP) paradigm, a paradigm more relevant to addiction, Bowling and Bardo, and then Green and colleagues reported that enrichment produces enhanced CPP to both amphetamine and cocaine in rats (Bowling and Bardo, 1994b; Green et al., 2010). These results lead to the hypothesis that enrichment *increases* the risk for addiction; however, in the paradigm with the greatest face validity for addiction, the intravenous drug self-administration paradigm, the reinforcing effects of amphetamine, cocaine, ethanol, and methylphenidate are decreased by enrichment and the rats self-administer less of these drugs (Alvers et al., 2012; Bardo et al., 2001b; Deehan et al., 2007; Gill et al., 2013; Green et al., 2010; Green et al., 2002; Stairs et al., 2006).

Enrichment also alters drug taking in treatment models. Exposure to enrichment *after* exposure to cocaine reduces locomotor activity, eliminates cocaine CPP and reduces cocaine-induced reinstatement of CPP, and decreases cocaine-seeking behavior during extinction and cue-elicited reinstatement (Chauvet et al., 2012; Ranaldi et al., 2011; Solinas et al., 2008; Thiel et al., 2009). Although cue-elicited reinstatement is reduced in the treatment model, enrichment does not alter cocaine-primed reinstatement, suggesting that enrichment reduces the salience of drug-associated environmental cues which could lead to an effective therapy for craving elicited by drug cues in humans (Thiel et al., 2009).

2.4 The protective antidepressant phenotype

Along with the protective addiction phenotype, environmental enrichment also produces a protective antidepressant-like phenotype. In humans, three of the hallmark symptoms of depression are anhedonia, social withdrawal and behavioral despair. Our prior research and others found that, compared to IC rats, EC rats consume more sucrose in a sucrose preference test, indicating decreased anhedonia-like behavior; longer grooming time in the social contact test, suggesting decreased social withdrawal; and greater mobility time in the forced swim test (FST), suggesting reduced “behavioral despair” (Brenes et al., 2008; Brenes Saenz et al., 2006; Green et al., 2010).

2.5 Anxiety

Similar to addiction- and depression-like behavior, multiple labs have demonstrated reduced anxiety-like behavior resulting from environmental enrichment. For example, EC rats display lower basal locomotor activity, yet increased distance traveled in the center of the arena in the open field test, indicating an anxiolytic effect (Urakawa et al., 2013). In addition, enriched rats and mice were found to spend more time in the open arms in the elevated plus maze (EPM), and showed lower amounts of defensive burying and less defensive behavior when in close proximity to a predator, also suggesting reduced anxiety

(Friske and Gammie, 2005; Leal-Galicia et al., 2007; Roy et al., 2001). Enriched mice show reduced anxiety in response to social defeat stress, an effect that was abolished by lesioning the infralimbic region of the prefrontal cortex prior to environmental enrichment exposure (Lehmann and Herkenham, 2011), suggesting a role of the prefrontal cortex in anxiety and environmental enrichment.

However, not all are convinced that enrichment is anxiolytic. Our own research demonstrated greater sucrose neophobia in EC rats and more fecal boli in the cold-stress defecation test; both suggesting *increased* anxiety (Green et al., 2010). In addition, latency to ejaculation has been used as a measure of anxiety (Wallace et al., 2009), and EC rats exhibit increased latency to ejaculate, again suggesting increased anxiety (Urakawa et al., 2014). It is noteworthy that the last three tests (sucrose neophobia, cold-stress defecation, and latency to ejaculation) are not a function of exploratory activity in a novel environment. Because environmental enrichment involves extended exposure to novel environments whereas IC rats have very little to no experience in novel environments, this confounds the results of behavioral tests involving exploration of a novel environment. In addition, environmentally enriched rats show less social withdrawal than isolated rats, which may confound the results of social defeat tests on enriched animals (Green et al., 2010). Taken as a whole, the benefits of enrichment on anxiety-like behavior are not as clear as other areas, but further analysis of anxiety-like behavior in enriched animals is an interesting avenue for future experimentation.

2.6 Other disorders

Environmental enrichment has also been shown to have beneficial effects in rodent models of neurodegenerative diseases. For example, in a mouse model of Huntington's disease, enrichment delays the onset and slows disease progression by minimizing the loss of cerebral volume and by rescuing protein deficits (Hockly et al., 2002; Spires et al., 2004; van Dellen et al., 2000). Enrichment can also prevent neurodegeneration in C57BL/6 mice caused by a neurotoxin that causes Parkinson's disease-like symptoms in humans by regulating expression of the dopamine transporter (DAT) (Bezard et al., 2003). In addition, an increasing number of studies reported the beneficial effect of environmental enrichment in improving learning and memory in behavioral and molecular aspects of Alzheimer's disease (Bouet et al., 2011; Jankowsky et al., 2005; Jankowsky et al., 2003; Levi et al., 2003; Wolf et al., 2006). A study on Tg2576 mice, a model of Alzheimer's, found that environmental enrichment counteracts the deleterious effects of chronic unpredictable stress in Alzheimer's disease progression (Jeong et al., 2011). Further, a recent proteomic study found that the microtubule-associated protein tau was upregulated in EC rats compared to IC rats (Fan et al., 2013a). That paper and two other proteomics papers also identified huntingtin, presenilin 1, tau and amyloid precursor protein as major upstream regulators for environmental enrichment (Fan et al., 2013b; Lichti et al., 2014). These results warrant further investigation of neurodegenerative disorders using the environmental enrichment paradigm.

2.7 Species differences

Although environmental enrichment has been studied extensively in rats, other species have garnered considerably less attention, save for the work of Harlow in monkeys described above (Harlow, 1958; Harlow and Suomi, 1971). However, the available evidence from the primate literature suggests that enrichment is a protective factor for stress, as well as for addiction-related behavior (Harlow, 1958; Harlow and Suomi, 1971; Kozorovitskiy et al., 2005; Nader et al., 2012). There are, however, some important rat/mouse differences in the effects of environmental enrichment. For example, rats show the unusual phenotype where environmental enrichment increases responsiveness to stimulants such as cocaine or amphetamine in locomotor, CPP and neurochemical studies, while these same rats show decreased drug self-administration (Bardo et al., 2001a; Bardo et al., 1999; Bowling and Bardo, 1994a; Bowling et al., 1993; Green et al., 2010; Green et al., 2002); mice, on the other hand, show decreased sensitivity to stimulants after environmental enrichment (Solinas et al., 2009). Regardless, environmental enrichment produces a net benefit in addiction related behavior in both species (Solinas et al., 2010).

3.1 What is inoculation stress?

As described above, the environmental enrichment paradigm is a non-drug, non-surgical preclinical animal model useful for studying various psychiatric and neurological conditions. Environmental enrichment produces protective phenotypes in addiction and depression models, which are robust and replicable. The major question is *how* environmental enrichment can produce such robust individual differences in a wide range of behaviors associated with addiction, depression and anxiety and beneficial effects even for animal models of neurodegenerative diseases. Our hypothesis is that enriched rats undergo inoculation stress. In short, chronic very mild stress from living in a complex environment and interacting non-aggressively with conspecifics inoculates enriched rats against subsequent stressors and/or drugs of abuse.

Inoculation stress, described previously in human studies, is a process of developing resilience to future stressful events by first being exposed to mildly stressful experiences early in life (Dienstbier, 1989; Fox et al., 2006; Khoshaba and Maddi, 1999; Lyons et al., 2009; Meichenbaum, 2007; Parker et al., 2004; Rutter, 2006). Exposure to stress or adversity that toughens an individual is protective, much like a vaccination that exposes an individual to a non-harmful version of a disease in order to develop immunity to that illness for the future (Lyons et al., 2009; Rutter, 2006). For example, adults who are exposed to work stress as adolescents have fewer negative health effects from work-related stress as adults (Mortimer and Staff, 2004). Unlike a vaccine, however, inoculation stress does not protect only against a single disease, but toughens the individual in general and prepares them to cope with a variety of stressors later in life. For example, individuals that have successfully coped with some adverse events in adolescence have overall better mental health and are better able to cope with serious illness, spousal loss, or a major accident as adults (Khoshaba and Maddi, 1999; Lyons et al., 2009; Seery et al., 2013).

An important distinction to make is that inoculation stress is not simply exposure to any stress early in life, but rather having positive and adaptive responses to mild stressors is critical. Severe stress early in life often causes the individual to be more vulnerable to stress later in life whereas mild stress exposure with an adaptive response can protect the individual (Khoshaba and Maddi, 1999; Parker and Maestripieri, 2011). Stress that is inoculating conditions the individual, and provides specific coping strategies for exposure to future stressors, ultimately leading to resilience.

Stress inoculation has also been described in non-human primates and rodents. Squirrel monkeys and rodents exposed to a manageable stress early in life are less stress-reactive in the future (Lyons et al., 2009; Lyons et al., 2010). In particular, environmental enrichment in adolescence is able to provide protection from the deleterious effects of subsequent stressors (Fox et al., 2006; Larsson et al., 2002; Lyons et al., 2009; Parker et al., 2004), providing the impetus for the hypothesis that environmental enrichment is a chronic mild stress environment leading to stress inoculation.

4.1 Allostasis and allostatic load

In trying to make sense of how mild/moderate stressors are adaptive yet severe stressors are maladaptive, it helps to frame the picture with respect to allostasis and allostatic load. For the purposes of this paper, the term “allostasis” will be used in its broadest sense, disregarding the narrow application of this term only to energy regulation (McEwen and Wingfield, 2010). Thus, allostasis is defined as the process of returning a dynamic system to its stable set point after a challenge to that system. In a conceptual sense, homeostasis is maintaining stability in a non-dynamic system while allostasis is regulating stability of a dynamic system.

The genome is chock full of allostatic mechanisms for maintaining stability in hundreds of dynamic systems. However, maintaining stability of a dynamic system is not without cost to the organism. This cost to the organism is termed “allostatic load” and can be paid in a currency as diverse as energy usage, ion concentrations (e.g. neuronal activity), or protein turnover. Regulatory systems have evolved to deal with allostatic load to a certain extent, but most systems are susceptible to “allostatic overload” if the demands on the system outpace the allostatic capacity. At that point, the system fails to maintain stability and pathology develops. In the arena of psychiatric conditions, allostatic overload is evident when severe stressors surpass the allostatic capacity of the person and induce pathological conditions such as post-traumatic stress disorder, major depression, addiction, and anxiety disorders (among others).

One thing that differentiates allostasis from homeostasis is that allostasis, by virtue of regulating a dynamic system, can *predict* future allostatic load and adjust its capacity accordingly in anticipation of that future load. For example, forcing the average person to run 12 miles will result in allostatic overload of many systems (energy, oxygen, joints, muscles) whereas the seasoned runner’s body has predicted the possibility of a 12 mile run and adjusted the allostatic capacity of these systems to match the high load. As a result, instead of being pathogenic, subsequent stressors can even be motivating to the individual,

allowing them to thrive in adverse conditions. Thus, individual responses to the same stressful event can be highly variable, and the resulting effect on the individual depends on that individual's allostatic capacity to handle stress.

Allostatic overload of stress response systems can lead to psychiatric disorders, but how can inoculation stress or exposure to mild stress become protective against developing psychiatric conditions? Unfortunately stress has garnered a negative connotation, making experiments on stress with humans and animal models sometimes ambiguous. In 1976, Hans Selye defined stress as "the nonspecific response of the body to any demand made upon it" (Selye, 1976). The term "stress" does not inherently specify whether the stimulus is adaptive (i.e. inoculation stress) or maladaptive (i.e. allostatic overload), but even in Selye's own work it was often assumed that "stress" meant maladaptive stress. However, whether a stressor is positive or negative chiefly depends on the *allostatic capacity* of the individual. Most individuals exposed to stress are resilient to psychiatric conditions but those that are susceptible have a lower allostatic capacity for stress. Inoculation stress causes the organism to predict future stress and increase the allostatic capacity of the individual accordingly, providing resilience to subsequent stressors that might otherwise produce psychiatric conditions. Thus, environmental enrichment is hypothesized to increase allostatic load capacity from the repeated exposure to very mild stresses, inoculating against subsequent stressors.

Since allostatic capacity for stress is associated with psychiatric disorders, it is useful to assess allostatic load capacity for stress in rodents. There is a wide range of stressors from which to choose to preclinically examine allostatic load capacity. Mildly stressful stimuli include brief periods of restraint stress, mild footshock, brief exposure to temperature changes as in the cold stress-induced defecation paradigm, and handling by an experimenter (Gärtner et al., 1980; Green et al., 2010; Gregus et al., 2005; Rabasa et al., 2011b). Some severe stressors are long periods of immersion in cold water, long periods of food or water deprivation, and intense footshock (Rabasa et al., 2011b; Willner, 1997). One very severe stressor is social defeat by an aggressive conspecific (Covington and Miczek, 2005; Koolhaas et al., 1996; Müller et al., 2000). It is important to note that individual stressors can be considered mild or severe depending on duration and intensity. For example, brief maternal separation in rats is adaptive whereas lengthy maternal separation leads to allostatic overload and susceptibility in models of psychiatric conditions (Lyons et al., 2009; Sih, 2011). Thus, footshock, maternal separation, physical restraint, and temperature changes can be considered mild stressors or severe stressors. Predictability also seems important; the so-called chronic unpredictable mild stress paradigm (CMS), despite the label of "mild", can be a strong enough insult to induce allostatic overload and prevent inoculation (see below). Therefore, evaluating the response of the rodent to various stressors can aid in gauging the allostatic capacity of the rodent (i.e. whether they are inoculated against future stressors).

5.1 Stress, addiction and depression

As mentioned in the previous section, severe stress can lead to various psychiatric disorders; therefore, understanding the consequences of adaptive and maladaptive stress is

translationally relevant. Stress is not only implicated in conditions such as post-traumatic stress disorder, major depression, and anxiety disorders, but also drug use disorders. Severe stress in adolescence (such as being abused as a child) is associated with higher risk for alcoholism, substance abuse, depression, suicide attempts, obesity and poorer general health (Felitti et al., 1998; McEwen, 2000). Evidence suggests there is overlap in the underlying mechanisms for mental health disorders and drug use disorders (Levin et al., 2008; Worley et al., 2012), and psychological stress may be the common link. The environmental enrichment paradigm produces robust protective phenotypes for depression-like and drug abuse behaviors. Therefore, our hypothesis is not only does maladaptive stress increase the likelihood of psychological disorders and drug abuse, but also the protective effects of environmental enrichment may be due to adaptive responses to stress.

Regarding stress and depression, stress is the leading factor for both the development of and relapse to major depression (Hardeveld et al., 2013; Kendler et al., 1998; Kendler et al., 1999; Morris et al., 2010). Stress in the workplace and major life events, such as the death of a spouse, can trigger depressive episodes and increase the risk of major depression (Heim et al., 1997; Tennant, 2001). Evidence suggests that alterations in the hypothalamo-pituitary-adrenocortical (HPA) axis, which is involved in coordinating the body's response to various stressors (i.e. managing allostatic load), are involved in depression. Cortisol is a glucocorticoid released by the adrenal cortex in response to environmental stimuli and individuals with higher cortisol reactivity to low stress conditions had more depressive symptoms over time than those with low cortisol reactivity (Morris et al., 2012). In order to study this phenomenon preclinically alterations in the hypothalamo-pituitary-adrenocortical axis should be evaluated.

Regarding addiction, stress is a leading factor contributing to relapse to drug use in humans (Pohorecky, 1991; Sinha, 2001; Sinha et al., 1999; Sinha et al., 2006), an effect modeled by self-administration studies in rats (Ahmed and Koob, 1997; Erb et al., 1996). Stress exposure in humans, particularly to a severe stressor, significantly increases cocaine craving during abstinence (Pohorecky, 1991). In addition to contributing to relapse, there is increasing preclinical evidence that stress contributes to the initial development of addiction (Burke and Miczek, 2014; Covington and Miczek, 2005; Goeders, 2002; Piazza and Le Moal, 1998). For humans, stress increases the likelihood that someone will start smoking along with increasing the risk of relapse to cigarette smoking (Bruijnzeel, 2012). Additionally, exposure to stress increases cocaine craving (Sinha et al., 1999) and more stress-induced cocaine craving increases the likelihood of relapse in cocaine-dependent individuals (Sinha et al., 2006).

Beyond stress/addiction and stress/depression interactions, there is evidence linking addiction directly to depression (Levin et al., 2008; Worley et al., 2012). For example, individuals with major depression are more likely to smoke than the average person (Bruijnzeel, 2012). Major depression and substance use disorders are often comorbid in humans, and the symptoms are often more severe together than with only one disorder (Kessler et al., 2005; Pettinati et al., 2013). The rate of comorbidity of major depression with alcohol use disorders is 40.3%, and major depression with a drug use disorder is 17.2% (Pettinati et al., 2013). The high rate of comorbidity makes developing an effective

treatment very complicated. If the mood disorder is solely substance-induced, controlling the drug use would solve both disorders, but if the mood disorder has some other etiology, then antidepressants may be required (Pettinati et al., 2013). Often treatment for individuals with these comorbid disorders is focused on one disorder or the other and disregards the fact that stress may underlie both disorders.

Depression and addiction phenotypes often go hand in hand not only in humans but also in rodents (Green et al., 2006; Green et al., 2010; Green et al., 2008; Pettinati et al., 2013). For example, overexpression of a dominant negative inhibitor of CREB (inducible cAMP early repressor/ICER or a mutant CREB/mCREB) or knockdown of CREB in the NAc produces an antidepressant-like phenotype and also decreases cocaine self-administration in a similar manner to environmental enrichment (Green et al., 2006; Green et al., 2010). Similarly, the transcription factor ΔFosB in the NAc is associated with stress and also cocaine-taking behavior (Vialou et al., 2010; Zhang et al., 2014). Thus, it is very difficult to tease apart addiction and depression phenotypes in humans and in rodent models. Therefore analysis of an animal model that addresses both disorders simultaneously, such as environmental enrichment, is very valuable. Further, the close ties between depression and addiction are hypothesized to explain how inoculation to stress can affect drug-taking behavior.

As a whole, the evidence linking stress, depression and addiction provides a plausible rationale for how the repeated mild stress of environmental enrichment can protect against addiction-related behavior.

6.1 Does cortisol/corticosterone equal stress?

Because stress is linked with psychological disorders and drug dependence disorders, assessing stress in preclinical models is translationally relevant but can prove to be difficult without a good objective measure of stress in animals. Often, measurements of plasma cortisol, or the rodent equivalent corticosterone (CORT), have been used as a measure of stress in a variety of experiments. CORT is the primary glucocorticoid released by the adrenal cortex as a final product of the HPA axis. The HPA axis is activated in response to environmental stressors and activation of this system releases CORT. As a result, many scientists use CORT as a de facto indication of a subject's stress level. However, it is important to note that CORT does not equal stress. The following paragraphs will argue: (1) CORT levels fluctuate throughout the day independent of stress, (2) rewarding stimuli induce CORT release, (3) CORT induction is *attenuated* with chronic stress, (4) CORT itself is reinforcing and (5) behavioral responses to CORT administration alone do not mimic responses to stress. In addition, there are several caveats when attempting to extrapolate emotional and behavioral state from plasma CORT levels, not the least of which is that the mere act of acquiring a blood sample to measure CORT can be stressful itself, especially with high frequency sampling (Abelson 2005). Therefore, evaluating stress in environmentally enriched animals based on CORT levels has contributed to differing hypotheses on whether enrichment or isolation is inherently stressful because of inconsistent findings of corticosterone levels between enriched and isolated animals (Konkle et al., 2010). However, this is not to say that CORT is not important, or that corticosterone is not involved in the beneficial effects of environmental enrichment. In fact, CORT and the HPA

axis may be involved in the inoculation stress of environmental enrichment (see Section 8.1 below for elaboration). However, measured CORT levels do not provide a complete picture of the adaptive or maladaptive nature of the stress responses of an animal.

6.2 CORT levels fluctuate throughout the day

CORT measurements taken at different times during the circadian cycle will vary because CORT has a characteristic circadian rhythm. Regardless of stress level, CORT generally peaks as rats awaken just prior to the dark cycle and is lowest at the beginning of the light cycle (Allen and Kendall, 1967; Butte et al., 1976). The dark cycle is the period where the animals are awake and highly active versus the light cycle when the animals are mostly sleeping. Thus, if one were to use CORT as a de facto measure of stress, the circadian rhythm of CORT will likely produce confounding results depending on when blood is collected.

6.3 Rewarding stimuli induce CORT

Phasic CORT release subsequent to an environmental stimulus is generally assumed to be an indication that the animal is in a negative emotional state and that the stimulus had a negative impact. For example, social defeat, where a test subject is physically dominated by a more aggressive conspecific, is a noxious stressor and causes release of CORT in the defeated male (Buwalda et al., 2012). However, assumptions of a negative state are not always true. For example, sexual activity, a positive and rewarding stimulus, releases similar amounts of CORT as social defeat (Buwalda et al., 2012). Another stimulus, exercise, which is regarded as positive and rewarding, can also cause an increase in cortisol in humans (Buono et al., 1986; Deinzer et al., 1997; Rojas Vega et al., 2006). Voluntary exercise can also increase circulating CORT in Sprague-Dawley rats (Fediuc et al., 2006). In addition, rewarding drugs, including cocaine, cause CORT release (Moldow and Fischman, 1987; Torres and Rivier, 1992). Both rewarding and noxious stimuli cause alterations in CORT; therefore, CORT levels alone cannot differentiate between negative and positive stimuli.

6.4 CORT induction is attenuated with chronic stress

If CORT is a de facto measure of stress, one would expect repeated stress to increase CORT. Multiple studies have shown, however, that induction of CORT after a mild/moderate stressor attenuates with repeated exposure to the stressor in rats (Barnum et al., 2007; Carter et al., 2004; De Boer et al., 1990; Magarinos and McEwen, 1995; Mizoguchi et al., 2001; Natelson et al., 1988; Rabasa et al., 2011a). In humans, cortisol release also habituates with repeated exposures to the same stressor although there are individual differences in cortisol responses to stress (Deinzer et al., 1997; Gerra et al., 2001). This evidence suggests that repeated stress increases the allostatic capacity to future stressors, which is a possible mechanism for the inoculation stress underlying environmental enrichment effects.

6.5 CORT is reinforcing

Additional evidence CORT is not the same as maladaptive stress is demonstrated by the fact that CORT itself has reinforcing properties. Rats will intravenously (Piazza et al., 1991) and

orally self-administer CORT (Deroche et al., 1993), causing release of dopamine in the nucleus accumbens (NAc) (Graf et al., 2013). CORT injections also potentiate amphetamine self-administration at medium and high doses of amphetamine (10 and 30 μg/infusion) (Piazza et al., 1991). These experiments and others show that CORT is reinforcing at circulating levels similar to that released by mild stress (Piazza et al., 1991).

6.6 Responses to CORT differ from responses to stress

If CORT equals stress, then CORT administration should produce the same responses as stress. MacDougall and Howland found that rats injected with CORT versus rats exposed to 30 minutes of restraint stress (a mild stressor) had the same amount of circulating CORT, but only restrained rats showed changes in short- and long-term synaptic plasticity in the subiculum (MacDougall and Howland, 2013). Retana-Marquez and colleagues (1998) found that CORT injections were not able to mimic the behavioral effects of social defeat stress even at very high circulating plasma levels. Social defeat causes decreases in male sexual behavior and decreases in testosterone, whereas CORT injections do not (Retana-Marquez et al., 1998). Conversely, rats restrained for 6 hours a day for 21 days did not show an increase in depression-like behavior in the forced swim test but rats injected with CORT did show an increase in depression-like behavior, suggesting that in some cases, elevated CORT can cause more maladaptive changes than mild restraint stress (Gregus et al., 2005). Thus, CORT and stress sometimes produce different behavioral effects, and CORT administration alone cannot reproduce the behavioral effects of stress; therefore CORT does not equal stress.

All told, it is clear that although stress usually releases CORT, the circulating level of CORT is not a direct measurement of stress level. Further, it is important to remember that animals in a chronically mild-stress environment show attenuated rather than potentiated CORT induction to stress or drugs. If CORT is not an adequate measure of stress, how can we determine if enriched rats are actually more stressed than isolated rats?

7.1 Are enriched rats really stressed?

The inoculation stress hypothesis of environmental enrichment proposes that enriched rats are repeatedly stressed. However, at first sight, it is exceedingly difficult to make this case. Young male Sprague-Dawley rats (unlike mice) typically establish dominance hierarchies through play behavior and, as long as there are no female rats in the vicinity, typically do not feel the need to challenge these hierarchies over time. Thus, in this rat enrichment utopia, fighting is rare, food is plentiful, space is expansive, and rats get all of the novelty, social contact (rats are social creatures), and exercise they desire. Additionally, it has repeatedly been proposed that enrichment is the “functional opposite of stress” (Fox et al., 2006; Solinas et al., 2010; Wright and Conrad, 2008). If true, how can one make the case that enriched rats are chronically stressed?

Although there are multiple good lines of evidence suggesting enrichment produces the functional opposite of stress (Fox et al., 2006; Solinas et al., 2010; Wright and Conrad, 2008), none of the reports gives a possible explanation for how enrichment produces this effect. The inoculation stress hypothesis of environmental enrichment outlined here posits

that enrichment is a chronic mildly stressful condition that induces neuronal and neuroendocrine plasticity leaving enriched rats more resistant (i.e. greater allostatic capacity) to overtly stressful stimuli. Environmental enrichment exposes animals to novelty, social contact, and exercise and multiple studies have found that these variables cause stress-like responses. Acute voluntary exercise induces the secretion of CORT (Fediuc et al., 2006) and exposure to novelty will induce secretion of both CORT and adrenocorticotropic hormone (ACTH) in rats (Hennessy 1979, Ostrander 2006, Piazza 1991, Larsson 2002). Finally, rats housed together with conspecifics had higher circulating CORT levels than isolated rats suggesting that social contact also causes a stress-like response (Raz, 2013). However, in the absence of a truly reliable and objective measure of stress, one must rely on circumstantial evidence comparing the effects of enrichment with the effects of repeated mild stress (see Table 1).

As summarized in Table 1, there are numerous parallels between the effects of environmental enrichment and repeated mild stress, adding strength to the idea that enriched rats are chronically stressed. Due to the importance of stress as a contributing factor to depression and addiction (see above), the effects of chronic stress on the body have been studied in depth. Results of these studies have produced a clear picture of the endocrine, neurobiological and behavioral sequelae of chronic stress in humans and in rat models. Environmentally enriched animals have also been assessed for the same endocrine, neurobiological and behavioral effects. There is much evidence that repeated mild stress blunts CORT induction in response to subsequent stressors (Barnum et al., 2007; Carter et al., 2004; De Boer et al., 1990; Magarinos and McEwen, 1995; Natelson et al., 1988; Rabasa et al., 2011b) and environmental enrichment also results in blunted CORT induction to stress (Skwara et al., 2012; Stairs and Bardo, 2009; Stairs et al., 2011). Repeated exposure to severe stressors (i.e. those producing allostatic overload), such as social defeat stress, do not show reductions in CORT induction to subsequent stress (Barnum et al., 2007). Repeated stress also blunts stress-induced adrenocorticotropic hormone (ACTH) induction (Gadek-Michalska and Bugajski, 2003) and stress-induced release of adrenaline (Dobrakovova et al., 1990). Environmental enrichment also blunts ACTH induction (Belz et al., 2003; Skwara et al., 2012) and reduces stress-induced release of adrenaline (Moncek et al., 2004). In addition, enlarged adrenal glands have been found in environmentally enriched animals (Mlynarik et al., 2004; Moncek et al., 2004) and in animals exposed to repeated mild stress (Marti et al., 1993; Swanson and van de Poll, 1983). Environmental enrichment and repeated mild stress also both produce lower body weights (Harris et al., 2004; Pena et al., 2009) and following stress, the animal's heart rate returns to baseline more quickly (Carter et al., 2004; Chen and Herbert, 1995; Sharp et al., 2002).

Not only do environmental enrichment and repeated mild stress show the same endocrine consequences, they also show concordant neurobiological consequences in the nucleus accumbens. Environmental enrichment reduces induction of immediate early genes (IEGs) (Zhang et al., 2014), as does repeated stress (Alibhai et al., 2007; Green et al., 2008; Shoji and Mizoguchi, 2010). In contrast to other IEGs, environmental enrichment causes accumulation of basal ΔFosB protein in the nucleus accumbens (Lobo et al., 2013; Solinas et al., 2009), which also occurs in repeatedly stressed animals (Lobo et al., 2013; Perrotti et al., 2004).

Environmental enrichment also produces robust effects on behavior that show similarities to the behavior of repeatedly stressed animals. For example, enriched animals show increased defecation to a mild stressor (novel cage under cold conditions) (Green et al., 2010), and rats that were alternately restrained for 1 hour or placed on a platform surrounded by water for 1 hour a day for 5 days also show increased defecation (Jorge et al., 2010). Environmentally enriched animals are more sensitive to the locomotor activating effects of amphetamine (Bowling and Bardo, 1994a; Bowling et al., 1993) and cocaine (Smith et al., 2009). Repeatedly stressed animals are also more sensitive to the locomotor activating effects of amphetamine and cocaine (Deroche et al., 1992; Lepsch et al., 2005). Despite being more sensitive to locomotor stimulants, environmental enrichment decreases spontaneous locomotor activity in response to a novel environment (Bowling et al., 1993; Green et al., 2010; Green et al., 2003). After mild stress, animals also show a similar decrease in locomotor activity when placed in a novel environment (Cruz et al., 2012).

As described above and illustrated in Table 1, environmental enrichment and repeated mild stress have matching endocrine, physiological, neurobiological, and behavioral effects. Although this is circumstantial evidence, it supports the argument that environmentally enriched animals are in a state of chronic mild stress and this mild stress in adolescence inoculates against future stressors.

The mild stress of environmental enrichment, however, is distinct from the stress of paradigms such as the chronic mild stress (CMS) paradigm or the chronic unpredictable stress paradigm in that environmental enrichment constitutes predictable stress that produces adaptive responses. The chronic mild stress paradigm typically involves stressors such as food deprivation, water deprivation, brief exposure to another subject, lights on during the dark cycle, periods of titling the cage by 30 degrees, and long periods of wet bedding material which occur randomly throughout the week for several weeks (Murison and Hansen, 2001; Willner et al., 1992; Willner et al., 1987). The CMS procedure can reduce sucrose and saccharine preference after several weeks of this unpredictable stress exposure (Hatcher et al., 1997; Willner et al., 1987), an anhedonic-like effect that can be reversed by several weeks of treatment with a tricyclic antidepressant (Willner et al., 1987). However, the CMS paradigm does not typically have other depression-like effects, and under some conditions this paradigm can actually increase sucrose consumption, suggesting inconsistencies in the paradigm (Murison and Hansen, 2001). An inoculation stress interpretation of these inconsistent data would posit that the unpredictable nature of the CMS can elevate the “mild” stress to a level that induces allostatic overload and that the inconsistencies in anhedonic-like behavior are a function of degree. The CMS by Murison and Hansen (2001) may not have induced allostatic overload, thus producing an inoculating effect whereas the other two reports induced a more severe stress (Hatcher et al., 1997; Murison and Hansen, 2001; Willner et al., 1987). Indeed, Hatcher and Hansen reported only finding an anhedonic saccharin response when the CMS paradigm included food deprivation.

8.1 CORT as a possible mediator of the protective EC phenotype

The sections above argue that CORT is not the same as stress; however, that is not to say that CORT is irrelevant. In fact, CORT responses may contribute substantially to the environmental enrichment protective phenotypes. For example, the environmental enrichment protective addiction phenotype fits well with what is known of the influences of CORT on stimulant self-administration. As mentioned above, CORT itself can be self-administered by rats (Deroche et al., 1993; Piazza et al., 1991), but there also exists evidence that CORT plays a significant role in stimulant self-administration. For example, higher CORT induction was associated with greater self-administration of low unit doses of cocaine regardless of whether the rats were stressed with contingent footshock, noncontingent footshock or no footshock (Goeders and Guerin, 1996b). Next, acquisition of cocaine self-administration can be completely blocked by bilateral adrenalectomy, partially reduced by pharmacological inhibition of corticosterone release by metyrapone in rats, and self-administration can be partially recovered by adding CORT to the drinking water (Goeders and Guerin, 1996a). Given that stimulants induce CORT release and blocking that release blocks self-administration, it is likely that the *amount* of CORT release from a stimulant determines the ability of low doses of that stimulant to engender or maintain self-administration. Blunted CORT release from stimulants in EC rats (Stairs and Bardo, 2009) could be the underlying molecular mechanism whereby inoculation stress produces the protective EC addiction phenotype. Further investigations are warranted to test this hypothesis.

9.1 Stress influences on self-administration

As discussed above, acute stress in humans is a major factor in relapse to addiction, so it is not surprising that acute stress in rodents produces reinstatement of cocaine seeking (Erb et al., 1996). However, the question at hand is how prior stress (i.e., not during or immediately before the session) affects subsequent stimulant self-administration. The logic in the above sections suggests that inoculation stress blunts CORT induction and that a blunted CORT response leads to less stimulant self-administration. Hence, one would predict that prior repeated mild stress (i.e. inoculation stress) would decrease drug self-administration. Two reports show that rats exposed to short-term maternal separation stress as pups later show reduced acquisition of cocaine self-administration at low unit doses (Matthews et al., 1996; Moffett et al., 2006). Despite these reports however, there are several reports showing that repeated stress *increases* stimulant self-administration (Carroll and Meisch, 1984; Goeders and Guerin, 1994; Kosten et al., 2000; Miczek and Mutschler, 1996; Piazza et al., 1990; Shaham and Stewart, 1994). Multiple factors could account for this discrepancy. First, the stressors in some of these papers are severe stressors that would exceed the allostatic capacity of the rats. Second, it is possible (even likely) that inoculation stress is most pronounced in very young animals (as with maternal separation and environmental enrichment). Third, it is possible that many other factors affect self-administration and that one or more of these factors are at play in some of these experiments. Regardless, if the inoculation stress hypothesis of environmental enrichment is correct, one would predict that environmental enrichment would decrease stimulant self-administration, which is undoubtedly the case (Alvers et al., 2012; Bardo et al., 2001b; Green et al., 2010; Green et

al., 2002; Puhl et al., 2012; Stairs et al., 2006; Thiel et al., 2009). In any case, the fact remains that EC rats have blunted CORT responses, blunted CORT responses are associated with less self-administration, and EC rats self-administer stimulants less readily than IC rats which supports the inoculation stress hypothesis.

10.1 What is the best control for enrichment?

An important problem in the enrichment field is the difficulty of being able to compare results between labs because of inconsistencies in enrichment protocols and the use of different control groups. Environmental enrichment is a compound manipulation whereby rats are chronically exposed to novelty, social contact, and exercise. The most rigorous scientific approach would be to study each aspect individually and then in combination. For example, it would be nice to know the relative contributions of social contact vs. object novelty vs. exercise, and if the combination of these factors is redundant, additive or synergistic. However, it is not possible to fully separate these aspects because social interaction is a form of novelty and greatly increases activity (i.e. exercise). In addition, this approach would entail studying eight different conditions, and we as scientists have an ethical obligation to reduce as much as possible the number of animals used in biomedical research (Council, 2011). Additionally, as the number of conditions increases, there rapidly comes a point of diminishing returns where the cost (in money and time) of running an increasing number of conditions exceeds the small incremental benefit of the knowledge gained. Thus, the number of conditions must be limited. The fact that novelty, social contact and exercise all fall under the umbrella term “environmental enrichment” presupposes some commonality among the three constituents of enrichment. Indeed, each of these factors is rewarding to rats, and each releases dopamine in the nucleus accumbens, a critical brain region involved in stress, addiction, and depression (Greenwood et al., 2011; Louilot et al., 1986; Rebec et al., 1997). Accordingly, the many different conditions can now be reduced to as few as two: environmental enrichment and the appropriate control group.

Identifying the appropriate control group for cocaine administration is easy—an injection with no cocaine. Logic would dictate that the appropriate control for environmental enrichment (composed of novelty, social contact and exercise) would be a group with the absence of novelty, social contact, and exercise (*i.e.* isolation). Because pair housing is a form of enrichment (Council, 2011), comparing EC to pair-housed “control” rats would be akin to comparing a 20 mg/kg cocaine group to a 10 mg/kg “control”. The problem is that “standard” laboratory housing for rats is two per cage in most research laboratories, and as such, these pair-housed rats are viewed by many scientists to constitute the “normal”, or control condition. From this viewpoint, instead of seeing a continuum of enrichment ranging from isolation to pair-housed to full enrichment, environmental enrichment is seen as one manipulation (compared to pair-housed rats) and environmental isolation is seen as a different kind of manipulation.

At first sight, the case for isolation being a different kind of manipulation might seem to have merit. The case that many researchers make is that isolation itself is a stressor, and as such should not be used as a control. Indeed, many studies have shown that maternal separation (isolating pups from dam) and neonatal isolation (isolating pups from dam and

other pups) are significant stressors, evoking ultrasonic vocalizations and inducing CORT release in pups (Hennessy and Weinberg, 1990; Kehne et al., 1991; Kuhn et al., 1990; Levine et al., 1991; McCormick et al., 1998; Viau et al., 1996). In addition, acute isolation of group-housed rats also induces CORT, which is taken as a clear indication of stress (Takatsu-Coleman et al., 2013). Many researchers thus make the leap of considering isolation rearing a condition of chronic stress, citing large CORT induction from acute stress as evidence. However, the inoculation stress hypothesis states that enriched animals are in a state of chronic very mild stress. As described above, *blunted* CORT induction is a sign of chronic stress, which is seen in EC rats and chronic mildly stressed animals alike. Table 1 further illustrates the many other signs of chronic stress that EC rats show that IC rats do not show. It is important to make the distinction that exhibiting a greater response to a stressor is not the same as being chronically stressed—quite the opposite. The isolated animals essentially have a lack of daily stimulation (i.e. stress) and therefore show a greater response to stress than EC rats, which are constantly stimulated (i.e. stressed) and therefore show a lesser response to subsequent stress.

Arguments frequently used in favor of pair-housed controls over isolated controls is that the IC group is not a “natural” condition, nor a “normal” condition, and has less “translational relevance” than pair-housed rats. Pair-housing rats in a laboratory is certainly not more translationally relevant than any other housing condition. The only place it is normal for two humans to be confined in the same small space is in prison. As for “normal”, even wild rats are social animals and are found in groups rather than pairs that stick together constantly. Regardless, is a control group itself supposed to be “normal”? In the case of a pharmacological control group, would it be “normal” for a human to take an injection of saline rather than a drug? A control group should be the lack of a manipulation, but the pair-housed or social condition contains some of the variables of enrichment, namely social interaction and elements of exercise or play, and therefore is an intermediate level of enrichment capable of producing some behavioral effects of full enrichment, but not all (Bardo et al., 2001b; Green et al., 2010; Rosenzweig, 2003; Zakharova et al., 2012). For as described above in the inoculation stress hypothesis, environmental enrichment is a combination of exercise, novelty, and social contact with conspecifics, all of which are mild stressors resulting in inoculation against future stressors. Neither social interaction, novelty, nor any other single variable can account for all of the effects of enrichment (van Praag et al., 2000). However, the goal in using the environmental enrichment paradigm is not to tease apart the different aspects of enrichment or different gradations of enrichment but rather to determine differences between enrichment and the lack of enrichment to find the molecular determinants of the resilience to addiction and depression. Therefore, in our opinion in light of the inoculation stress hypothesis, the isolated condition is the correct control for enrichment and the inclusion of an intermediate group such as the social condition is unnecessary.

11.1 Conclusions

The inoculation stress hypothesis is an under-appreciated framework for understanding many of the complexities of stress and an organism’s response to that stress. This hypothesis also has utility as a scaffold for which to build other novel hypotheses concerning

susceptibility and resistance to psychiatric conditions such as addiction and major depression. Accordingly, application of the inoculation stress hypothesis to the environmental enrichment paradigm helps clarify the nature of nurture (i.e. role of environment) and its contribution to the resilience to addiction- and depression-related behavior. Future research designed from a better understanding of stress and environment will help to identify novel targets for the treatment of addiction and depression. Current research is already making headway (Fan et al., 2013a; Fan et al., 2013b; Pavlovsky et al., 2013).

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Highlights

- Enrichment produces protective phenotypes for addiction and depression
- Enriched rats have a chronic mild stress-like phenotype
- This chronic mild stress inoculates enriched rats against subsequent stressors or drugs of abuse
- CORT is not the same as stress
- Isolated rats are the best control for environmental enrichment

Table 1

Parallels between repeated stress and enrichment

	Effect	Repeated mild stress	Environmental enrichment
Endocrine	Blunted CORT induction	Natelson et al., 1988, De Boer et al., 1990, Magarinos and McEwen 1995, Barnum et al., 2007, Rabasa et al., 2011b, Carter et al., 2004	Stairs et al., 2011, Stairs and Bardo 2009, Skwara et al., 2012
	Blunted ACTH induction	Gadek-Michalska and Bugajski, 2003	Skwara et al., 2012, Belz et al., 2003
	Enlarged adrenals	Swanson and van de Poll, 1983, Marti et al., 1993	Mlynarik et al., 2004, Moncek et al., 2004
	Blunted adrenaline release	Dobrakovova et al. 1990	Moncek et al., 2004
Neurobiology	ΔFosB accumulation in NAcc	Perrotti et al., 2004, Lobo et al., 2013	Zhang et al., 2014, Lobo et al., 2013, Solinas et al., 2009
	Attenuated immediate-early gene induction in NAcc	Shoji and Mizoguchi, 2010 (cFos), Green et al. 2008 (ATF3),	Zhang et al. 2014, manuscript in preparation
Behavior	higher sensitivity to locomotor activating effects of amphetamine and cocaine	Deroche et al., 1992, Lepsch et al., 2005	Bowling et al., 1993, Bowling and Bardo 1994, Smith et al., 2009, Green et al., 2010
	decreased stimulant self-administration	Matthews et al., 1996, Moffett et al., 2006, *however, see:* Carroll and Meisch 1984, Piazza et al., 1990a, Goeders and Guerin 1994, Shaham and Stewart 1994, and Miczek and Mutschler 1996, Kosten et al., 2000	Bardo et al., 2001, Green et al., 2002, Stairs et al., 2006, Thiel et al., 2009, Green et al., 2010, Alvers et al., 2012, Puhl et al., 2012
	increased defecation under stress conditions	Jorge et al. 2010	Green et al., 2010
	decreased locomotor activity to novelty	Cruz et al., 2012	Bowling et al, 1993, Green et al., 2003, Green et al. 2010
Physiology	heart rate returns to baseline more quickly after stress	Carter et al., 2004, Chen and Herbert, 1995	Sharp et al., 2002
	lower body weight	Harris et al., 2004	Pena et al., 2009