MODELING THE EFFECTS OF STRESS ON RISK FOR PSYCHIATRIC DISORDERS

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ABSTRACT

The adverse effects of stress on health have long been known and there is a growing appreciation of the effects of stress on the risk for psychiatric disorders. In this report, we set out to add to our understanding of these issues by using mice to model the effects of stress on the brain and behavior. In the first series of studies, we demonstrate that mild chronic variable stress impairs cognitive function and that aged mice are particularly susceptible. Interestingly, we also find that stress exposure is associated with changes in the expression of several Alzheimer's disease-related genes including a 1.5 to 2 fold increase in *Bace1* in the hippocampus of young adult mice and the hippocampus, prefrontal cortex, and amygdala of aged mice. Finally, we find that exposure to environmental enrichment during stress prevents the changes in cognition, gene expression, and DNA methylation. In a second series of studies, we show that social defeat stress results in anxiety-like behaviors, depression-like behaviors, and increased baseline stress hormone levels. We then show that these effects persist long after the withdrawal of stressors thus confirming previously reported effects of chronic social stress on mood, anxiety, and HPA-axis function while also suggesting that the social defeat paradigm may be useful as a model of chronic, unremitting mental illness. Finally, we demonstrate that exposure to environmental enrichment after stress effectively reverses the changes in depression-like behavior, anxiety-like behavior, and HPA axis hyperactivity. Together, these studies reaffirm the role stress and the HPA axis in the pathogenesis of Alzheimer's disease and affective disorders. Further, the data presented here suggest that understanding the mechanisms by which environmental enrichment effectively prevented or reversed the observed effects of stress on cognition, mood,

anxiety, HPA-axis activity, gene expression, and DNA methylation will be a critically important area of future study that may ultimately provide insights into novel therapeutic targets for the treatment of Alzheimer's disease, major depression, and other stress-related psychiatric disorders.

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CHAPTER 1: INTRODUCTION

The effects of stress on the brain and risk for psychiatric disorders.

The link between stress and disease has likely been known since at least the time of Hippocrates who acknowledged the effects of 'toil' on both physical and psychological health (Lloyd, 1978). However, the modern attempt to understand 'stress' as it applies to health began in 1936 with the publication of Hans Selye's classic paper on the 'generalized adaptation syndrome' in which he described how diverse noxious stimuli lead to a syndromic cluster of adrenal hypertrophy, thymic atrophy, and gastric ulceration (Selye, 1936). Then, sometime during the 1940's, Selye used the term 'stressor' for the first time in reference to the various stimuli capable of inducing the generalized adaptation syndrome and in 1950 he published a landmark textbook on 'The Physiology and Pathology of Exposure to Stress' (Selye, 1950; Szabo, 2012). Coincidentally, prednisone was synthesized the same year and glucocorticoids were being rapidly adopted as powerful immunosupressants. Then, in 1952, Howard Rome published what is probably the first description of the adverse psychological response to steroids (Rome, 1952). More than two decades later, 'steroid psychosis' was first used to describe the many psychiatric symptoms (including among others, emotional lability, anxiety, depression, insomnia, hallucinations, delusions, hypomania, and memory impairment) that are associated with long-term prednisone treatment (Hall, 1979).

In the nearly 80 years since Selye first described the generalized adaptation syndrome, an enormous body of literature including both human studies and animal models have demonstrated that depression, anxiety, mania, schizophrenia, motivated behaviors, eating disorders, post-traumatic stress disorder (PTSD), and cognitive impairment can be

precipitated or exacerbated by stress, activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis, and exposure to glucocorticoids (McEwen, 1995 and 2007; Malkoff-Schwartz, 1998, Sapolsky, 1999; Lucas, 2004; Hammen, 2005; Lupien, 2009; Markham, 2011). While there are obviously many complicated factors linking specific stressors to specific psychiatric disorders, it is clear that the timing, duration, and severity of stressors are involved. For example, the prenatal period appears to be a time during which individuals are particularly susceptible, likely due to the effects of stress on neurodevelopment and programming of the HPA axis. Animal studies suggest that maternal exposure to even relatively mild stressors during pregnancy increases the offspring's risk for depression-like and anxiety-like phenotypes, drug-seeking behavior, and cognitive impairment (Lupien, 2009) while exposure to more severe 'stressors' such as infection, malnutrition, or pregnancy in harsh environments appear to increase the risk for severe developmental delay and schizophrenia (Markham, 2010) though, when such severe stressors are involved, the relative contributions of HPA axis disruption versus other mechanisms of disease are difficult to determine.

Interestingly, the aging brain also appears to be particularly susceptible to the adverse effects of stress. Many studies have demonstrated that even brief exposure to stress levels of glucocorticoids can induce cognitive deficits and hippocampal atrophy (Sapolsky, 1999) while sustained stress exposure may activate the β-amyloid pathway and increase the risk for Alzheimer's disease (Wilson, 2003; Norton, 2010; Green, 2006).

Alternatively, the early postnatal period seems to be a time of relative resistance to the effects of stress on the brain and risk for future psychiatric disorders. Despite the hyporesponsiveness of the HPA axis during this time, maternal separation and low parental care have been robustly associated with increased behavior problems, fear response, and risk of depression during adolescence (Lupien, 2009). Rodent studies suggest that the long-term behavioral consequences of maternal separation may be due, at least in part, to alterations in the sensitivity of the pituitary, hippocampus, hypothalamus, prefrontal cortex, and amygdala to corticotropin-releasing factor (CRF) (Anisman, 1998). However, translatability of these studies is difficult as the early post-natal period in rodents corresponds roughly with the human third trimester.

Adolescence, on the other hand, is a time of relatively high basal activity, hyper-responsivity, and slow habituation of the HPA axis. Numerous reports indicate that exposure to stressful life events during this period often precede first episodes of depressive and anxiety disorders (Lupien, 2009). The underlying neurobiology of these observations may be related to increased expression of glucocorticoid receptors in the adolescent prefrontal cortex (Perlman, 2007). Further, the widely observed sex differences in risk for depression and anxiety disorders may be explained, at least in part, by estrogens activating effects on the HPA axis (McCormick, 2007). Additionally, rodent studies suggest that stress exposure during adolescence may have long-lasting effects that may not emerge until adulthood. For example, exposing rats to chronic stress during adolescence increases the risk of adult-onset anxiety-like behavior (Avital, 2005), drug-seeking behavior (McCormick, 2007), and cognitive dysfunction (Isgor, 2004). The

findings related to learning and memory are difficult to translate, however, as the human hippocampus reaches developmental maturity earlier than in rodents (Rice, 2000).

By far, the effects of stress on the brain are best known in adulthood. Careful studies using stressors of varying duration and severity have led to an 'inverted-U' model (Diamond, 1992) which suggests that acute exposure facilitates vigilance and learning due to activation of pathways connecting the amygdala and hippocampus (Vouimba, 2007) while chronic or severe stressors increase the risk for a number of psychiatric disorders including cognitive impairment, depressive episodes, anxiety, and PTSD (Lupien, 2009). Additionally, it is widely recognized that stressful life events and disruption of circadian rhythms often precipitates both depressive and manic episodes among patients with bipolar disorder (Malkoff-Schwartz, 1998; Wulff, 2010). Insights into the mechanisms underlying these adverse effects on the adult brain come mostly from rodent studies which suggest that chronic and severe stressors result in impaired hippocampal neurogenesis (Gould, 1997), dendritic loss in the hippocampus (Watanabe, 1992; Magarin, 1995) and prefrontal cortex (Joels, 2007), and dendritic hypertrophy in the amygdala (Vyas, 2002).

Together, prior studies demonstrate a strong association between stress exposure at various points across the lifespan and risk for psychiatric disorders. However, much work remains to be done regarding understanding mechanisms, especially as a means towards developing novel treatment targets. In the chapters that follow, we will describe our studies using rodent models in order to provide a better understanding of the effects of

stress on the risk for cognitive and affective disorders, insights into underlying mechanisms, and potential interventions. But first, we will briefly review the most commonly used stress paradigms and the techniques most often used to assess cognition and affective-behavior in rodent models.

Stress Paradigms in Rodent Models

A tremendous variety of approaches have been taken to induce a 'stressed' state in rodent models. However, surprisingly little work has been done to compare the effects of various paradigms. Here, we will briefly describe several of the most commonly used techniques and the psychiatric phenotypes they are most commonly thought to produce. While this is certainly not an exhaustive list, it is intended to reflect the diversity of paradigms used in rodent studies of stress-related psychiatric disorders.

Prenatal Stress

In this paradigm, dams are usually exposed to variable stressors (such as restraint, forced swimming, cold, light, etc.) once to three times per day and many studies restrict stress exposure to specific developmental periods during pregnancy (for example, the third week of gestation to coordinate maternal stress exposure with rapid development of the fetal HPA axis). Consistently, studies have found that prenatal stress exposure results in long-lasting hyperactivity and hyper-responsiveness of the offspring's HPA axis. Impairment of hippocampal-dependent negative feedback resulting from decreased glucocorticoid receptor expression and binding has been proposed as an underlying mechanism (Welberg, 2001; Cottrell, 2009). Multiple studies have also found decreased *Bdnf* expression among offspring exposed to prenatal stress (Roth, 2010). Additionally, maternal glucocorticoids produced in response to prenatal stress may affect the offspring due to decreased placental expression of the glucocorticoid metabolizing enzyme, 11β-HSD-2 (Pena, 2012). Interestingly, changes in the expression of the glucocorticoid receptors (Cottrell, 2009), *Bdnf* (Roth, 2010; Boersma, 2014a), and 11β-HSD-2 (Pena,

2012) may be epigenetically regulated. Behaviorally, prenatal stress has been associated with increased anxiety-like and depression-like behaviors, alterations in coping style, impaired fear extinction, and cognitive impairment among adult offspring (Welberg, 2001; Boersma, 2014b, 2014c).

Maternal Separation

Maternal Separation is one of most commonly used stress paradigms during the early post-natal period. Typically, maternal separation involves the temporary removal of an entire litter from a dam each day for several days. By reducing maternal care, the paradigm is though to model both the physical and psychological aspects of parental neglect. Alternatively, some studies have taken advantage of naturally occurring variation in maternal care and separated offspring into groups based on the extent of maternal licking, grooming, or nursing (Schmidt, 2010). Both maternal separation and low-maternal care have been associated with long-term hyperactivity of the HPA axis signaling (Ladd, 1996; Liu, 1997) and altered *Bdnf* expression (Scaccianoce, 2006; Chatterjee, 2007). Behaviorally, maternal separation has been shown to increase the risk for depressive-like (Lee, 2007) and schizophrenia-like phenotypes (Ellenbroek, 2000) while naturally occurring low-maternal care has been associated with anxiety-like behavior (Caldji, 1998) as well as impaired learning under non-stressed conditions but enhanced fear-related learning (Champagne, 2008).

Social Isolation & Impoverished Environment

From around the time of weaning throughout the adolescent period, two closely related stress paradigms- social isolation and impoverished environments- appear to be the most commonly used. In both models, rodents are usually individually housed in cages without enrichments for several days to several weeks (Niwa, 2011). Alternatively, at least a few studies have exposed intact litters to impoverished environments (Avishai-Eliner, 2001). In most studies, rodents are exposed to social isolation or impoverished environments early in life while the behavioral and physiological effects are not assessed until adulthood. Behaviorally, both paradigms have been associated with depression-like, anxiety-like, schizophrenia-like phenotypes (Niwa, 2011, 2013). Cognitively, early social isolation has been associated with impairment during early adulthood (Niwa, 2011), while rats exposed to an early impoverished environment did not develop cognitive deficits until later in life (Brunson, 2005). Regarding potential underlying mechanisms, both paradigms have been found to increase baseline corticosterone levels (Avishai-Eliner, 2001, Niwa, 2011) and both may alter neurotransmitter levels in the prefrontal cortex and amygdala (Niwa, 2011, 2013). Additionally, exposure to an early impoverished environment has been found to impair hippocampal long-term potentiation (Brunson, 2005).

Chronic Restraint

Chronic restraint was widely used in the early investigations of habituation and sensitization of the HPA axis to stressors. In most cases, rodents are restrained for 1 to 6 hours a day for 7 to 21 days, though single episodes of restraint have also been used. These studies found that acute restraint results in a rapid increase in corticosterone while

repeated restraint results in increased baseline corticosterone and habituation to acute stressors (Pitman, 1988). Chronic restraint was also used as a model of major depression in early studies demonstrating that deficits in serotonin may play a role in phenotype and that antidepressant drugs may act to increase serotonergic signaling (Kennett, 1986 and 1987). Further, chronic restraint was one of the first paradigms to show that stress can induce dendritic atrophy in the hippocampus (Watanabe, 1992) and dendtritic hypertrophy in the amygdala (Vyas, 2002). Behaviorally, some studies have reported cognitive impairment after chronic restraint (Luine, 1994), though the phenotype does not appear to clearly correlate with length of stress exposure or changes in hippocampal dendritic morphology (McLaughlin, 2007). Others have found enhanced cognitive performance (Bowman, 2001), increased exploratory behavior (Marin, 2007), and decreased depression-like behavior (Platt, 1982) after chronic restraint. A number of studies have commented on the difficulty in interpreting results following habituation to chronic restraint (Melia, 1994; Ortiz, 1996; Marin, 2007).

Foot Shock

Many early studies of chronic, inescapable stress used foot shock to induce a state of 'learned helplessness' (Maier, 1976). In most cases, animals are exposed to multiple, brief, inescapable shocks to the foot or tail on a single day (often ~100 shocks of 1-2 mA, each lasting a few seconds). This approach results in a dramatic increase in corticosterone across various species and strains of rodents (Maier, 1976) with relatively little habituation (Shanks, 1990). Behaviorally, learned helplessness is characterized by failure to avoid shocks when the opportunity for escape is provided (Maier, 1976; Anisman,

1990). This paradigm has been widely used as a model of major depression, and has been shown to respond to chronic treatment with antidepressants (Murua, 1991a; Cyran, 2002), and exercise (Greenwood, 2003). Additionally, increased locomotor activity (Fadda, 1979) and increased drug seeking behavior (Goeders, 1994) have been observed in response to chronic foot shock. Others have suggested that the observed failure to avoid shocks despite baseline hyperactivity represents impaired cognitive ability to pair a behavioral response with a likely outcome rather than 'learned helplessness' (Anisman, 1990). The translatability of the 'learned helplessness' phenotype to major depression may be further confounded by other studies that have robustly demonstrated an acute analgesic effect of chronic shock (Jackson, 1979; Maier, 1983). While the extent to which learned helplessness represents depression-like behavior may be unclear, the impact of chronic shock on contingency learning (Maier, 1976) and hippocampal neurogenesis has been repeatedly shown (Malberg, 2003; Vollmayr, 2003).

Social Defeat Stress & Subordination

While chronic restraint and unavoidable foot shock clearly elicit strong stress responses, translatability of these and other similar paradigms may be limited. In order to better model the large psychosocial component of most human stressors, a number of groups have studied the effects of social defeat or subordination. In the social defeat paradigm, a test subject (the 'intruder') is placed in the home cage of an aggressive, dominant male (the 'resident'). The resident and intruder are usually allowed to physically interact for several minutes a day for a few days to a few weeks. After each period of physical interaction, the resident and intruder are most often separated by a barrier that allows the

intruder to see, smell, and hear the resident, thereby allowing persistent psychosocial distress (Martinez, 1998). Alternatively, the subordination model takes advantage of a more naturalistic social structure within a colony of male and female rodents. After several days, dominance hierarchies are established and it's thought that subordination results in a state of chronic stress (Blanchard, 1995). Both social defeat and subordination models have been found to markedly increase the production of glucocorticoids and there is some suggestion that baseline glucocorticoid levels remain elevated after withdrawal of stressors, which may be due in part to impaired hippocampal-dependent negative feedback (Martinez, 1998; Tamashiro, 2005). Further, even brief exposure to social stress appears to dramatically decrease hippocampal dendritic length, neurogenesis, and LTP (Buwalda, 2005), decrease serotonergic signaling within the hippocampus (Martinez, 1998; Tamashiro, 2005), and decrease *Bdnf* expression (Tsankova, 2006). In cortical and limbic structures, social stress appears to increase serotonergic signaling (Martinez, 1998; Tamashiro, 2005), and increase *Bdnf*-dependent dopaminergic signaling (Berton, 2006). Behaviorally, socially stressed rodents display depression-like and anxiety-like phenotypes, increased submissiveness, increased drug-seeking, decreased sexual behavior, and impaired hippocampal-dependent memory (Blanchard, 2001). However, socially stressed rodents are most often used as a model of major depression due, at least in part, to robust responsiveness of the depression-like phenotype to chronic treatment with antidepressant drugs (Golden, 2011).

Chronic Variable Stress

Chronic variable stress is a commonly used paradigm designed to introduce recurrent physical, psychological, and social stress that is unavoidable and unpredictable in timing (Katz, 1981a). While there is a large degree of variability in the methods used, most studies expose rodents to relatively mild stressors 1 to 3 times per day for 1 to 2 weeks. Some commonly used stressors include bright lights, loud noise, forced swimming, wet bedding, cold, a moving platform, social isolation, overcrowding, and restraint. Initially, chronic variable stress was specifically designed as an animal model of major depression (Katz, 1981a). The behavioral phenotype was characterized by decreased exploratory behavior (Katz, 1981a) that could be reversed with a number of commonly used antidepressant drugs (Katz, 1981b; Roth, 1981; Murua, 1991b; Willner, 1997). Mechanistically, these early studies largely contributed support for the monoamine hypothesis of major depression (Willner, 1997). While the first chronic variable stress protocols involved inescapable foot shock, food and water deprivation, and tail pinches (Katz, 1981a, Murua, 1991b), these severe stressors seem to have been largely replaced by milder stressors in more recent studies. Milder versions of chronic variable stress have been used to show that stress exposure increases CRF expression in the hypothalamic paraventricular nucleus (PVN) while also decreasing glucocorticoid receptor expression in the cortex and hippocamps, thus providing insights into the neurobiology underlying dysregulation of the HPA axis in response to chronic stress (Herman, 1995, Ostrander, 2006). Behaviorally, milder versions of chronic variable stress has also been associated with depression-like and anxiety-like behaviors that are reliably reversed by antidepressants (Willner, 2005). Further, several recent reports have focused on the cognitive effects of mild chronic variable stress. These studies have consistently found

impaired hippocampal-dependent learning and memory (Isgor, 2004; Song, 2006; Maras, 2014, Baglietto-Vargas, 2015), impaired hippocampal LTP (Alfarez, 2003), decreased hippocampal neurogenesis (Isgor, 2004), and loss of hippocampal synapses (Maras, 2014).

Behavioral Assays in Rodent Models

First, it should be noted that in rodent studies of the effects of stress on behavior, there is tremendous diversity in the choice of animal strain, age, stressor, length of exposure, and method of assessing outcomes with few studies using multiple behavioral tests. Further, many studies generalize findings from various tests despite considerable variation in methods, specific behavioral phenotypes assessed, and brain regions involved. In order to provide a rationale for the behaviors assessed in our own studies and also avoid over-generalization of findings, we will first review some of the most common behavioral tests of cognition, mood, and anxiety. For each test, we begin by summarizing the most common methods employed and then discuss the brain regions or pathways that are thought to be involved.

Assays of Learning & Memory

Morris Water Maze

The Morris Water Maze is perhaps the most well-known and commonly used test of spatial learning and memory in rodents. The standard protocol requires rodents to swim from a start location to a previously unknown escape platform that is submerged below the surface of opaque water, and therefore hidden from sight. The test requires rodents to orient themselves and navigate to the hidden escape platform using cues located on the perimeter or outside of the arena. Spatial learning can be assessed by measuring latency to finding the escape platform across multiple trials, and memory is most often assessed by removing the platform and measuring a preference for the quadrant in which the platform had previously been located (Morris, 1982, 1984; Vorhes, 2006). Performance

in the Morris Water Maze is correlated with hippocampal function, and has been specifically associated with hippocampal NMDA receptor function by two studies using NMDA receptor antagonists (Morris, 1986, 1989). Similarly, performance in the Morris Water Maze has been correlated with hippocampal long-term potentiation (LTP) (Morris, 1986, 1989, Jeffery, 1993). Reversal learning, which is a common addition to the standard protocol, requires rodents to learn a new location of the platform and, based on lesion studies, is thought to be more heavily dependent on the prefrontal cortex and striatum (deBruin, 1994; D'Hooge, 2001). Additional lesion studies suggest that other brain areas including the prefrontal cortex, basal forebrain, striatum, and cerebellum are involved in various aspects of the Morris Water Maze (Vorhees, 2006; D'Hooge, 2001).

Barnes Maze

The Barnes Maze is closely related to the Morris Water Maze in that the test requires rodents to find a hidden escape using external spatial cues. The primary variation from the Morris Water Maze is that, rather than relying on swimming, the Barnes Maze uses a dry, elevated circular platform with multiple potential escape holes located at the periphery. A hidden escape box is placed under only one hole at any given time. Also like the Morris Water Maze, learning, memory, and cognitive flexibility can be assessed by measuring latency to completion of the task across multiple trials, time spent in the area that had previously contained the escape box, and reversal learning, respectively. Based on early electrophysiological recordings from live animals, performance in the Barnes Maze is also thought to be largely hippocampal dependent (Barnes, 1979) though lesion studies of Morris Water Maze performance suggest that other brain regions such as the

prefrontal cortex and striatum are likely more involved in reversal learning tasks (deBruin, 1994; D'Hooge, 2001). While used less frequently than the Morris Water Maze, the Barnes Maze may have an advantage in cases where swimming speed, motivation, or motor coordination is impaired. Also, use of the Barnes Maze may avoid confounding factors associated with stress responses that are known to be activated by the Morris Water Maze. This is supported by at least one study which found that, while stress hormone levels are increased in both the Barnes Maze and Morris Water Maze, the stress response is significantly greater in the Morris Water Maze and test performance is correlated with stress hormone level only in the Morris Water Maze (Harrison, 2009).

Radial Arm Maze

The Radial Arm Maze consists of an elevated platform with several equally spaced arms (most often 8) radiating from a small, open central area and visual cues positioned around the maze. The test usually involves 'baiting' all or a subset of the arms with a food pellet and measuring latency to retrieval of pellets and errors over multiple trials. Latency to completion of the task across trials is thought to assess spatial learning while entry to a non-baited arm is usually considered an error of reference memory and re-entry to a previously baited arm is considered an error of working memory (Olton, 1976; Hodges, 1996). In another commonly used set-up known as the Radial Arm Water Maze, 6 arms radiate from a central area and only one arm contains an escape platform that is hidden under the surface of opaque water. Performance in the Radial Arm Water Maze is assessed in much the same way as the Morris Water Maze (Diamond, 1999). Also like the Morris Water Maze and Barnes Maze, the Radial Arm Maze is usually thought of as a

test of hippocampal function based on lesion studies (Diamond, 1999) and a study using NMDA receptor antagonists linked Radial Arm Maze performance to hippocampal LTP and NMDA receptor function (Ward, 1990). However, several studies using reversible, region specific sodium channel blockade have reported that Radial Arm Maze performance may be dependent on more widely distributed neural network involving the hippocampus, prefrontal cortex, nucleus accumbens, striatum, and thalamus (Floresco, 1997; 1999).

Y-Maze

The Y-Maze consists of a Y-shaped platform with three equally spaced, enclosed arms. Many variations of the test have been published but most rely on the tendency for rodents to explore novel environments and thus prefer entering a new arm rather than returning to an arm that has just been explored. Most commonly, the primary outcomes are the total number of arm entries and number of 'spontaneous alternations' or 'triads' which are defined as entering into each of the three arms without returning to a previously explored arm (Bailey, 2009). Alternatively, a single arm can be initially blocked, then unblocked in subsequent trials. In this case, the outcome can simply be whether or not the rodent first enters the previously blocked arm (Dellu, 1992; Bailey, 2009). The precise brain regions associated with Y-Maze performance likely vary depending on the experimental set-up. Lesion studies (Biggan, 1991; Lalonde, 2002), morphological studies (Koo, 2003), computational modeling (Atallah, 2004), selective induction of oxidative stress (Dean, 2009), and transgenic mice (Lalonde, 2002) have suggested that networks including the hippocampus, septum, prefrontal cortex, basal forebrain, striatum, and

cerebellum are involved.

T-Maze

The T-Maze consists of an elevated, T-shaped platform with three enclosed arms. Like the Y-Maze, many variations of the T-Maze are in common use. Also like the Y-Maze, the most common set-ups involve measuring tendency to enter a previously unexplored arm (i.e. 'spontaneous alternation'), tendency to enter a previously blocked arm, or latency to retrieval of a reward in a baited arm. In contrast to the Y-Maze, which measures 'spontaneous alternations' within a single trial, 'spontaneous alternations' in the T-Maze are usually measured in separate trials. That is, a cognitively intact rodent that choses to explore the left arm of a T-Maze in one trial is expected to explore the right arm on the next trial and doing so would be counted as a spontaneous alternation (Deacon, 2006). A much more complex 14-unit T-Maze has also been used in which a rodent is required to learn a series of right and left turns to reach a goal box. In the 14-unit T-Maze, rodents are motivated to find the escape by foot shocks that are administered upon failure to complete sections within a certain amount of time. Successful navigation of the 14-unit T-Maze is thought to rely more on procedural memory than spatial memory and thus may depend on striatal function with the hippocampus playing a subtler role (Goodrick, 1968; Pistell, 2009). Again like the Y-Maze, brain regions involved in T-Maze performance likely depend on the precise experimental set-up. Insights from many studies of brain lesions and transgenic mice indicate that the hippocampus, septum, prefrontal cortex, basal forebrain, thalamus, striatum, and cerebellum may all be involved (Lalonde, 2002; Deacon, 2006).

Novel Object Recognition

Several variations of the Novel Object Recognition test are in common use. Generally, the test involves a memory acquisition phase and a recall phase. In the acquisition phase, a rodent is allowed to explore a chamber containing two identical objects. The recall phase then takes place after an interval (usually ranging from several hours to a few days), during which one object is either moved to a new location or replaced with a novel object and the rodent is again allowed to explore. The ratio of time spent exploring the novel versus familiar object is measured. Alternatively, rodents can be presented with a series of objects at different times during the acquisition phase, then, during the recall phase, the 'familiar' object is considered the one that was most recently seen. Cognitively intact animals are expected to discriminate between novel and familiar objects and preferentially interact with novel objects. Performance in the Novel Object Recognition test and similar tests of recognition memory are thought to involve function of the hippocampus as well as cortical areas. Taken together, the results of several different lesion studies indicate that the hippocampus appears to be involved when tests involve recall of an object's place or object recency, but the prefrontal and perirhinal cortex are more involved in novel object preference (Mumby, 1994; Bussey, 1999, 2000; Barker, 2011).

Conditioned inhibition

Several tests of learning and memory including discrimination reversal, feature negative discrimination (FN), and the Variable Interval Delayed Alternation (VIDA) task, measure

behavioral inhibition and seem to be largely dependent on the ventral hippocampus with potential roles for the cortex and hypothalamic reward circuits (Davidson, 2005).

Both discrimination reversal and FN involve variations of classical conditioning. In discrimination reversal, rodents are first trained in a simple discrimination task such that one conditioned stimulus (CS1) is paired with an unconditioned stimulus (US), often a food or sucrose pellet, while another conditioned stimulus (CS2) is not. After asymptotic performance is reached, the pattern is reversed such that CS2 is paired with the US. Learning the pattern after reversal is related to hippocampal and prefrontal cortex function (Kanoski, 2007). During FN, a CS1 is paired with an US, but no US is delivered when CS1 is preceded by CS2. Learning to preferentially respond to CS1 alone is thought to be hippocampal-dependent (Kanoski, 2010).

Finally, a few studies of cognition in response to various stressors used the VIDA test, which is a modified go/no-go task. In the standard protocol, rodents are first trained to lever press for a food pellet using a continuous reinforcement schedule before introducing a simple alternating pattern between go and no-go trials. At first, the go and no-go trials are not separated by an intertrial interval and rodents quickly learn to lever press preferentially during go trials. After the rodents have successfully learned the alternating go/no-go pattern, a variable intertrial interval is introduced with longer intervals requiring more sustained memory of the previous trial. Memory can then be measured by comparing latency to lever press during go versus no-go trials with various intertrial intervals (Greenwood, 2001; Winocur, 2005). Learning the simple alternation rule is

thought to principally involve the frontal cortex while performance in trials with longer intertrial intervals is thought to be more hippocampal dependent (Winocur, 1991, 1992).

Assays of Mood & Anxiety

Open Field

The open field test may be the most commonly used assay in behavioral studies. Initially developed as a test for 'emotionality' (Hall, 1932) and later as a test for depression-like behavior (Katz, 1981a), the open field is now largely used as a test of locomotor activity, exploratory behavior, and novelty induced anxiety/ fear (Gould, 2009). Generally, the test involves tracking the behavior of a rodent placed in a novel square or circular arena. Distance traveled, time immobile, time in the center of the arena, rearing, defecation, and stereotypic behaviors are commonly assessed outcomes (Walsh, 1979). When the test is performed for a short time (often 5 or 10 minutes), behavior likely reflects response to a novel environment and increased immobility, increased thigmotaxis, and increased defication are thought to indicate an anxiety-like phenotype (Simon, 1994; Gould, 2009). In longer trials (sometimes lasting for several hours to a few days), the open field can be used as a measure of general locomotor activity and circadian cycles. Mechanistically, immobility and thigmotaxis in short open field trials respond reliably to benzodiazepines while most studies find no effect of monoamine-acting antidepressants. These anxiety-like behaviors are thus thought to be largely related to GABA signaling deficits (Prut, 2003) though it is also clear that cortical dopamine transmission also plays a role in exploration of novel environments (Fink, 1980; Dulawa, 1999).

Elevated Plus Maze & Elevated Zero Maze

The elevated plus maze is one of the most robustly validated assays of rodent behavior (Gould, 2009). Relying on a rodent's natural tendency to avoid open, elevated, brightly lit spaces (Pellow, 1985), the test has remained in wide use and largely unaltered since it was first introduced. In virtually all protocols, the apparatus consists of two open arms and two closed arms connected by an open center platform. Each mouse or rat is placed at the center of the elevated plus maze, facing an open arm, and allowed to freely explore for 5 minutes. Rodents naturally prefer the closed arms while increased time on or increased entries onto the open arm is thought to represent 'anti-anxiety' behavior (Walf, 2007). Alternatively, numerous studies have also used the zero maze which consists of an elevated, ring shaped platform with two open and two enclosed segments. Conceptually identical to the elevated plus maze, the zero maze eliminates the ambiguous center platform (Shepherd, 1994). The elevated plus maze was used in some of the first studies of the behavioral effects of benzodiazepines and related GABA-acting compounds (Pellow, 1986). Numerous other studies have demonstrated that benzodiazepines and 5-HT1A serotonin receptor agonists increase open arm time/ entries and that the effects are largest in models of anxiety (Hogg, 1996). Regarding neurobiology, studies using c-fos labeling after an elevated plus maze have been useful for understanding the brain regions involved in anxiety-like behavior including the amygdala, hippocampus, dorsal raphae nucleus, and limbic pathways. A similarly large rodent literature using the elevated plus maze has suggested the involvement of GABA, glutamate, serotonin, and the HPA axis in the mechanisms underlying anxiety (Walf, 2007).

Light-Dark Box

The light-dark box test is based on the conflict between rodents' natural tendencies to explore novel environments but also avoid bright, open spaces, and is thought to be an assay of anxiety-like behavior (Crawley, 1980). In principle, protocol, outcome measures, and responsiveness to anxiolytic drugs, the light-dark box is closely related to the elevated plus maze. In most studies, rodents are allowed to freely explore a chamber consisting of a large, brightly lit compartment and a smaller, dark compartment, though the details of different set-ups vary widely. Also, while the protocol has been adapted for rats, nearly all studies to date have been conducted with mice (Bourin, 2003). Regarding outcomes, the light-dark box test was initially designed to assess anxiolytic drug effects (Crawley, 1980). As such, most light-dark box data are framed such that decreased time in the dark compartment and increased transitions between compartments are thought to represent 'anti-anxiety' behavior. And like in the elevated plus maze, effectively all benzodiazepines and 5-HT1A serotonin receptor agonists have been found to increase time in the open compartment of the light-dark box, while more mixed results are found with SSRIs (Gould, 2009). The light-dark box has also been used to assess the behavioral phenotype of genetic models of psychiatric disorders, though apparently far less often than the elevated plus maze.

Forced Swim Test

The forced swim test is a frequently used assay of depressive-like behavior. In nearly all protocols, immobility is assessed after rodents are placed in an inescapable cylinder of water. In mouse studies, behavior is always scored in the last 4 minutes of a 6 minute trial

while most rat studies include a 15 minute habituation trial one day prior to a 5 minute test trial. Decreased latency to immobility and increased time spent immobile are thought to represent measures of 'behavioral despair' (Gould, 2009; Slattery, 2012). Beginning with the very first report, immobility in the forced swim test has been shown to respond to virtually all antidepressant drugs as well as ECT (Porsolt, 1977; Gould, 2009). While the forced swim test remains in wide use, some have questioned the validity of the model, suggesting that immobility could indicate adaptive learning/ coping rather than behavioral despair (De Pablo, 1989; West, 1990), though this may only be relevant for rat studies that usually include a habituation trial (Cyran, 2004). Additionally, thought the forced swim test has become a 'gold standard' rodent model for antidepressant drug response, some have questioned the face validity of the model as nearly all studies report immediate 'antidepressive' effects despite clear clinical evidence that the drugs currently in use have few acute effects on mood (Slattery, 2012). Apart from detection of antidepressant response, the forced swim test has also been widely used to validate genetic and stress-induced rodent models of depression which have provided tremendous insights into the roles of serotonin receptors, monoamine oxidase, GABA, glutamate, BDNF, noradrenergic signaling, and HPA axis signaling in depressive-like behavior (Cyran, 2004).

Tail Suspension Test

The tail suspension test, much like the forced swim test, assesses immobility in the presence of an inescapable stressor as a measure of 'behavioral despair' (Steru, 1985). Used almost exclusively in mice, the protocol involves suspending each animal from near

the tip of the tail for 6 minutes. In most studies, the primary outcome is immobility where increased time spent immobile is thought to represent depression-like behavior (Gould, 2009). Also like the forced swim test, immobility in the tail suspension test has been shown to reliably respond to acute treatment with effectively all classes of antidepressants (Cryan, 2005). In distinguishing the tail suspension test from the forced swim test, some have argued that tail suspension avoids hypothermia associated with the forced swim, and that immobility may be more easily scored in the tail suspension test (Steru, 1985; Cyran, 2005). Further, the tail suspension test appears to respond to lower doses of antidepressants and the response tends to be linear, whereas most dose-response studies using the forced swim test have reported an inverted-U response cure (Steru, 1985; Cyran, 2005). In addition to the rapid behavioral response to antidepressants, which is incongruent with the long duration of treatment required to elicit anti-depressive effects in humans (Gould, 2009), several other limitations to the tail suspension have been noted. Most importantly is the tendency for mice on a C57BL/6 background to rapidly learn to climb their tail and thus 'escape' (Mayorga, 2001). Also, immobility in the tail suspension test is closely related to general motor function. In fact, others have adapted the protocol to assess immobility and clasping postures as measures of motor coordination (Mangiarini, 1996). More recently, the tail suspension test has been used in a series of genetic studies in mice aiming to identify quantitative trait loci underlying differences in depression-like behavior and response to antidepressants. Results from these studies have strengthened evidence suggesting that GABA receptors (Yoshikawa, 2002), monoamine transport, and adrenergic signaling (Crowley, 2006) may play roles in the mechanisms underlying depression.

Sucrose Intake & Preference Tests

Intake of and preference for sweet-tasting solutions have been used as measures of hedonic response to a highly salient and rewarding stimulus (Katz, 1982; Willner, 1987). Conceptually, the detection of hedonic response to sucrose solution has a high degree of face validity as a potential measure of depression-like and mania-like behaviors. However, the specific methods used across studies vary greatly with regard to the specific sweetener, concentration, length of exposure, water access, and food access, making comparison of results difficult. Generally, after an initial habituation period, mice or rats are given access to either sucrose solution alone (1% sucrose appears to be the most commonly used concentration) or sucrose and water for between a few hours and a few days. Long-lasting decreases in preference for sucrose has been reported in models of depression, and the phenotype appears to be reversible with chronic antidepressant treatment (Willner, 1987; 1997). Additionally, the sucrose preference test has been proposed as a method for consistently identifying prone versus resistant mice in a stress-induced model of depression (Strekalova, 2004). In a model of mania, increased intake and preference for sucrose has been reported. Interestingly, the mania-like phenotype responded to the mood stabilizers lithium and valproate but not an antidepressant (Flaisher-Grinberg, 2009). A few mechanistic studies have suggested that sucrose preference may be predominately driven by dopaminergic signaling (Hsiao, 1995) with a minor role for the opioid system (Delamater, 2000).

Summary

Together, prior human studies clearly demonstrate a strong association between stress exposure at various points across the lifespan and risk for psychiatric disorders while rodent models have provided invaluable tools to both further our understanding of the mechanisms underlying these associations and to develop novel treatment approaches. However, there are few if any reviews in the rodent literature that compare the effects of different stress paradigms, and there seems to be a tendency towards over-simplification such that different stressors are discussed as though they produce discrete phenotypes (for example, conceptualizing social defeat as a 'model of major depression,' foot shock as a 'model of learned helplessness,' or maternal separation as a 'model of anxiety'). Similarly regarding behavioral testing, there is an emerging trend to move away from more comprehensive batteries of behavioral tests in favor of single, high throughput, automated screens (Tecott, 2004), and the interpretations of data from individual assays are often over-generalized (for example, viewing the Morris water maze as a test of 'hippocampal function' or the forced swim as a test of 'mood'). Careful review of the literature, however, suggests a great deal of phenotypic overlap can be seen across the various stress paradigms that are in common use and that much caution should be taken to avoid overgeneralizing the results of any single behavioral test. In the experiments described in the following chapters, we avoid using specific stress paradigms as models of particular psychiatric disorders, relying on behavioral assays to confirm those models, and assessing molecular changes as a means to elucidate mechanisms underlying the models. Rather, we take an approach that attempts, more simply, to better understand links between stress exposure and psychiatric-related phenotypes. We then investigate

molecular changes that might be relevant for understanding these links, and how the effects of stress on risk for psychiatric disorders might be prevented.

CHAPTER 2:

EFFECTS OF CHRONIC VARIABLE STRESS ON COGNITION

Introduction

A number of medical conditions including neurodegenerative disorders, psychiatric disorders, and cardiovascular disease are know to be precipitated or exacerbated by stressful life events and activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis (McEwen, 1995; Sapolsky, 1999; Black, 2002; Hammen, 2005). In Alzheimer's disease (AD), the most common age-related neurodegenerative disorder accounting for 60-80% of all dementias and currently affecting 5.4 million individuals in the United States, it is now thought that glucocorticoids may play a role in the development of cognitive impairment (Wilson, 2005; Norton, 2010; Alzheimer's Association, 2015). In transgenic animal models, prior studies indicate that stress exposure might exacerbate the characteristic beta-amyloid (Aβ) and tau neuropathological features of AD (Green, 2006; Carroll, 2011; Baglietto-Vargas, 2015) and it is now known that two genes whose products are centrally involved in Aβ pathology, *App* and *Bace1*, contain elements that are directly bound by glucocorticoid receptors (Green, 2006; Sambamurti, 2004). Additionally, increased cortisol levels in the cerebrospinal fluid (CSF) have been found among individuals who carry an *ApoE4* allele, which is well known to increase AD risk (Peskind, 2001). Further evidence suggesting a central role of stress and the HPA axis in the development of neurodegenerative disorders comes from a number of animal studies that have successfully prevented or slowed the development of cognitive impairment and neuropathology by means of stress-reducing environmental enrichment or drugs directed against a hyperactive HPA axis (Jankowsky, 2005; Lazarov, 2005; Mayer, 2006; Llorens-Martin, 2011).

Although the underlying mechanisms remain poorly understood, chronic activation of the HPA axis has been consistently associated with changes in brain morphology including neuronal loss, decreased neurogenesis, and altered connectivity in both animal and human studies (Lupien, 1996; Montaron, 2006; Klempin, 2007). Further, it is thought that critical developmental periods exist during which individuals are most vulnerable to these detrimental effects of stress exposure and old age has been identified as particularly sensitive time (Lupien, 2009). Conversely, human studies and animal models have indicated that neurodegenerative disorders result in further dysregulation of the HPA axis due to impaired hippocampal feedback (Sapolsky, 1999). In addition to its effects on neuronal morphology, chronic stress exposure has also been strongly linked to alternations in epigenetic markers which may ultimately lead to changes in gene expression. As just one example, it has now become clear that exposure to glucocorticoids can induce epigenetic modifications of the *Fkbp5* gene, which codes for a protein that normally functions as a co-chaperone of the glucocorticoid receptor (Tissing, 2005; Binder, 2009; Lee, 2010), but, in AD, it may also facilitate tau aggregation (Jinwal, 2010; Salminen, 2011; Blair, 2013). In addition to *Fkbp5*, epigenetic modifications have been strongly implicated in the regulation of *Bdnf* (Roth, 2009; Boersma, 2014a), which is critical for the survival and differentiation of neurons, and decreased expression is thought to be involved in a number of neurodegenerative disorders (Duman, 2006). Finally, several studies have found global changes in epigenetic markers in AD (Chouliaras, 2010; Lunnon, 2014), and recent studies using a mouse model of AD indicate that expression of the *Bace1* gene may be regulated by chromatin modification (Marques, 2012).

Taken together, prior evidence suggests a role for stress and potential underlying epigenetic mechanisms in the pathogenesis of neurodegenerative disorders including AD. In this study, we report that Chronic Variable Stress (CVS) results in mild impairments among young adult mice and dramatic impairment among aged mice in two tests of learning and memory. Further, in the hippocampus, we found that CVS is associated with increased expression of *Bace1*, which may be regulated by promoter region DNA methylation (DNAm). Finally, we show that environmental enrichment is able to prevent stress-related cognitive deficits as well as the changes in *Bace1* expression and DNAm.

Materials & Methods

Animals

60 male CD-1 mice (Charles River, Raleigh, NC) were used in this experiment. All mice were individually housed in standard tub cages on a 12h:12h light-dark cycle with *ad libitum* access to water and standard chow (Harlan 2018). Mice were randomly assigned to experimental groups. All protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Chronic Variable Stress (CVS)

CVS is a commonly used paradigm that is designed to introduce recurrent physical, psychological, and social stress that is unpredictable and unavoidable (Katz, 1981a). In the CVS paradigm used here, mice were exposed to one stressor each day for 14 days. Stressors included restraint, swim, cold, a moving platform, overcrowding, white noise, and light. To prevent habituation, stressors were introduced at varying times and for varying durations (Table 1). In the first experiment, mice were maintained under control conditions (CTRL) or exposed to CVS (Stress) at either 6 months (Young) or 18 months of age (Aged), which resulted in four groups: Young CTRL (n=6), Young Stress (n=6), Aged CTRL (n=8), Aged Stress (n=8).

Environmental Enrichment (EE)

In a separate cohort, a group of mice were exposed to control conditions, CVS, or CVS and EE. EE included a large tub cage filled with extra bedding, nesting sheets, and polycarbonate tunnels, balls, and housing domes (Bio-Serv, Frenchtown, NJ). EE was

started one week prior to CVS and continued throughout the 14 days of stress. This resulted in six groups: Young CTRL (n=5), Young Stress (n=5), Young Stress+EE (n=6), Aged CTRL (n=5), Aged Stress (n=5), Aged Stress+EE (n=6).

Plasma Corticosterone (CORT)

In order to determine CORT levels at baseline and in response to stress, blood was collected from all mice by making a small nick at the tip of the tail on day 1 and day 14 of CVS. Stressed mice were then exposed to 30 minutes of restraint (unstressed mice were returned to their home cage) after which another sample was collected from all mice. A third sample was collected 1 hour later. Plasma CORT concentration was determined by radioimmunoassay (MP Biomedicals, Solon, OH) and area under the curve was calculated for day 1 and day 14.

Open Field

The open field consists of an opaque plastic box (60cm square chamber, 60cm high walls) with a clearly marked central zone (circle with a 35cm diameter). Each mouse was allowed to freely explore the open field for 10 minutes. Behavior was recorded by a digital camera and later coded by a blinded observer for time spent in the inner zone and time spent exploring, immobile, assessing risk, and grooming.

Novel Object Recognition (NOR) Test

For 3 days before objects are introduced, all mice were habituated to the testing arena for 10 minutes each day. On day 4, two objects were placed in the arena and each mouse was

allowed to freely explore for 10 minutes. On day 5, one 'familiar' object was replaced with a 'novel' object and each mouse was again allowed to freely explore for 10 minutes. Behavior was recorded by a digital camera and later coded by a blinded observer for time spent exploring the novel and familiar objects.

Barnes Maze

The Barnes Maze consists of a plastic circular, elevated platform (122cm diameter) with 40 small holes (5cm diameter) around its periphery. A hidden escape box is fixed in place under one hole. Additionally, 3 visual cues and a bright light are also fixed around the perimeter of the maze. Mice were allowed to explore the maze during 4 trials a day for 4 consecutive days with 30 minutes between trials. If a mouse failed to find the escape box within 180 seconds it was gently guided to the escape. 24 hours after the final training trial, each mouse was given a single probe trial. Latency to entering the escape box was measured for each trial.

Tissue Collection

After behavioral testing, all mice were killed by rapid decapitation. The adrenal glands were weighed. Brains were removed, immediately frozen on powdered dry ice and stored at -80°C. The hippocampus, prefrontal cortex (PFC) and amygdala were later isolated from 300uM thick frozen coronal sections using a blunted 16-guage needle. Within each mouse, tissue from the right side of the brain was used for gene expression analysis and tissue from the left side of the brain was used for DNAm analysis.

Gene Expression

Tissue punches were initially placed in Qiazol. Total RNA was then extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Quantitative real-time PCR reactions were carried out in triplicate using 1xTaqMan master mix (Applied Boisystems, Foster City, CA), 1xTaqMan probes for each gene (Table 2), and 2ug of cDNA in a total of 20uL. Real-time PCR reactions were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system under standard conditions for 40 cycles. Expression levels relative to *Actb* were determined by the -**ΔΔ**Ct method.

Bisulfite Pyrosequencing

Genomic DNA (gDNA) was isolated using the Masterpure DNA Purification Kit (Epicentre, Madison, WI). gDNA concentration was then determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL). Bisulfite conversion was carried out on 500ng of gDNA using the EZ-DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). Nested PCR was then carried out using 25ng of bisulfite treated gDNA. Nested PCR products were then mixed with streptavidin coated sepharose beads (GE Healthcare, Waukesha, WI), isolated with a vacuum prep workstation, and released into a PSQ HS 96-well plate containing pyrosequencing primers. PyroMark Gold Q96 Reagents and a PyroMark MD System (Qiagen, Valencia, CA) were used for pyrosequencing. Quantification of CpG methylation was performed with Pyro Q-CpG Software v1.0.9 (Qiagen, Valencia, CA). Sequences of primers are listed in Table 3.

Statistical Analysis

Statistical analysis was completed using Statistica 7 (StatSoft, Inc., Tulsa, OK). Data are expressed as averages +/- standard error of the mean. In the first cohort, differences between groups were assessed by factorial or repeated measures ANOVA with 'Age' and 'Stress' as between subject factors followed by Tukey post hoc analysis. In the second cohort, differences between groups were assessed by one-way or repeated measures ANOVA followed by Tukey post hoc analysis. Correlations between levels of gene expression and DNAm or adrenal weight were assessed by Pearson's correlation. For all statistical tests, $p<0.05$ was considered significant.

Results

CVS results in cognitive impairment, and aged mice are especially susceptible.

On day 1 and day 14 of CVS, plasma CORT levels were determined at baseline, immediately following 30 minutes of restraint (control mice remained in their home cages), and after a 60-minute recovery period. AUC analysis shows that restraint resulted in elevation of plasma CORT among stressed mice on day 1 (Stress: $F(1,24)=56.37$, $p<0.0001$) and day 14 of CVS (Stress: $F(1,24)=37.77$, $p<0.0001$). Repeated measures ANOVA revealed that CORT responses were slightly lower on day 14 (Time: $F(1,24)=24.15$, $p<0.0001$) (Figure 1a). The chronic overproduction of stress hormones in response to CVS is further reflected by adrenal hypertrophy among stressed mice at the end of the experiment (Stress: $F(1,24)=40.24$, $p<0.0001$) (Figure 1b).

After CVS, behavior was assessed in the open field, NOR test, and Barnes maze. There was no effect of age or stress on behavior in the open filed (Figure 2). In the NOR test, young adult control and aged control mice were indistinguishable. However, CVS impaired the performance of only aged mice (Age*Stress: $F(1,24)=19.12$, $p=0.002$) (Figure 3a). In the Barnes maze, young adult control and aged control mice were indistinguishable in their ability to learn and remember the location of the escape box. CVS resulted in increased escape latency among both young adult and aged mice (Stress: $F(1,24)=66.36$, $p<0.0001$). However, aged mice exposed to CVS required significantly more time to complete the maze compared to all other groups (Age*Stress: ($F(1,24)=11.74$, $p=0.002$). Post hoc analysis of individual trials indicated that young adult mice exposed to CVS had increased escape latency compared to both control groups on

trials 13, 14, and 16 whereas significant differences between controls and aged mice exposed to CVS were found on trials 3, 4, and 6-17 ($p<0.05$). Moreover, aged mice exposed to CVS required significantly more time to find the escape box than young adult mice exposed to CVS during trials 3, 8, 12, and 14-17 ($p<0.05$) (Figure 3b). Together, the behavioral data suggests that, under control conditions, the performance of aged mice is indistinguishable from that of young adult mice. After exposure to CVS, cognitive impairment is evident in both young adult and aged mice, however, aged mice appear to be more greatly affected.

CVS increases the expression of *Bace1*.

In order to understand the effects of age and CVS on stress-related and AD-related gene expression, mRNA levels of *Bace1*, *Gsk3b*, *Bdnf*, *Fkbp5*, and *App* were measured in the hippocampus, PFC, and amygdala. In the hippocampus, CVS was associated with increased expression of *Bace1* among both aged and young adult mice (Stress: $F(1,24)=16.43$, $p=0.006$). CVS was also associated with increased expression of *Gsk3b* (Stress: $F(1,23)=6.24$, $p=0.02$) and *Bdnf* expression was significantly lower among aged mice (Age: $F(1,22)=4.72$, $p=0.04$) (Figure 4a).

In the PFC, CVS was associated with increased expression of *Bace1* only among aged mice (Age*Stress: $F(1,24)=4.60$, $p=0.04$). There was also an effect of age on the expression of *Gsk3b* (Age: $F(1,24)=5.57$, $p=0.03$). As in the hippocampus, the expression of *Bdnf* was significantly lower among aged mice (Age: $F(1,22)=4.65$, $p=0.04$) (Figure 4b).

In the amygdala, the expression of *Bace1* was increased only in aged mice exposed to CVS (Age*Stress: $F(1,23)=5.54$, $p=0.03$) (Figure 4c).

Taken together, these data suggest that stress exposure may impact the brain expression of several stress and AD-related genes. Perhaps most interesting is the consistent effect of stress on *Bace1* expression which was increased in the hippocampus of young adult mice exposed to CVS and in the hippocampus, PFC, and amygdala of aged mice exposed to CVS.

The stress related increase in *Bace1* expression may be epigenetically regulated.

In order to determine whether the observed changes in gene expression in response to CVS might be epigenetically mediated, bisulfite pyrosequencing was performed using primers designed to target CpGs in the promoter regions of *Bace1* and *Gsk3b*, as well as the region immediately upstream of *Bdnf* exon 4 where changes in DNAm have been previously reported (Boersma, 2014a). In the hippocampus, PFC, and amygdala, there was a consistent pattern of stress-related demethylation of several CpGs in the promoter region of *Bace1*. Specifically, in the hippocampus, CVS was associated with demethylation of CpGs located at 554 bases upstream of the transcription start site (tss-554) (Stress: $F(1,24)=14.72$, $p=0.0008$) and tss-506 (Stress: $F(1,24)=31.54$, $p<0.0001$) (Figure 5a). In the PFC, CVS was associated with demethylation of tss-506 (Stress: $F(1,24)=19.70$, $p=0.0002$). Also, methylation of tss-518 was lower among aged mice (Age: $F(1,23)=4.58$, $p=0.04$) (Figure 5b). In the amygdala, CVS was again associated

with demethylation of tss-506 (Stress: $F(1,24)=18.29$, $p=0.0003$). Additionally, aged control mice had higher methylation at tss-554 (Age*Stress: $F(1,24)=5.64$, $p=0.03$) (Figure 5c).

Regarding *Gsk3b*, there were no effects of age or stress on methylation of any of the CpGs that were assessed (Figure 6). For the *Bdnf* exon 4 region, aged mice had higher methylation at tss-109 in the hippocampus (Age: $F(1,23)=10.10$, $p=0.004$). Otherwise, there were no effects of age or stress on methylation of other CpGs (Figure 7).

Because gene expression and DNAm were assessed within each subject, we were able to correlate the two measures. Interestingly, we found significant inverse correlations between the expression of *Bace1* and methylation at the tss-506 CpG in the hippocampus ($r=-0.747$, $p<0.0001$) and PFC ($r=-0.622$, $p=0.0004$) and a similar, but non-significant trend in the amygdala ($r=-0.343$, $p=0.07$) (Figure 8). Together, these data suggest that *Bace1* expression may be epigenetically regulated and that changes in DNAm may underlie the stress-related increase in *Bace1* expression.

Environmental enrichment prevents the effects of CVS on cognition and *Bace1* expression.

Because EE has been previously shown to both moderate the stress response and enhance cognitive performance in rodent models, we sought to understand weather EE might prevent the observed effects of CVS on behavior, gene expression, and DNAm. In a separate cohort, young adult and aged mice were exposed to control conditions, CVS, or

CVS+EE. Interestingly, while all mice exposed to CVS demonstrated an elevation of CORT in response to restrain stress, there was a trend towards a blunted CORT response among mice exposed to CVS+EE. Among young adult mice, there was a significant group effect on plasma CORT during a 30 minute restraint test on day 1 ($F(2,13)=7.17$, $p=0.01$) and day 14 of CVS ($F(2,13)=5.37$, $p=0.02$). However, post hoc analysis revealed differences only between Young CTRL and Young Stress mice on day 1 ($p=0.01$) and day 14 ($p=0.02$) (Figure 9a). Among aged mice, there was also a significant group effect on day 1 ($F(2,13)=12.05$, $p=0.001$) and day 14 of CVS ($F(2,13)=6.50$, $p=0.02$) and post hoc analysis revealed differences only between Aged CTRL and Aged Stress mice on day 1 ($p=0.002$) and day 14 ($p=0.01$) (Figure 9b). The potentially protective effects of EE were further supported by measurement of adrenal weight at the end of the experiment. Among young adult mice, there was a significant group effect on adrenal weight ($F(2,13)=15.92$, $p=0.0004$) and post hoc analysis revealed significant adrenal hypertrophy among Young Stress mice compared to both the Young CTRL group ($p=0.0006$) and Young Stress+EE group ($p=0.003$) (Figure 9c). Similarly, among aged mice, there was a significant group effect on adrenal weight ($F(2,13)=9.84$, $p=0.003$) and post hoc analysis revealed significant adrenal hypertrophy among Aged Stress mice compared to both the Aged CTRL group ($p=0.002$) and Aged Stress+EE group ($p=0.004$) (Figure 9d).

We next assess the effects of EE on behavior. First, in the open field, there were no effects among young adult mice (Figure 10a). Among aged mice, there was a group effect only on exploration time ($F(2,13)=6.24$, $p=0.01$). Post hoc analysis revealed slightly greater exploratory behavior among Aged CTRL mice compared to both the Aged Stress

group ($p=0.04$) and the Aged Stress+EE group ($p=0.01$) (Figure 10b). In the NOR test, as in the first cohort, there was no group effect on the behavior of young adult mice (Figure 11a). There was, however, a group effect on the performance of aged mice in the NOR test ($F(2,13)=7.73$, $p=0.008$). Post hoc analysis revealed that Aged Stress mice were significantly impaired compared to the Aged CTRL group ($p=0.007$) and the Aged Stress+EE mice ($p=0.05$) (Figure 11b). In the Barnes maze, there was an overall effect of time on the performance of young adult mice ($F(16,208)=7.22$, $p<0.0001$) and a trend for an overall group effect ($F(2,13)=3.08$, $p=0.08$) (Figure 11c). Among aged mice, there was a significant time by group interaction ($F(32,208)=2.12$, $p=0.001$). Post hoc analysis of individual trials revealed significantly greater escape latency among Aged Stress mice compared to Aged CTRL mice on trials 10, 11, 12, 15, 16, and 17 ($p<0.05$). Aged Stress mice also had significantly greater escape latency compared to Aged Stress+EE mice on trials 12, 15, 16, and 17 ($p<0.05$). There were no trials during which the performance of the Aged CTRL group differed significantly from the performance of the Aged Stress+EE group further suggesting that EE may protect against the effects of stress on cognition, especially among aged mice (Figure 11d).

After behavioral testing, we assessed gene expression and DNAm in the brain of all mice. Because the most consistent changes in the first cohort were found in the hippocampus, we focused on this brain region in the second cohort. As in the first set of experiments, there was a group effect on hippocampal *Bace1* expression among young adult mice ($F(2,13)=7.59$, $p=0.007$) and post hoc analysis revealed higher expression of *Bace1* among Young Stress mice compared to both Young CTRL ($p=0.01$) and Young

Stress+EE mice (p=0.02) (Figure 12a). Similarly, among aged mice, there was a group effect on hippocampal *Bace1* expression ($F(2,12)=6.18$, $p=0.02$). Post hoc analysis revealed higher expression of *Bace1* among Aged Stress mice compared to Aged CTRL (p=0.02) and a trend for higher *Bace1* expression in the Aged Stress group compared to the Aged Stress+EE group (p=0.08) (Figure 12b).

When we assessed DNAm of the *Bace1* promoter region, we again found that CVS was associated with demethylation of several CpGs and this pattern of demethylation was prevented by EE. Specifically, among young adult mice, there was a group effect on methylation of the *Bace1* promoter at tss-554 ($F(2,13)=45.32$, $p<0.0001$), tss-518 ($F(2,13)=34.49$, $p<0.0001$), and tss-506 ($F(2,12)=6.07$, $p=0.02$). For tss-554, post hoc analysis revealed significantly lower methylation among the Young Stress group compared to both the Young CTRL group (p=0.0002) and the Young Stress+EE group (p=0.0002). For tss-518, post hoc analysis revealed significantly lower methylation among the Young Stress group compared to both the Young CTRL group (p=0.0002) and the Young Stress+EE group (p=0.0002). For tss-506, post hoc analysis revealed significantly lower methylation among the Young Stress group compared to the Young Stress+EE group (p=0.01) and a trend compared to the Young CTRL group (p=0.1) (Figure 13a). Among aged mice, there was a group effect on methylation of the *Bace1* promoter at tss-554 ($F(2,13)=42.06$, $p<0.0001$) and tss-506 ($F(2,13)=10.63$, $p=0.003$). For tss-554, post hoc analysis revealed significantly lower methylation among the Aged Stress group compared to both the Aged CTRL group (p=0.0005) and the Young Stress+EE group (p=0.0002). For tss-506, post hoc analysis revealed significantly lower

methylation among the Aged Stress group compared to both the Aged CTRL group ($p=0.01$) and the Aged Stress+EE group ($p=0.004$) (Figure 13b).

Finally, as in the first cohort, we found a significant inverse correlation between *Bace1* expression and methylation at tss-506 ($r=-0.462$, $p=0.01$) (Figure 14a). In the second cohort, there was also a strong inverse correlation between the expression of *Bace1* and methylation of a CpG located at tss-554 ($r=-0.695$, $p<0.0001$) (Figure 14b) suggesting again that *Bace1* may be epigenetically regulated and that changes in DNAm may underlie the stress-related increase in *Bace1* expression. Finally, we found that hippocampal expression of *Bace1* is strongly correlated with adrenal weight ($r=-0.704$, $p<0.0001$), further suggesting a potential role for CORT in the regulation of *Bace1* (Figure 14c). Together, these data largely confirm the findings of the first cohort and further suggest that EE is effective in preventing the effects of CVS on cognition as well as the expression and DNAm of *Bace1*.

Discussion

Several studies have implicated stress in the pathogenesis of cognitive decline and AD and prior work has also suggested that the aging brain may be particularly susceptible to the effects of stress (McEwen, 1995; Sapolsky, 1999). However, the nature of these associations remains largely unknown. In this study we found, at baseline, no differences in the performance of young adult and aged wild type mice in two behavioral tests of learning and memory. Clear differences emerged, however, after exposure to two weeks of chronic variable stress. We found that stress induced moderate impairment in the Barnes maze, a largely hippocampal dependent task, among young adult mice. In aged mice, stress resulted in profound impairment in both the Barnes maze and NOR test, which likely involves both hippocampal and cortical function. Others have similarly reported on the cognitive effects of stress. For example, in one study, exposure of young adult C57BL/6 mice to chronically high levels of CORT through drinking water resulted in impaired performance in the NOR test, Barnes maze, and Morris water maze (Darcet, 2014). Other studies have used chronic mild stressors that are similar to the CVS procedure used here and found stress-related impairment in the Morris water maze (Song, 2006) and NOR (Elizalde, 2008) among young adult mice. Regarding age-dependent effects of stress, one study found that prolonged social stress or administration of CORT impairs Morris water maze performance in middle-aged but not young-adult rats (Bodnoff, 1995) and another study found that even unstressed levels of CORT might negatively affect cognition in aged rats (Montaron, 2006).

Regarding the effects chronic stress in the brain, here we show that CVS increases the

expression of *Bace1* by 1.5 to 2-fold in the hippocampus of young adult mice and in the hippocampus, PFC, and amygdala of aged mice. *Bace1*, which codes for the beta-secretase enzyme, is critically important for AD pathology as it cleaves APP in the first step of the pathway leading to Aβ peptides. In AD, it is predominantly the Aβ peptides that are thought to promote the development of "senile plaque" pathology and neurodegeneration (Glenner, 1983; Iwatsubo, 1994; Crews, 2010). Several studies using transgenic mouse models of AD have previously reported that exposure to chronic stress can increase β-amyloid plaque burden (Dong, 2004 and 2008; Jeong, 2008) and another found that delivery of exogenous glucocorticoids can increase the expression of both beta-secretase and APP (Green, 2006). A study using wild-type rats found that chronic, unpredictable stress could increase the protein levels of beta-secretase in the brain (Catania, 2009). However, these prior reports provided limited insight into mechanisms underlying the increase in beta-secretase expression.

Here, we provide novel insight into the potential epigenetic regulation of *Bace1* and suggest that changes in DNAm may drive the stress-related increase in *Bace1* expression. While at least one other study has reported that histone modification may regulate *Bace1* expression (Marques, 2012), and a recent genome wide methylation analysis of post-mortem brain tissue strongly implicated hypermethylation of another gene, *ANK1*, in AD (Lunnon, 2014), DNAm changes in *Bace1* have not been well studied. In addition to finding stress-related demethylation of several CpGs in two separate cohorts, we found that the expression of *Bace1* across all groups was strongly correlated with DNAm of a CpG at tss-506. While this study focused on a small number of CpGs in the *Bace1* promoter region and there are certainly other CpGs that could have regulatory functions,

we known from epigenetic studies of other genes that, in some cases, even single site changes in methylation can greatly affect gene expression (Robertson, 1995; Zou, 2006; Nile, 2008; Claus, 2012). Further supporting the possibility that the observed changes in DNAm may be mediating *Bace1* expression, the tss-506 CpG is located within a putative Sp1 transcription factor binding site. Intriguingly, Sp1 has been previously shown to regulate the expression of human *BACE1* (Christensen, 2004) and binding of the transcription factor to its target sequence is thought to be regulated by DNAm (Mancini, 1999).

Finally, we show that EE is able to prevent the effects of CVS on cognition, *Bace1* expression, and *Bace1* methylation. EE has been previously shown to improve cognitive performance of wild type rodents and transgenic AD models (Jankowski, 2005), reduce Aβ levels in a transgenic AD mouse model (Lazarov, 2005), and enhance neuronal plasticity (Van Praag, 2000; Nithianantharajah, 2006). Further, numerous clinical reports suggest that enriching/stimulating environments and cognitive exercise may be protective against cognitive decline (Valenzuela, 2009; Bavelier, 2010). However, the effects of EE on stress-induced cognitive impairment and *Bace1* expression have not been well studied. Finally, though the precise mechanism linking CVS and EE to changes in methylation of the *Bace1* promoter remains an area of ongoing investigation, we also found that expression of *Bace1* strongly correlated with adrenal weight suggesting a role for glucocorticoids, which have been previously associated with changes in DNAm (Jinwal, 2010; Yang, 2012; Ewald, 2014).

Together, the current findings confirm the adverse effects of stress on cognition and further suggest that aged mice are especially susceptible. Additionally, we report that

CVS can decrease methylation and increase the expression of *Bace1* in the brain, which may provide a novel link between stress, Aβ pathology, and AD. Moving forward, exploring the mechanisms that directly link stress exposure to cognitive impairment and changes in the methylation and expression of genes in the β-amyloid pathway clearly warrant further investigation which may serve to reaffirm the role the HPA axis in the β-amyloid pathway and emphasize the need to carefully monitor cognitive function among patients (especially elderly patients) who have been exposed to high levels of stress or treated with glucocorticoids. Finally, understanding the mechanisms by which EE effectively prevented the observed effects of stress on cognition and *Bace1* expression will be an important area of future study that may ultimately provide insights into novel therapeutic approaches for the treatment of Alzheimer's disease.

Tables & Figures

Table 1. Chronic Variable Stress (CVS) schedule.

Chronic Variable Stress (CVS) Schedule			
Day	Morning	12pm	Afternoon
1	Restraint- 30min		
2		Swim- 10min	
3			Lights on- overnight
4		Shaker- 30min	
5	White Noise- 6hr		
6		Cold- 30min	
7			Social Stress- overnight
8		Shaker- 30min	
9	White Noise- 6hr		
10			Social Stress- overnight
11		Swim- 10min	
12	Cold- 30min		
13			Lights on- overnight
14	Restraint- 30min		

Table 2. TaqMan assays used for gene expression analysis.

Gene Symbol	Gene Name	NCBI RefSeq	TaqMan Assay ID
Actb	Actin, beta	NM_007393.3	Mm00607939_s1
Bace1	Beta-secretase 1	NM_011792.5	Mm00478664_m1
Gsk3b	Glycogen synthase kinase 3 beta	NM_019827.6	Mm00444911_m1
Bdnf	Brain-derived neurotrophic factor	NM_001048139.1	Mm04230607_s1
Fkbp5	FK506 binding protein 5	NM_010220.4	Mm00487401_m1
App	Amyloid precursor protein	NM_001198823.1	Mm01344172_m1

Table 3. Bisulfite pyrosequencing primers.

	Bace1
Out forward	GATAGGGTTTTTTTGTGTAG
Out reverse	CTATAATTCTTACCACAATATA
In forward	GTTTTGGAATTTATTTTGTAG
In reverse	ATAAACTAAAAACCTACCTC
Sequencing	TAGGTTGGTTTGGAATTTAG

	Gsk3b
Out forward	ATGGGGAYGGTTTTAYGTTTTA
Out reverse	CTCACTACTATACAATCRCAA
In forward	GATYGGGTTGTATAGTTAGT
In reverse	CCTAACTACAATAAAATTAACCT
Sequencing	GAAAGTAGATGTAGGT

	***Bdnf* exon 4**
Out forward	GAATTTGTTAGGATTGGAAGTG
Out reverse	TCCACRCTACCTTAACRTAAAC
In forward	ATAAAGTATGTAATGTTTTGGA
In reverse	TATCATATAATACCTCCTCTA
Sequencing	AATAAAAGATGTATTATTTTAAATG

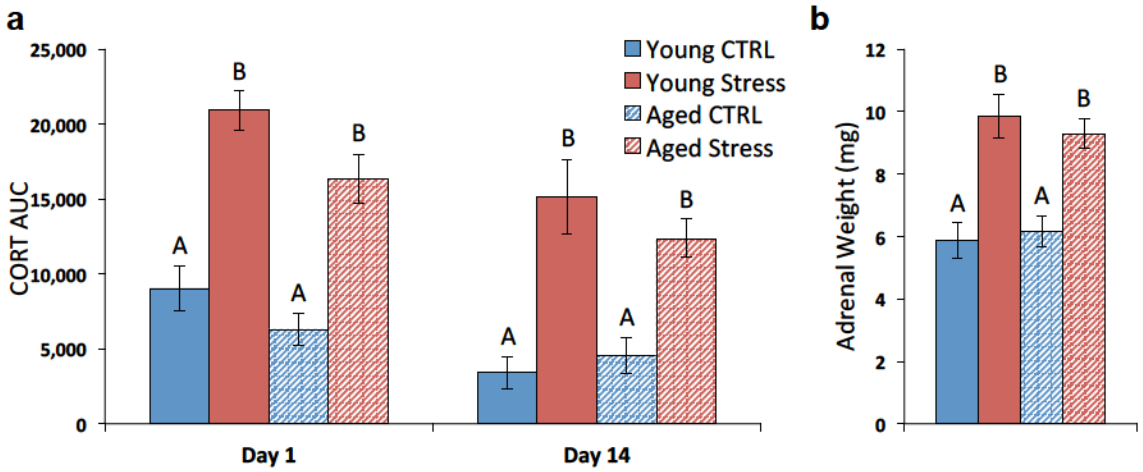


Figure 1. Stress response to CVS.

(**a**) Restraint stress tests on day 1 and day 14 resulted in elevation of plasma CORT among young adult and aged mice. (**b**) Chronic overproduction of stress hormones in response to CVS is reflected by adrenal hypertrophy among stressed mice. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

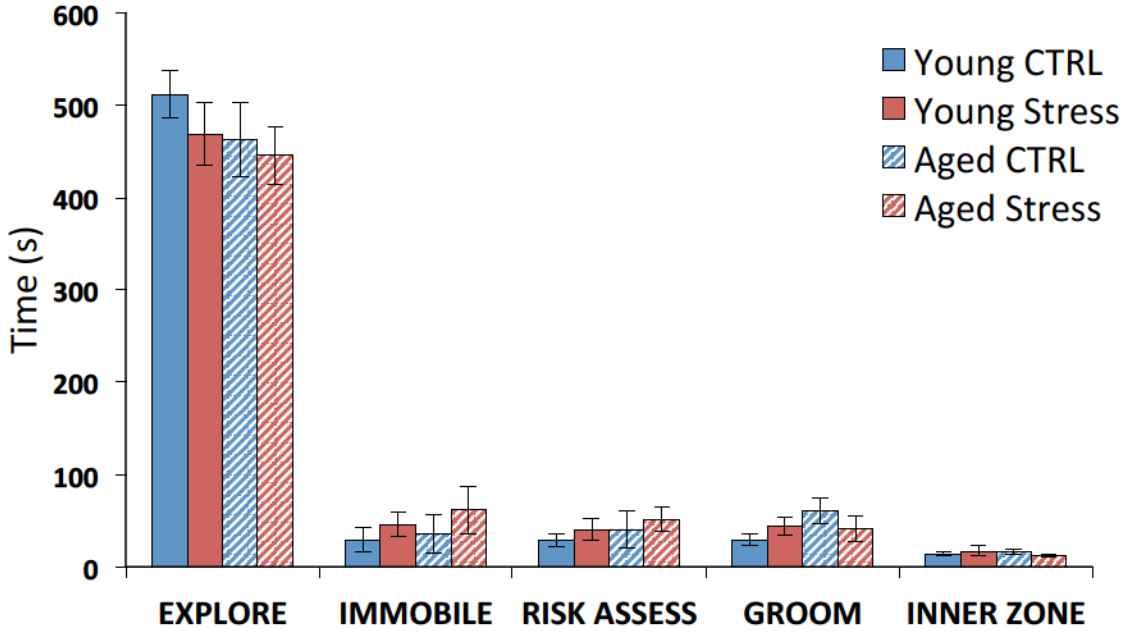


Figure 2. Open field behavior.

There was no effect of age or CVS on locomotor activity or exploratory behavior in the open field. Data represent mean ± SEM.

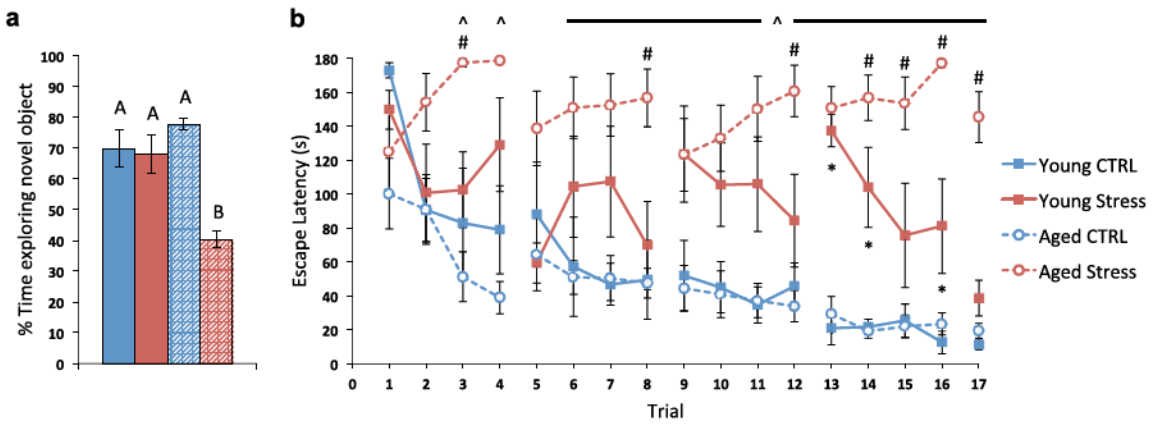


Figure 3. CVS impairs performance in tests of cognitive function; aged mice are particularly susceptible.

(**a**) CVS resulted in impaired performance in the NOR test only among aged mice. (**b**) In the Barnes maze, CVS resulted in increased escape latency among young adult and aged mice, but aged mice were more profoundly impaired. Data represent mean ± SEM. For post hoc analysis in **a**, groups that do not share letters are significantly different ($p<0.05$). For **b**, *$p<0.05$ Young Stress v. CTRL; ^$p<0.05$ Aged Stress v. CTRL; #$p<0.05$ Young Stress v. Aged Stress.

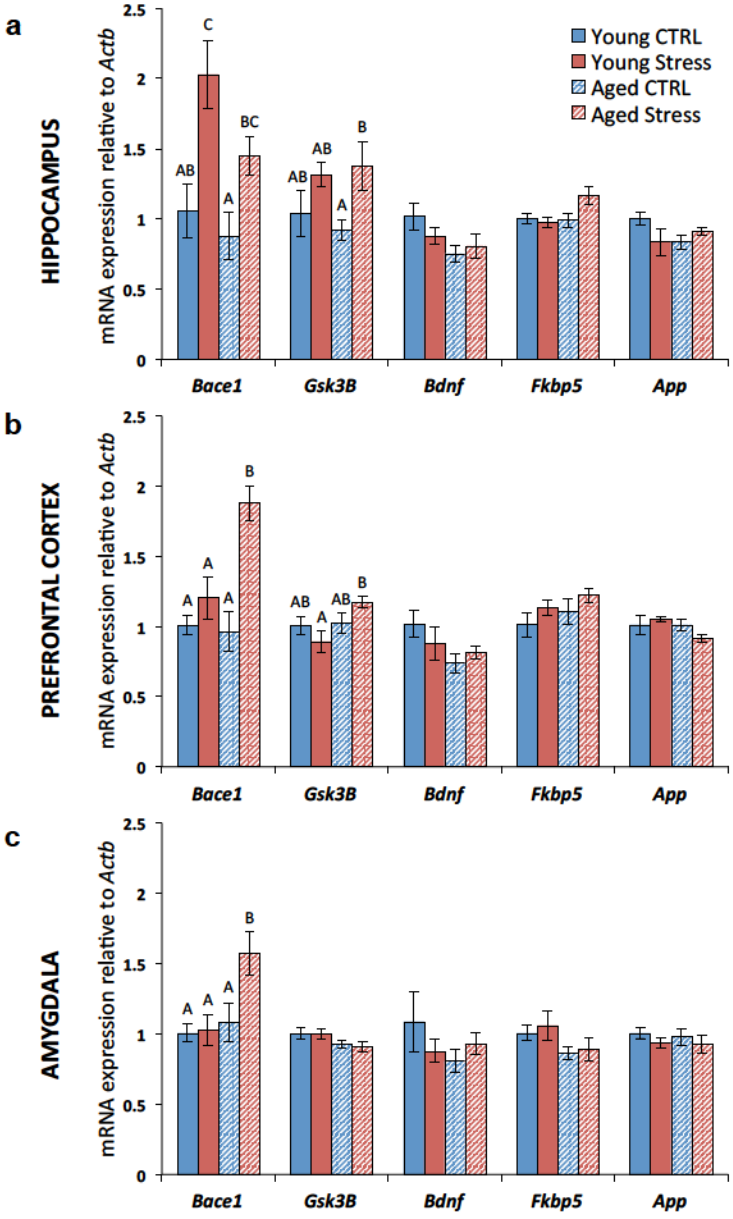

a
HIPPOCAMPUS
b
PREFRONTAL CORTEX
c
AMYGDALA
mRNA expression relative to Actb
Young CTRL
Young Stress
Aged CTRL
Aged Stress
Bace1
Gsk3B
Bdnf
Fkbp5
App

Figure 4. CVS results in increased expression of *Bace1*.

(**a**) In the hippocampus, CVS was associated with increased expression of *Bace1* among both aged and young adult mice. CVS was also associated with increased expression of *Gsk3b* and the expression of *Bdnf* was significantly lower among aged mice. There was a trend towards increased expression of *Fkbp5* among aged mice exposed to CVS. (**b**) In the PFC, CVS was associated with increased expression of *Bace1* only among aged mice. There was also an effect of age on the expression of *Gsk3b* and the expression of *Bdnf* was significantly lower among aged mice. (**c**) In the amygdala, the expression of *Bace1* was increased only in aged mice exposed to CVS. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

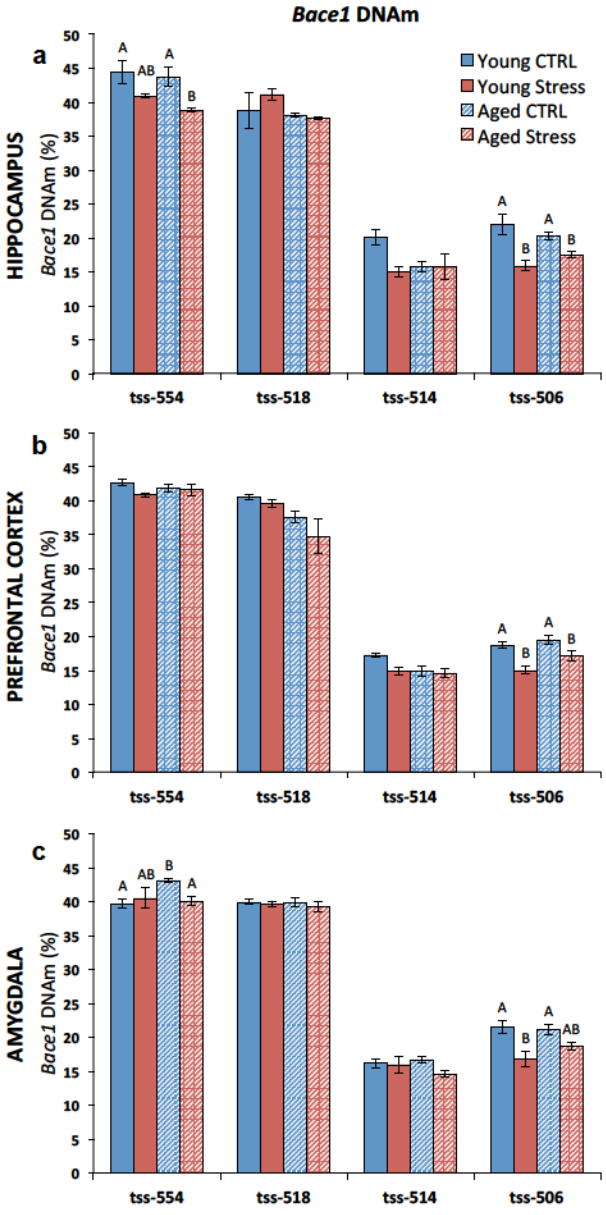

Bace1 DNAm
a
HIPPOCAMPUS
Bace1 DNAm (%)
Young CTRL
Young Stress
Aged CTRL
Aged Stress
tss-554
tss-518
tss-514
tss-506
b
PREFRONTAL CORTEX
c
AMYGDALA

Figure 5. CVS results in decreased DNA methylation of the *Bace1* promoter region. (**a**) In the hippocampus, CVS was associated with demethylation of CpGs located at tss-554 and tss-506. There was a trend towards higher methylation in the Young Control group at tss-514. (**b**) In the PFC, CVS was associated with demethylation of tss-506. Aged mice also had lower methylation at tss-518. (**c**) In the amygdala, CVS was associated with demethylation of tss-506 and Aged Control mice had higher methylation at tss-554. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

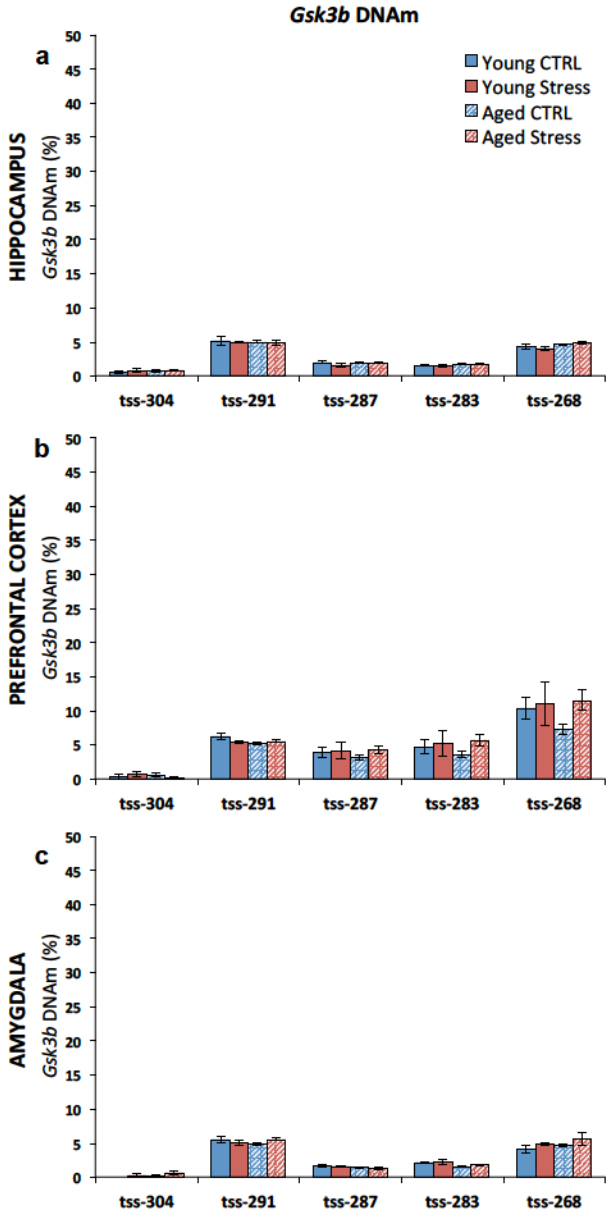


Figure 6. *Gsk3b* promoter region DNAm.

(**a**-**c**) In the hippocampus, PFC, and amygdala, there were no effects of age or stress on methylation of the *Gsk3b* promoter region CpGs that were assessed. Data represent mean ± SEM.

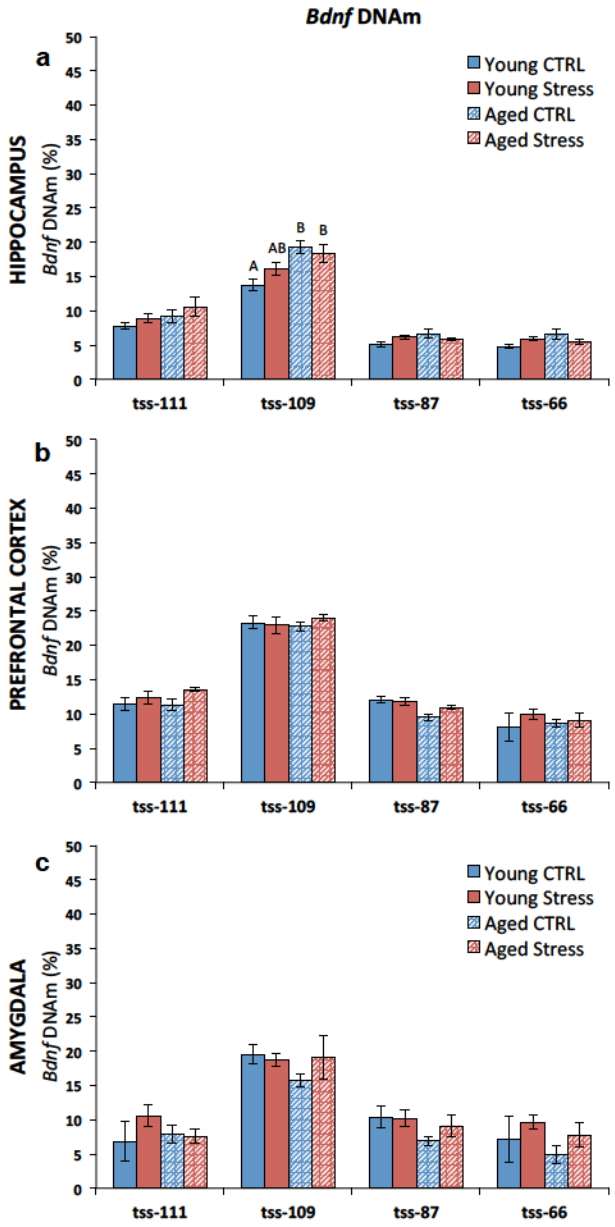


Figure 7. *Bdnf* exon 4 region DNAm.

(**a**) In the hippocampus, aged mice had higher methylation at a CpG located at tss-109. (**b**,**c**) In the PFC and amygdala, there were no effects of age or stress on methylation of the CpGs that were assessed. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

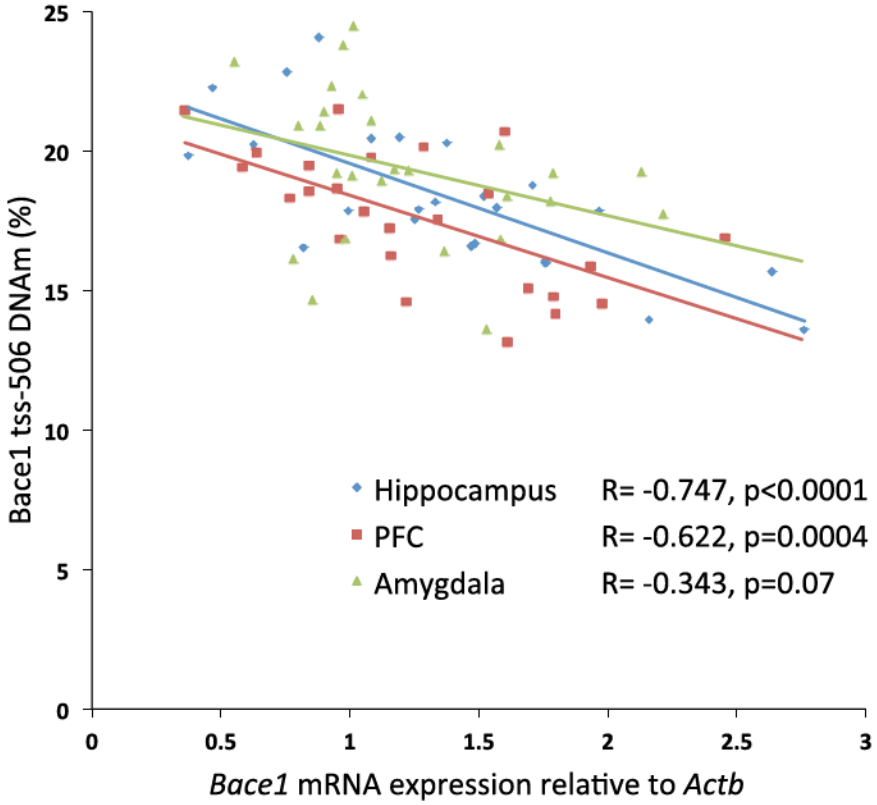


Figure 8. *Bace1* expression correlates with promoter region DNA methylation.

The expression of *Bace1* is inversely correlated with DNAm at tss-506 in the hippocampus and in the PFC. The correlation did not reach significance in the amygdala.

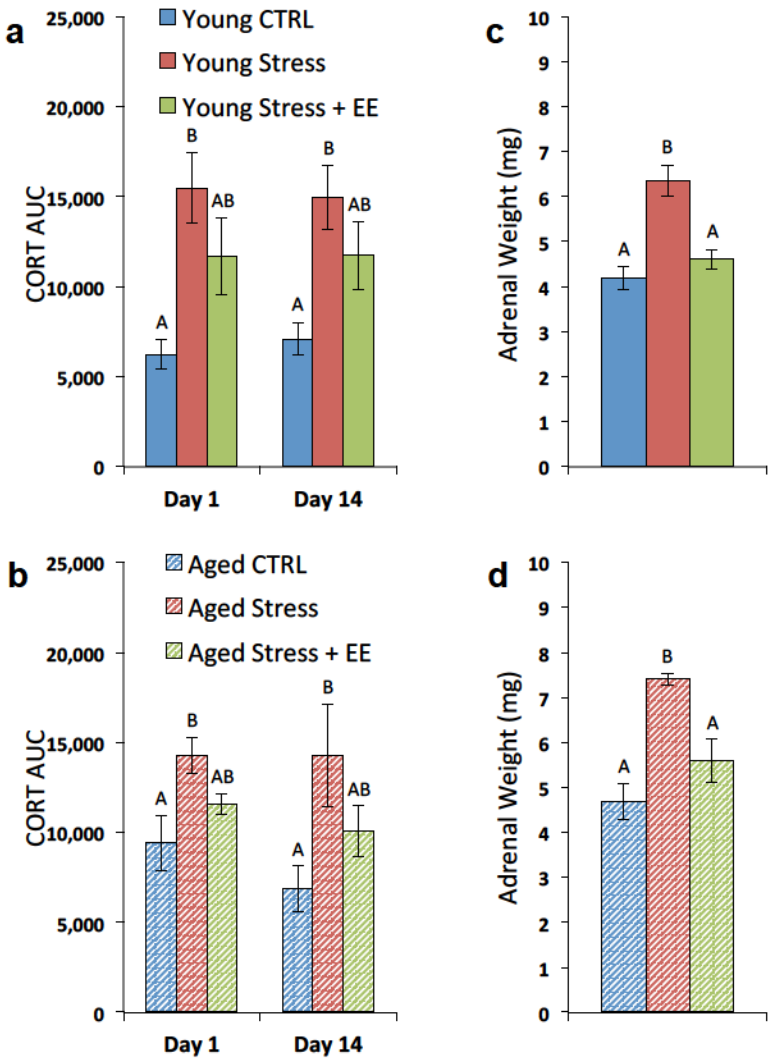


Figure 9. Environmental Enrichment blunts the stress response to CVS.

(**a**,**b**) Restraint stress tests on day 1 and day 14 resulted in elevation of plasma CORT among young adult and aged mice while EE appeared to blunt the stress response. (**c**,**d**) Chronic overproduction of stress hormones in response to CVS is reflected by adrenal hypertrophy among stressed mice. This effect was prevented by EE. Data represent mean ± SEM. For post hoc analysis in, groups that do not share letters are significantly different ($p<0.05$).

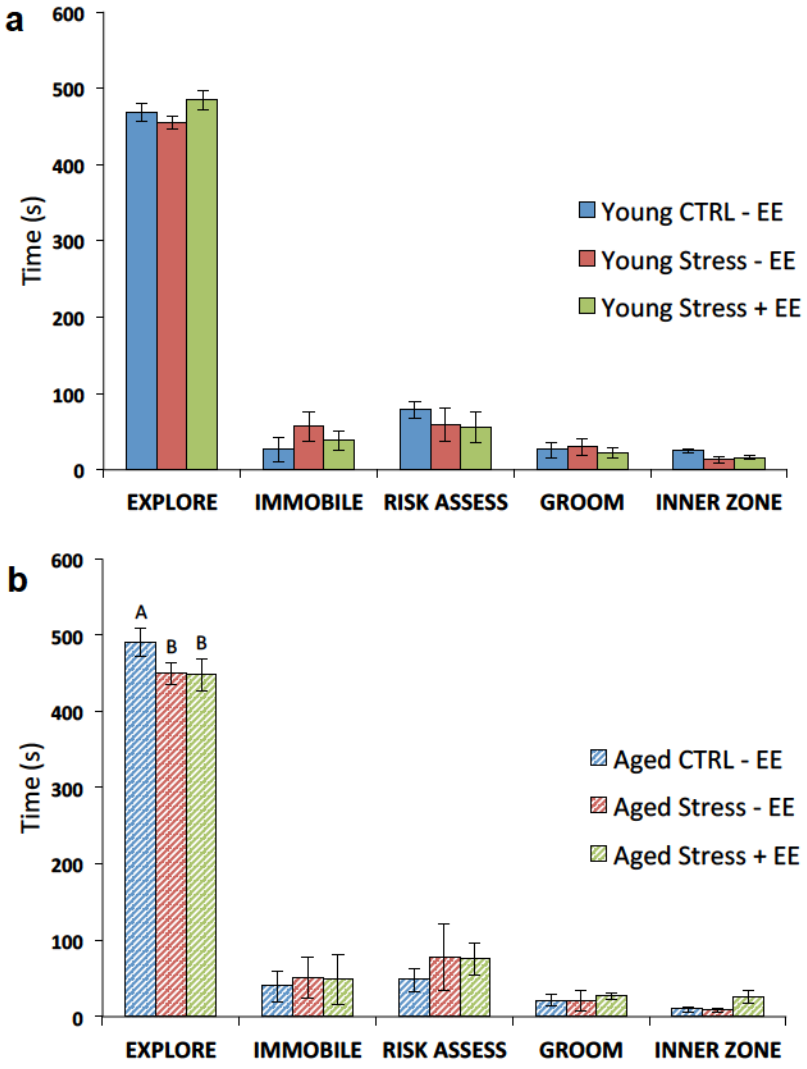


Figure 10. Open field behavior.

(**a**) There were no group differences in the behavior of young adult mice in the open field. (**b**) Aged CTRL mice spent slightly longer exploring the open field compared to both Aged Stress and Aged Stress+EE mice. There were no other group differences among aged mice in the open field. Data represent mean ± SEM. For post hoc analysis in, groups that do not share letters are significantly different ($p<0.05$).

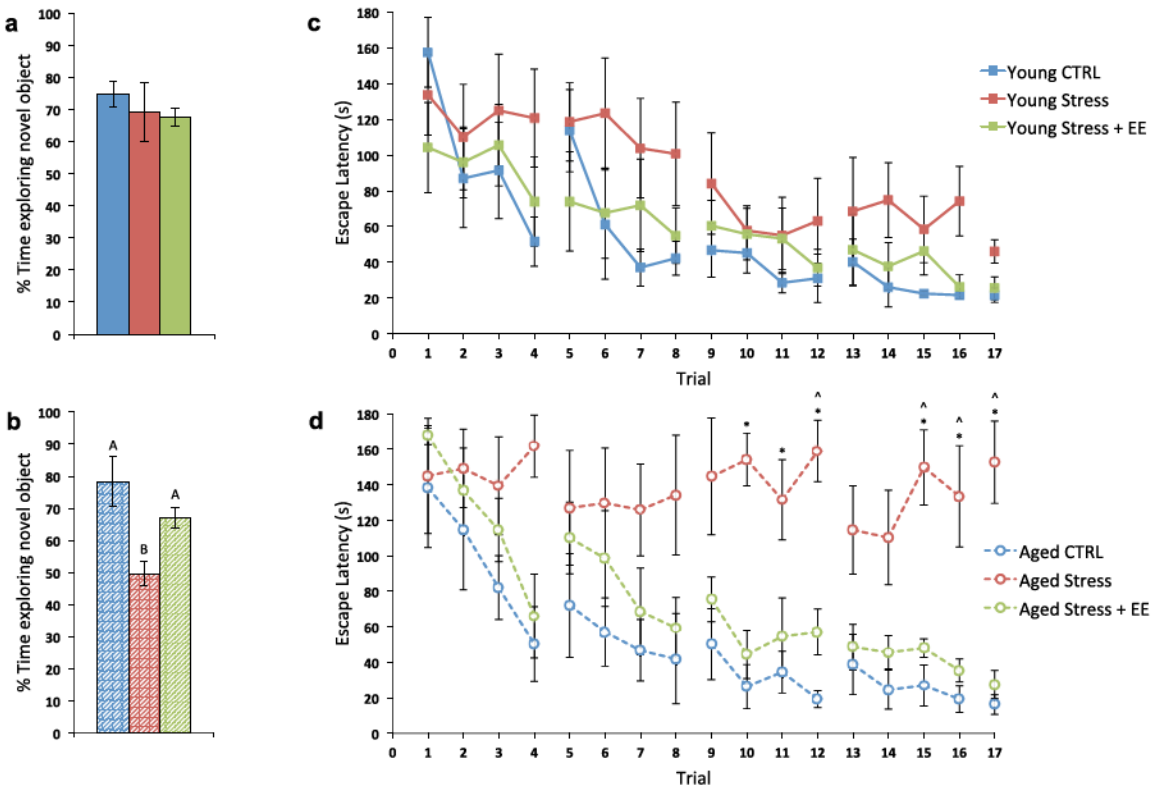


Figure 11. Environmental enrichment prevents the effects of CVS on cognitive performance among aged mice.

(**a**,**b**) CVS resulted in impaired performance in the NOR test only among aged mice. This effect was prevented by EE. (**c**,**d**) In the Barnes maze, there was a trend for increased escape latency in the Young Stress group. Aged Stress mice had significantly greater escape latency compared to both Aged CTRL and Aged Stress+EE mice. Aged mice exposed to CVS+EE were indistinguishable from controls. Data represent mean ± SEM. For post hoc analysis in **b**, groups that do not share letters are significantly different ($p<0.05$). For **d**, *$p<0.05$ Aged Stress v. Aged CTRL; ^$p<0.05$ Aged Stress v. Aged Stress+EE.

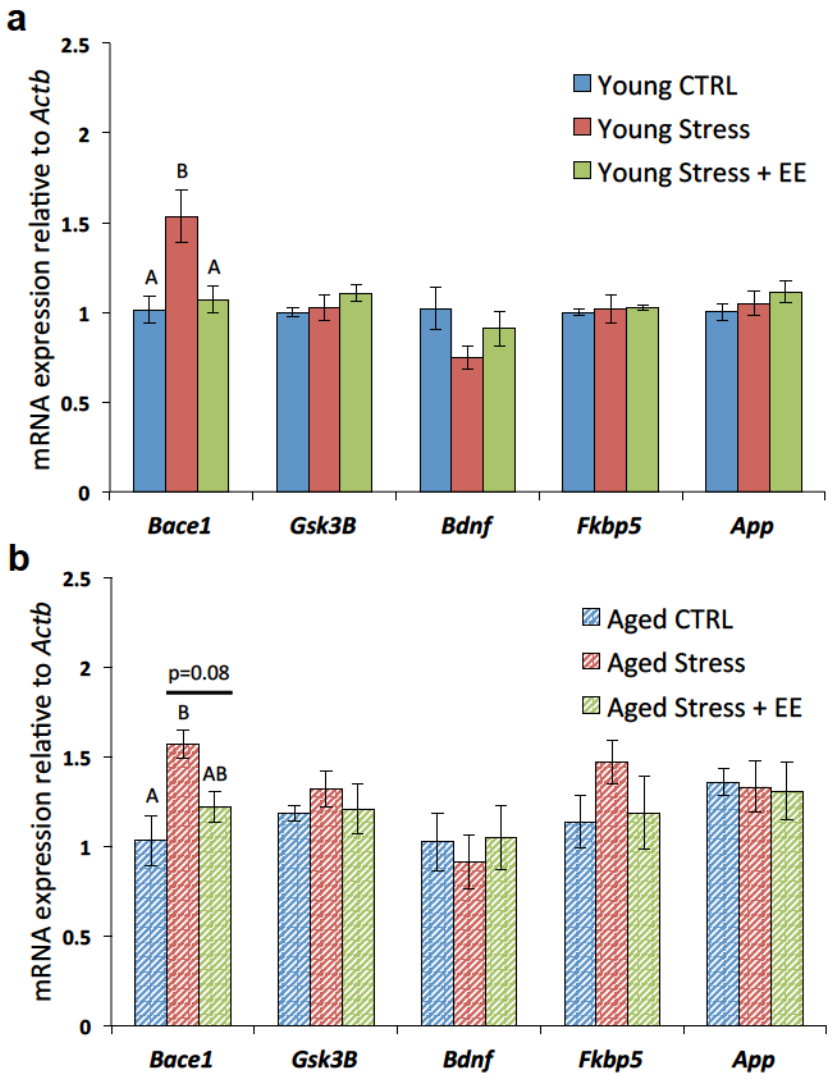


Figure 12. Environmental enrichment prevents the effects of CVS on *Bace1* expression.

(**a**,**b**) In the hippocampus, CVS was associated with increased *Bace1* expression among young adult and aged mice. This effect was prevented by EE. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

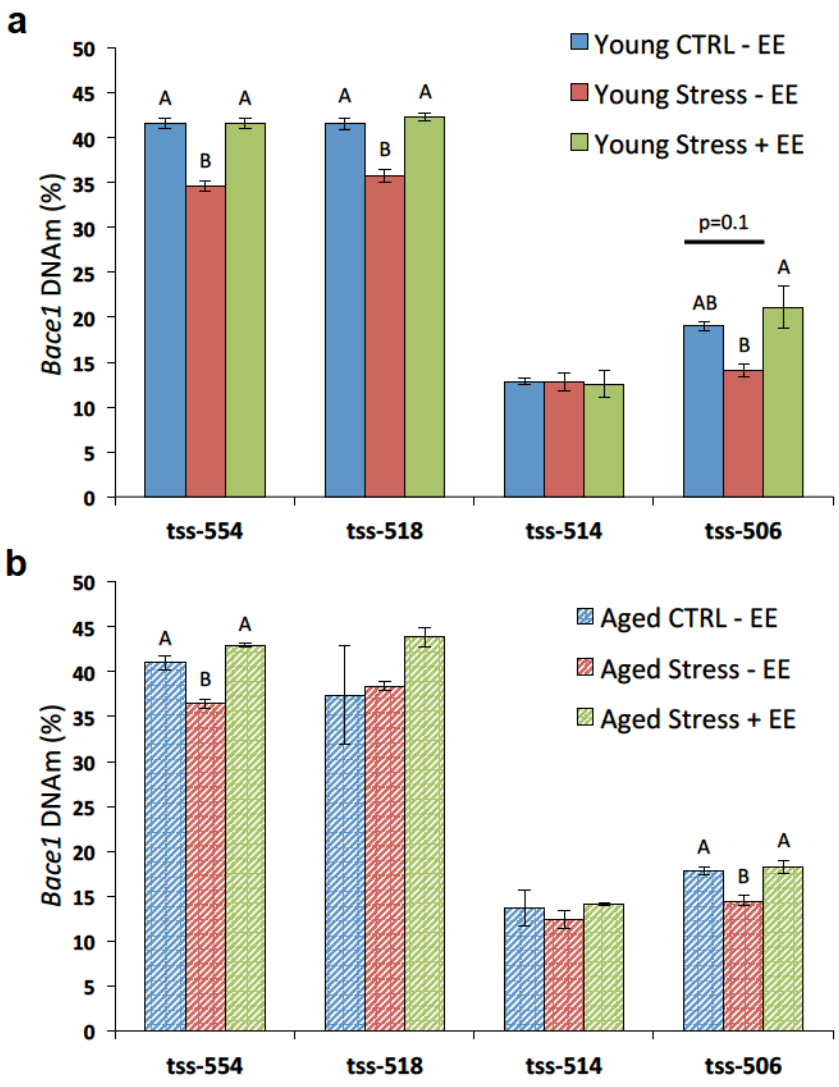


Figure 13. Environmental enrichment prevents the effects of CVS on *Bace1* promoter region DNAm.

(**a**,**b**) In the hippocampus, CVS was associated with demethylation of *Bace1* promoter region CpGs located at tss-554, tss-518, and tss-506 among young adult mice, and tss-554 and tss-506 among aged mice. All of these effects were prevented by EE. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

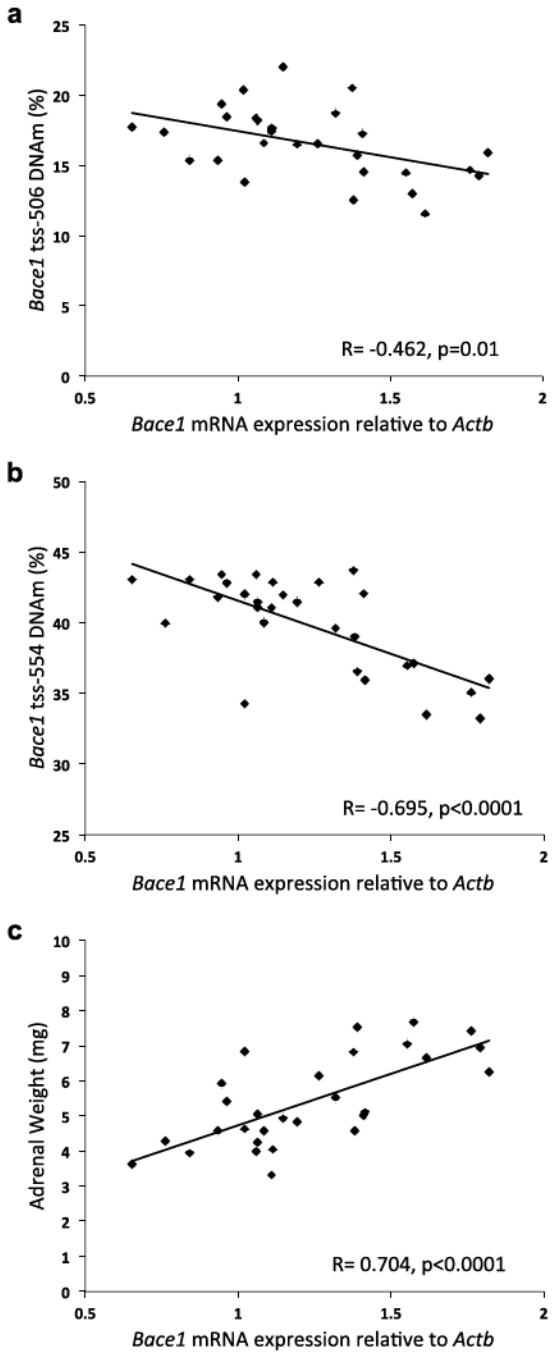

a
Bace1 tss-506 DNAm (%)
R= -0.462, p=0.01
Bace1 mRNA expression relative to Actb
b
Bace1 tss-554 DNAm (%)
R= -0.695, p<0.0001
Bace1 mRNA expression relative to Actb
c
Adrenal Weight (mg)
R= 0.704, p<0.0001
Bace1 mRNA expression relative to Actb

Figure 14. *Bace1* expression correlates with promoter region DNA methylation and adrenal weight.

(**a**,**b**) Across all groups, the hippocampal expression of *Bace1* is inversely correlated with DNAm at tss-506, and tss-554. (**c**) The hippocampal expression of *Bace1* is also positively correlated with adrenal weight.

CHAPTER 3:

EFFECTS OF CHRONIC SOCIAL STRESS ON MOOD & ANXIETY

Introduction

Across the lifespan, correlations between chronic activation or dysregulation of the HPA axis and risk for affective disorders have long been established. During the prenatal period, maternal exposure to chronic stressors, even if relatively mild, increases the offsprings' risk for depression and anxiety later in life and similar associations have been reported with either early maternal separation or low maternal care. During adolescence, stressful life events often precede first major depressive episodes and throughout adulthood stressors often precipitate recurrence. Then, in late life, interactions between stress exposure, depression, and cognitive decline emerge (Reviewed in Lupien, 2009).

To better understand the effects of stress on the brain and behavior, numerous rodent models of stress-related depression and anxiety have been established. These models, including foot shock, social isolation, and social defeat among others, have provided fundamental insights into the potential mechanisms underlying mental illness. For example, they have been used to dissect pathways connecting brain regions though to be involved in affective disorders, to demonstrate the effects of stress on neuronal morphology, neurogenesis, and electrophysiology, and to understand the roles of various neurotransmitters such as serotonin, dopamine, and glutamate in the development of depression-like phenotypes (Reviewed in Martinez, 1998; Krishnan, 2008). In closely related experiments, these models have been used to investigate the mechanisms of action of anti-depressant and anxiolytic drugs. In parallel, others have attempted to use these models as a means to identify novel treatments, though most efforts have largely focused on targeting familiar pathways such as the HPA axis, oxidative stress, dopamine, or Bdnf

(Berton, 2006). However, relatively few studies have used a rodent model of depression or anxiety and taken a broader approach to identifying novel treatments that go beyond the canonical pathways targeted by existing drugs. Additionally, despite great clinical interest in non-pharmaceutical treatment approaches, surprisingly few studies have investigated the effects of behavioral interventions on rodent models of affective disorders.

Here, we use a social stress paradigm in mice to model aspects of both depression-like and anxiety-like behaviors. In a separate cohort, we demonstrate the chronic nature of the phenotype and also investigate environmental enrichment as a potential treatment.

Materials & Methods

Animals

A total of 64 male C57BL/6 mice and 32 male CD-1 mice (Charles River, Raleigh, NC) were used in this study. At the start of each experiment, C57BL/6 mice were 8-10 weeks old and weighed 19g to 23g. CD-1 mice were 16-32 weeks old and weighed 33g to 42g. Throughout the study, all mice were on a 12h:12h light-dark cycle with *ad libitum* access to water and standard chow (Harlan 2018). Mice were randomly assigned to experimental groups. All protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Social Defeat Stress

Social defeat stress was conducted according to a previously published protocol (Golden, 2011). In this paradigm, test subjects (referred to as 'intruders') are pair-housed in the home cages of aggressive, dominant males (referred to as 'residents') for 14 days. In our study, 8-10 week old C57BL/6 mice were used as intruders and 16-32 week old CD-1 mice were used as residents. Throughout the 14 day social defeat period, residents and intruders were divided by a barrier except for 10 minutes each day when the mice were allowed to interact. Within the first 10 minute period of interaction, resident mice quickly establish dominance over and display aggression towards intruders. After each interaction, the mice were separated by a clear, ventilated barrier for the remainder of the 24 hour period thereby allowing persistent psychosocial distress (Martinez, 1998). On subsequent days, immediately prior to the 10 minute interaction period, each intruder was rotated to the home cage of a different resident in order to prevent habituation.

Unstressed C57BL/6 controls were pair-housed in divided cages throughout the experiment.

In the first cohort, C57BL/6 mice were maintained under control conditions (CTRL, n=16) or exposed to social defeat (SD, n=16). Behavioral testing began 1 day after the last social defeat interaction during which all C57BL/6 mice were pair-housed in a divided cage with another C57BL/6 from the same group.

Environmental Enrichment (EE)

In a separate cohort, C57BL/6 mice were exposed to control conditions (CTRL) or social defeat (SD) for 14 days. A recovery period began 24 hours after the last social defeat interaction. During the recovery period, half of the C57BL/6 mice in each group were pair-housed in divided cages while the other half were singly housed and given access to environmental enrichment (EE) for the remainder of the experiment. Behavioral testing began after 21 days of recovery. Housing conditions were maintained throughout behavioral testing. EE included a large tub cage filled with extra bedding, nesting sheets, and polycarbonate tunnels, balls, and housing domes (Bio-Serv, Frenchtown, NJ). This resulted in four groups: unstressed control without EE (CTRL - EE, n=8), unstressed control with EE (CTRL+EE, n=8), social defeat without EE (SD - EE, n=8), and social defeat with EE (SD+EE, n=8).

Body Weight

The body weights of each C57BL/6 mouse was recorded daily during the social defeat period. In cohort 2, body weight was also measured 3 times per week during the recovery period.

Fur Quality

The fur quality of each C57BL/6 mouse was recorded daily during the social defeat period. In cohort 2, fur quality was also measured 3 times per week during the recovery period. Fur quality scores were based on a subjective 1 to 4 scale where a score of 4 indicates a normal, smooth, well-groomed coat. Scoring criteria are listed in Table 4.

Open Field

The open field consists of an opaque plastic box (60cm square chamber, 60cm high walls) with a clearly marked central zone (circle with a 35cm diameter). Each mouse was allowed to freely explore the open field for 10 minutes. Behavior was recorded by a digital camera and later coded by a blinded observer. Horizontal activity was recorded by a computerized detection system (AccuScan, Columbus, OH).

Elevated Plus Maze

The elevated plus maze consists of a plastic platform with four arms (10cm x 50 cm) joined by a square intersection (10cm x 10 cm) to form a '+' shape that is elevated 50cm above the ground. Two opposing arms have walls that are 30cm high. The remaining two arms are open. Each mouse was placed at the center of the maze and allowed to freely

explore for 5 minutes. Behavior was recorded by a digital camera and later coded by a blinded observer.

Sucrose Preference Test

Mice were habituated to a 1% w/v sucrose solution for 3 hours on two consecutive days before the start of the test. During the preference test, mice were given *ad libitum* access to two bottles: one containing water, and one containing a 1% w/v sucrose solution. The test began immediately prior to onset of the dark period. Intake was measured after 12 and 24 hours of access and a preference ratio was calculated.

Forced Swim Test

Each mouse was placed in a cylinder filled with water (22cm diameter, 17cm water height, 22°C water temperature) for 6 minutes. The first 2 minutes were treated as a habituation period and only the last 4 minutes were scored. Behavior was recorded by a digital camera and later coded by a blinded observer.

Tissue Collection

Three days after behavioral testing, mice were deeply anesthetized with isoflurane, retro-orbital blood was collected and all mice were killed by rapid decapitation. The adrenal glands, spleen, and thymus of each mouse were weighed. Brains were removed, immediately frozen on powdered dry ice and stored at -80°C for future experiments. Carcasses were stored at -20°C and body composition analysis was later performed.

Plasma Corticosterone (CORT)

Plasma was isolated from retro-orbital blood and CORT concentration was determined by radioimmunoassay (MP Biomedicals, Solon, OH).

Body Composition

Prior to body composition analysis mouse carcasses were brought to room temperature and weight. Lean mass, adipose mass and total water mass were determined using EchoMRI-100TM analysis (EchoMRI, Houston, TX). The percentage body fat was calculated from the adipose weight and total carcass weight.

Statistical Analysis

Statistical analysis was completed using Statistica 7 (StatSoft, Inc., Tulsa, OK). Data are expressed as averages +/- standard error of the mean. In the first cohort, differences between groups were assessed by Student's t-test or repeated measures ANOVA. In the second cohort, differences between groups were assessed by factorial or repeated measures ANOVA with 'Stress' and 'EE' as between subject factors followed by Tukey post hoc analysis. Correlations were assessed by Pearson's correlation. For all statistical tests, $p<0.05$ was considered significant.

Results

Social defeat results in impaired body weight gain.

Because weight loss is a frequent symptom of depression that correlates with other classical depressive symptoms (Hamilton, 1960), we monitored the body weight of all C57BL/6 mice throughout the experiment. Over the 14 day social defeat period, stressed mice exhibited impaired weight gain relative to controls (Time*Stress, $F=3.97$, $p<0.0001$). Post hoc analysis of individual days revealed that socially defeated mice weighed significantly less than controls on days 3 and 5 through 14 ($p<0.05$) (Figure 15).

Social defeat results in impaired grooming behavior.

Impaired grooming has been widely reported in rodent models of depression and anxiety (Gould, 2009). Here, we found that the fur quality of stressed mice declined over time while control mice maintained normal grooming behavior (Time*Stress, $F=5.04$, $p<0.0001$). Post hoc analysis of individual days revealed that socially defeated mice had lower fur quality relative to controls on days 3-14 ($p<0.05$) (Figure 16).

Social defeat results a mixed depression and anxiety-like phenotype.

Behavioral phenotyping began 1 day after the last social defeat interaction. Tests included the open field, elevated plus maze, and sucrose preference. In the open field, socially defeated mice spent more time immobile ($p=0.04$) (Figure 17a). and less time in the inner zone ($p=0.03$) (Figure 17b).

In the elevated plus maze, socially defeated mice spent more time in the closed arm ($p=0.01$) and less time on the open, center platform ($p=0.01$) (Figure 17c).

In the 24 hour sucrose preference test, socially defeated mice displayed a decreased preference for the sucrose solution ($p=0.03$) (Figure 18a). There were no differences in total intake volume (Figure 18b).

Taken together, these behavioral data suggest that 14 days of social defeat induces a mixed depression-like and anxiety-like phenotype characterized by impaired grooming, immobility, decreased exploratory behavior, and anhedonic-like response to rewarding stimuli.

Social defeat results in increased baseline corticosterone.

At the conclusion of behavioral testing, a blood sample was collected to determine the effects of SD on baseline plasma CORT. We found a more than 2-fold increase in baseline CORT among socially defeated mice ($p=0.01$) (Figure 19a). Adrenal hypertrophy at the end of the experiment further reflected the chronic overproduction of CORT among socially defeated mice ($p=0.006$) (Figure 19b).

Recovery from social defeat results in rapid body weight gain that is moderated by environmental enrichment.

We again monitored body weight in the second cohort and found that, during the social defeat period, all mice tended to gain weight over time, but socially defeated mice gained

less weight compared to unstressed mice (Time*Stress: $F=4.07$, $p=0.001$). During the recovery period, all groups gained weight but mice exposed to EE gained less weight over time (Time*EE, $F=10.19$, $p=0.003$). Surprisingly, socially defeated mice without access to EE gained more weight over time than all other groups (Time*SD*EE: $F=2.25$, $p=0.02$). Post hoc analysis of individual days revealed that unstressed mice weighed significantly more than socially defeated mice from day 2 through day 14 ($p<0.05$). During the recovery period, the CTRL-EE and SD-EE group weighed significantly more than the CTRL+EE and SD+EE groups by day 23 (recovery day 9) and this difference remained significant until day 30 (recovery day 16) ($p<0.05$) (Figure 20a)

Despite the rapid weight gain among SD-EE mice and slow weight gain among mice given access to EE, we found no differences in body composition when measured at the conclusion of the experiment (Figure 20b). However, it should be noted that the body composition measurements were taken after behavioral testing and post-mortem tissue collection at which point there were no group differences in body weight.

Social defeat results in long-term impairment in grooming that is reversed by environmental enrichment.

Fur quality was assessed throughout the social defeat and recovery periods. During social defeat, stress exposure was again associated with a decrease in fur quality over time (Time*Stress: $F=3.39$, $p<0.0001$). Interestingly, during the recovery period, the SD-EE group continued to display impaired grooming while the fur quality of SD+EE group rapidly improved to that of unstressed mice (Stress*EE: $F=15.22$, $p<0.0001$; Time*EE:

F=2.16, p=0.03). Post hoc analysis of individual days revealed that socially defeated mice had significantly impaired fur quality beginning on day 2 ($p<0.05$). The CTRL-EE mice continued to display impaired grooming relative to controls on each day of the recovery period ($p<0.05$) while the SD+EE mice were indistinguishable from controls after day 23 (recovery day 9) (Figure 21). Together, these data suggest that exposure to social defeat has a long-lasting effect on grooming, which can be rapidly reversed by EE.

Social defeat results in long-term depression and anxiety-like behaviors that are reversed by environmental enrichment.

Behavioral phenotyping began after 21 days of recovery. Tests included the open field, elevated plus maze, sucrose preference, and forced swim. In the open field, we found no effect of stress. There was, however, an overall effect of EE such that mice with access to enrichment had greater total activity (EE: F=14.37, $p<0.0001$) and spent more time in the inner zone (EE: F=7.86, p=0.009) suggesting that EE exposure alone may increase exploratory behavior independent of stress past exposure (Figure 22a,b). There were no effects of stress or EE on time spent immobile in the open field (Figure 22c).

In the elevated plus maze, there was no significant effect of stress or enrichment on time spent in the closed arm, center, or open arm of the plus maze (Figure 23a). However, there was an overall effect of stress and an interaction between stress and enrichment on the number of entries into the open arm such that the SD-EE group made fewer entries into the open arm, while the SD+EE group was indistinguishable from controls (Stress: F=4.98, p=0.03; Stress*EE: F=6.03, p=0.021) (Figure 23b).

In the sucrose preference test, like in the elevated plus maze, we found an overall effect of stress, and an interaction between stress and enrichment such that the SD-EE group displayed decreased preference for a sucrose solution, while the SD+EE group was indistinguishable from controls (Stress: $F=15.70$, $p<0.0001$; Stress*EE: $F=11.26$, $p=0.002$) (Figure 24).

Likewise, in the forced swim test, we found an overall effect of stress and an interaction between stress and enrichment on both time spent immobile (Stress: $F=5.58$, $p=0.02$; Stress*EE: $F=16.12$, $p<0.0001$) and latency to immobility (Stress: $F=11.08$, $p=0.002$; Stress*EE: $F=5.26$, $p=0.03$) such that the SD-EE group displayed increased immobile behavior while the SD+EE group was indistinguishable from controls (Figure 25a,b).

Taken together, these behavioral data suggest that the depression-like and anxiety-like phenotype induced by social defeat stress persists for at least 3 weeks after last stress exposure. However, the phenotype is completely reversed by 3 weeks of access to environmental enrichment.

Social defeat results in a long-term increase in the production of corticosterone that is reversed by environmental enrichment.

After the recovery period and behavioral testing, a blood sample was collected to determine the long-term effects of SD and EE on baseline plasma CORT. We found overall effects of stress and enrichment (Stress: $F=107.18$, $p<0.0001$; EE: $F=6.19$,

p=0.001) such that baseline CORT was highest in the SD-EE group while CORT levels among SD+EE mice were indistinguishable from unstressed mice (Figure 26a).

Adrenal weight at the end of the experiment further reflected the chronic overproduction of CORT among socially defeated mice and the ability of enrichment to reverse this effect. We found an overall effect of stress and an interaction between stress and enrichment such that the adrenal glands were significantly hypertrophied among the SD-EE group while SD+EE mice were indistinguishable from controls (Stress: $F=35.89$, $p<0.0001$; Stress*EE: $F=9.67$, $p=0.004$) (Figure 26b). Among all mice, adrenal weight positively correlated with baseline plasma CORT ($r=0.667$, $p<0.0001$) (Figure 26c).

Finally, we found that adrenal weight negatively correlated with sucrose preference ($r=-0.692$, $p<0.0001$) as well as entries into the open arm of the elevated plus maze ($r=-0.379$, $p=0.03$) and positively correlated with immobility in the forced swim test ($r=0.512$ $p=0.003$) further suggesting a role for glucocorticoids in the mechanisms underlying the effects of SD on behavior (Figure 27a-c).

Together, these data extend the findings from the first cohort and suggest that the effects of social defeat stress on the HPA axis and behavior are long lasting though entirely reversible with access to environmental enrichment.

Discussion

Many rodent models of affective disorders have already been established and include widely used paradigms such as maternal separation, chronic restrain, and social defeat or subordination. These models have provided invaluable insight into the neurobiology of mental illness as well as the mechanisms linking chronic stress with depression and anxiety. Additionally, these models have been used to study the cellular and molecular mechanisms of action of virtually all commonly used anti-depressant and anxiolytic drugs. However, relatively few studies have taken a broader approach to identifying novel treatments that go beyond the canonical pathways targeted by existing drugs. Additionally, despite great clinical interest in non-pharmaceutical treatment approaches, surprisingly few studies have investigated the effects of behavioral interventions on rodent models of affective disorders.

Here, we first established the phenotype of wild type mice exposed to chronic social defeat stress. During social defeat, we found that mice failed to gain weight while fur quality, a marker of grooming/self-care, declined. Then, after 14 days of social defeat, stressed mice exhibited anxiety-like and anhedonia-like behaviors as well as elevated baseline CORT and adrenal hypertrophy. These data largely reflect the findings of other studies, which have demonstrated that chronic social stressors produce a robust phenotype characterized by features of depression, anxiety, and HPA axis dysfunction (Martinez, 1998; Krishnan, 2007).

In a separate cohort, we next demonstrated that the behavioral and endocrine phenotype resulting from social defeat stress is chronic in nature. Specifically, we found that the impaired grooming, anxiety-like behavior, anhedonia-like behavior, and learned helplessness-like behavior induced by social defeat persist for at least 3-4 weeks after the last stress exposure. Similarly, baseline CORT levels remained elevated and adrenal glands remained hypertrophied among socially defeated mice 4 weeks after the last stress exposure. Others have shown that components of the phenotype induced by social defeat is maintained after 3 weeks of social isolation housing (Schloesser, 2010), which itself can be used to induce depression-like and anxiety-like behaviors (Niwa, 2011). However, persistence of the stress-induced phenotype after several weeks of 'recovery' under standard, control conditions has not been previously reported and the data presented here may provide further support for social defeat as a model of chronic, unremitting mental illness.

In the second cohort, we also show that treatment with environmental enrichment reverses the effects of social defeat on grooming, anxiety-like behavior, and depression-like behavior. Others have found that exposure to enrichment prior to social defeat confers resilience to the effects of stress (Lehmann, 2011). In another report, socially defeated mice housed in an enriched environment displayed less anxiety-like and depression-like behavior than mice housed in an impoverished environment (Schloesser, 2010), however, no unstressed controls were included in this study. Interestingly, we also demonstrate that 4 weeks of environmental enrichment normalizes baseline CORT levels and adrenal weight. Enrichment has been previously shown to decrease baseline stress

hormone levels among unstressed rats (Belz, 2003), and reverse HPA-axis hyperactivity induced by early life stress (Francis, 2002; Morley-Fletcher, 2003), however the effects of enrichment or other treatments on HPA-axis function in the social defeat model has not been well studied. Finally, though the precise mechanisms linking social defeat, environmental enrichment, and behavioral phenotypes remains an area of ongoing investigation, we also found that CORT levels and adrenal weight across all groups correlated with behavior in tests of both anxiety and depression suggesting a role for glucocorticoids.

Together, the current findings confirm previously reported effects of chronic social stress on mood, anxiety, and HPA-axis function and further suggest that the social defeat paradigm may be useful as a model of chronic, unremitting mental illness. Finally, we show that the effects of social defeat can be reversed by environmental enrichment, thus providing support for non-pharmaceutical, behavioral interventions as treatments for affective disorders. Moving forward, understanding the mechanisms by which environmental enrichment prevented the observed effects of stress, and comparing these mechanisms to those of existing drugs, will be important areas of future study that may ultimately provide insights into novel therapeutic targets for the treatment of major depression and other stress-related psychiatric disorders.

Tables & Figures

Table 4. Fur quality scoring criteria.

Fur Quality Score	Criteria
4	Normal, smooth, well-groomed coat.
3	Wet, matted, un-groomed fur around the hind limbs.
2	Wet, matted, un-groomed fur over the entire body, excluding the head.
1	Wet, matted, un-groomed fur over the entire body and head and/or open bite wounds rostral to the hind limbs.

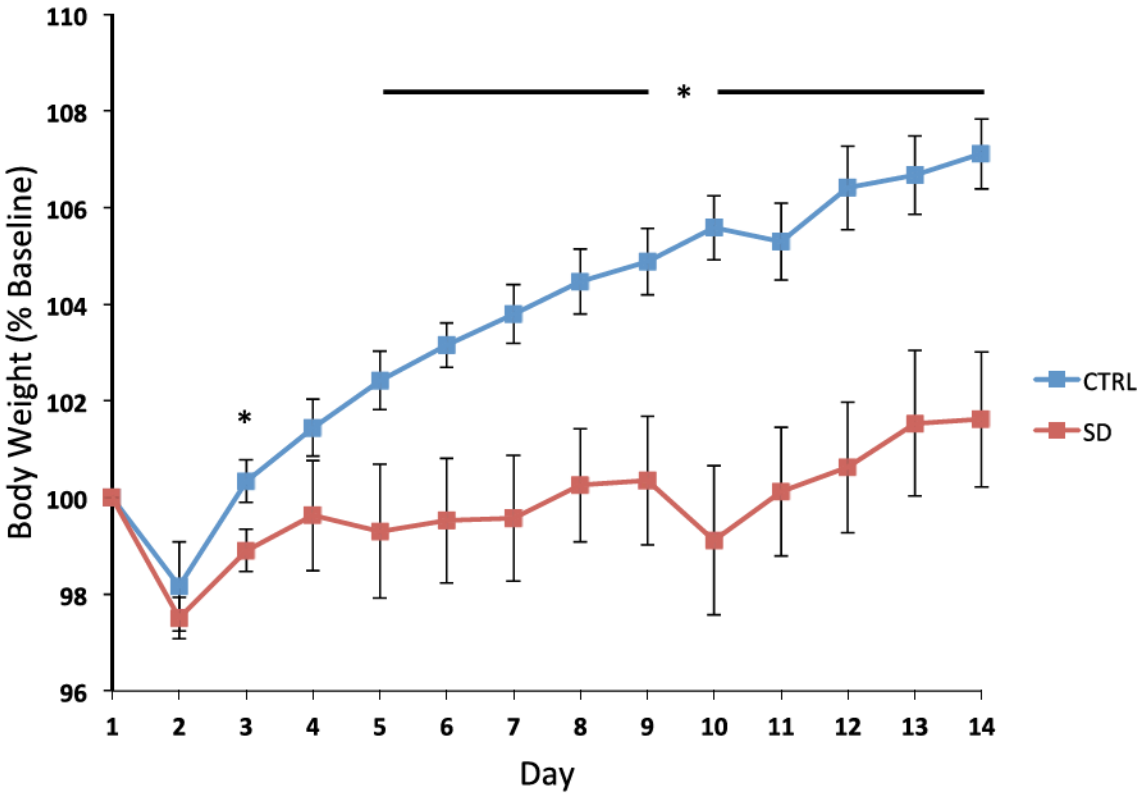


Figure 15. Social defeat stress impairs body weight gain.

Mice exposed to SD gained weight more slowly than unstressed controls throughout the experiment and weighed less than unstressed mice by day 3. Data represent mean ± SEM. For post hoc analysis on individual days, *$p<0.05$.

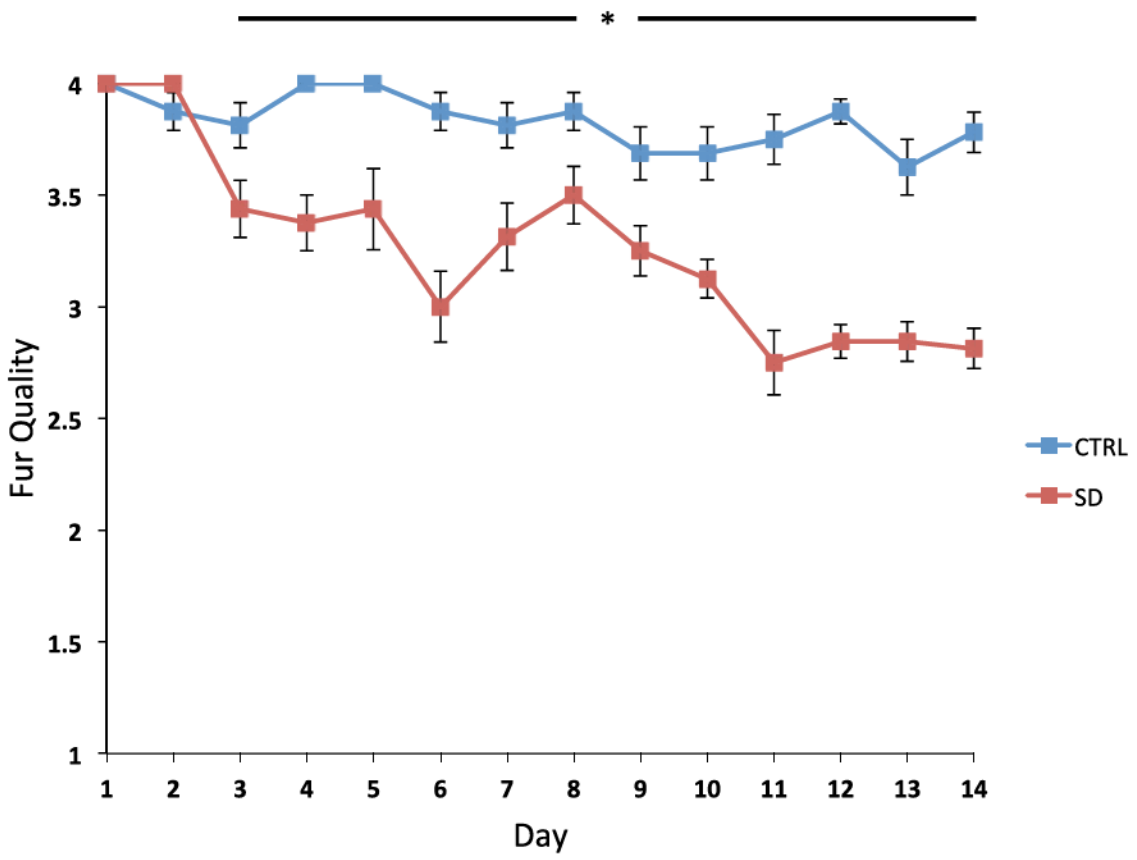


Figure 16. Social defeat stress impairs grooming.

Fur quality of socially defeated mice decreased over time and was significantly impaired relative to unstressed mice by day 3. Data represent mean ± SEM. For post hoc analysis on individual days, *$p<0.05$.

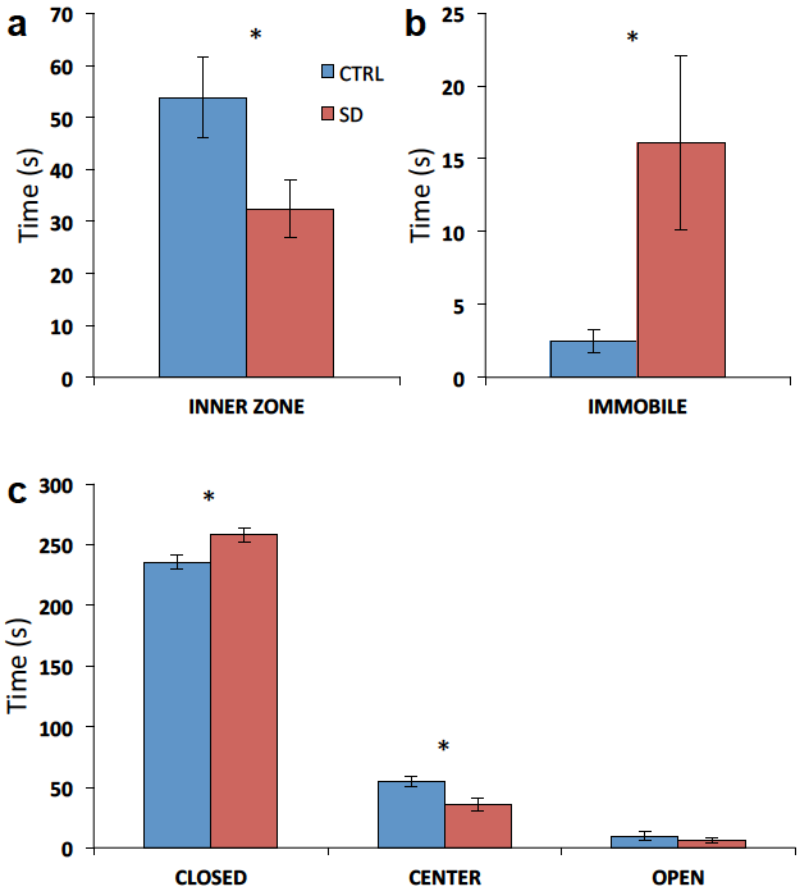


Figure 17. Social defeat stress results in anxiety-like behavior.

(**a**,**b**) In the open field, socially defeated mice spent less time in the inner zone and more time immobile than unstressed mice. (**c**) In the elevated plus maze, socially defeated mice spent more time in the closed arm and less time on the center platform. Data represent mean ± SEM, *p<0.05.

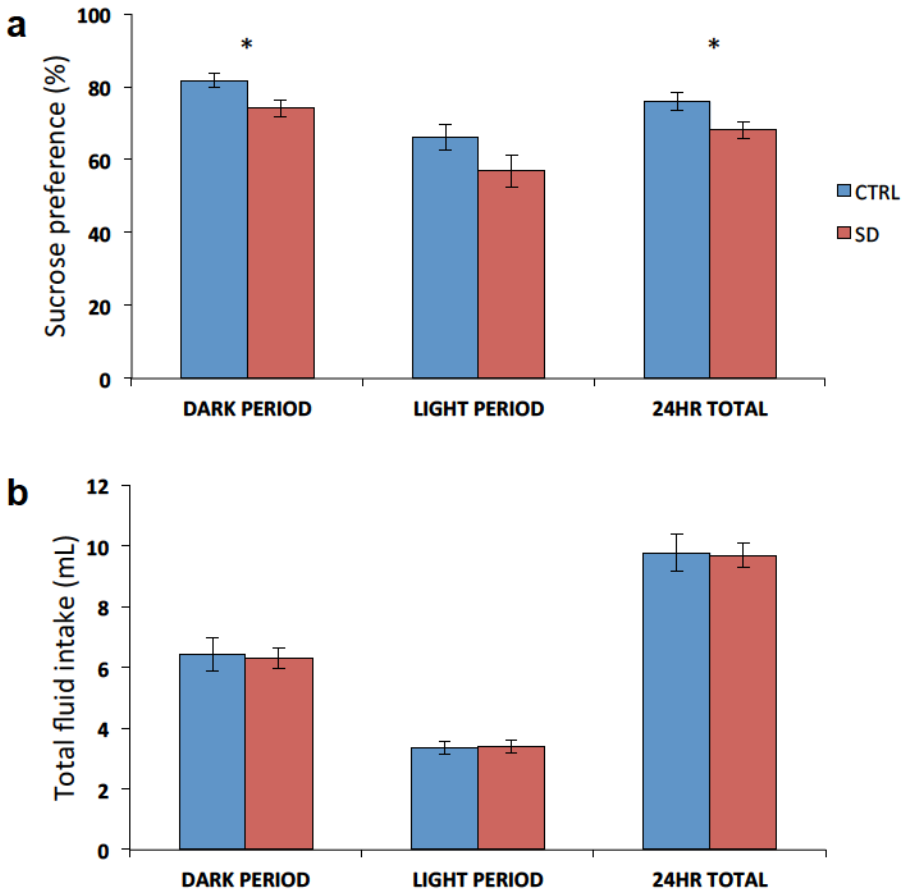


Figure 18. Social defeat stress results in anhedonic-like behavior.

(**a**) Socially defeated mice displayed a decreased preference for a sucrose solution relative to unstressed controls. (**b**) There were no effects of SD on total fluid intake. Data represent mean ± SEM, *p<0.05.

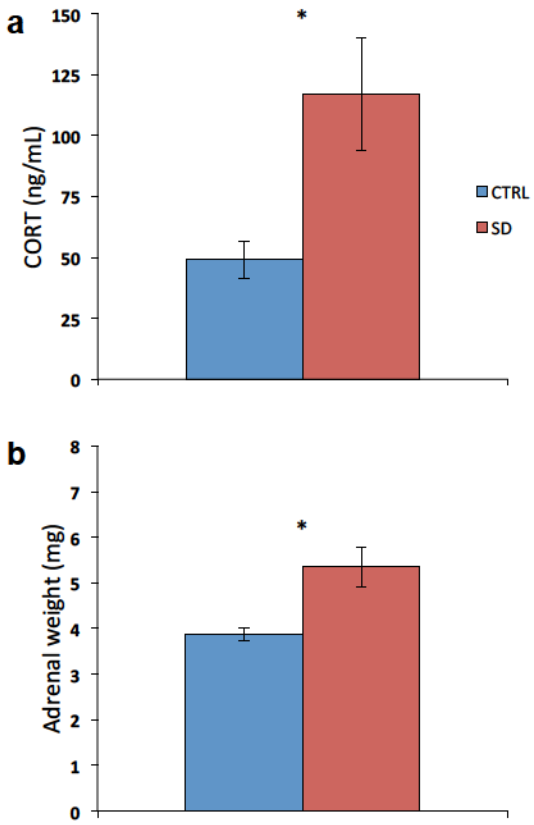


Figure 19. Social defeat stress results in increased baseline CORT and adrenal hypertrophy.

(**a**) Baseline CORT levels were elevated after 14 days of exposure to social defeat. (**b**) Chronic overproduction of stress hormones in response to SD is reflected by adrenal hypertrophy among stressed mice at the conclusion of the experiment. Data represent mean ± SEM, *$p<0.05$.

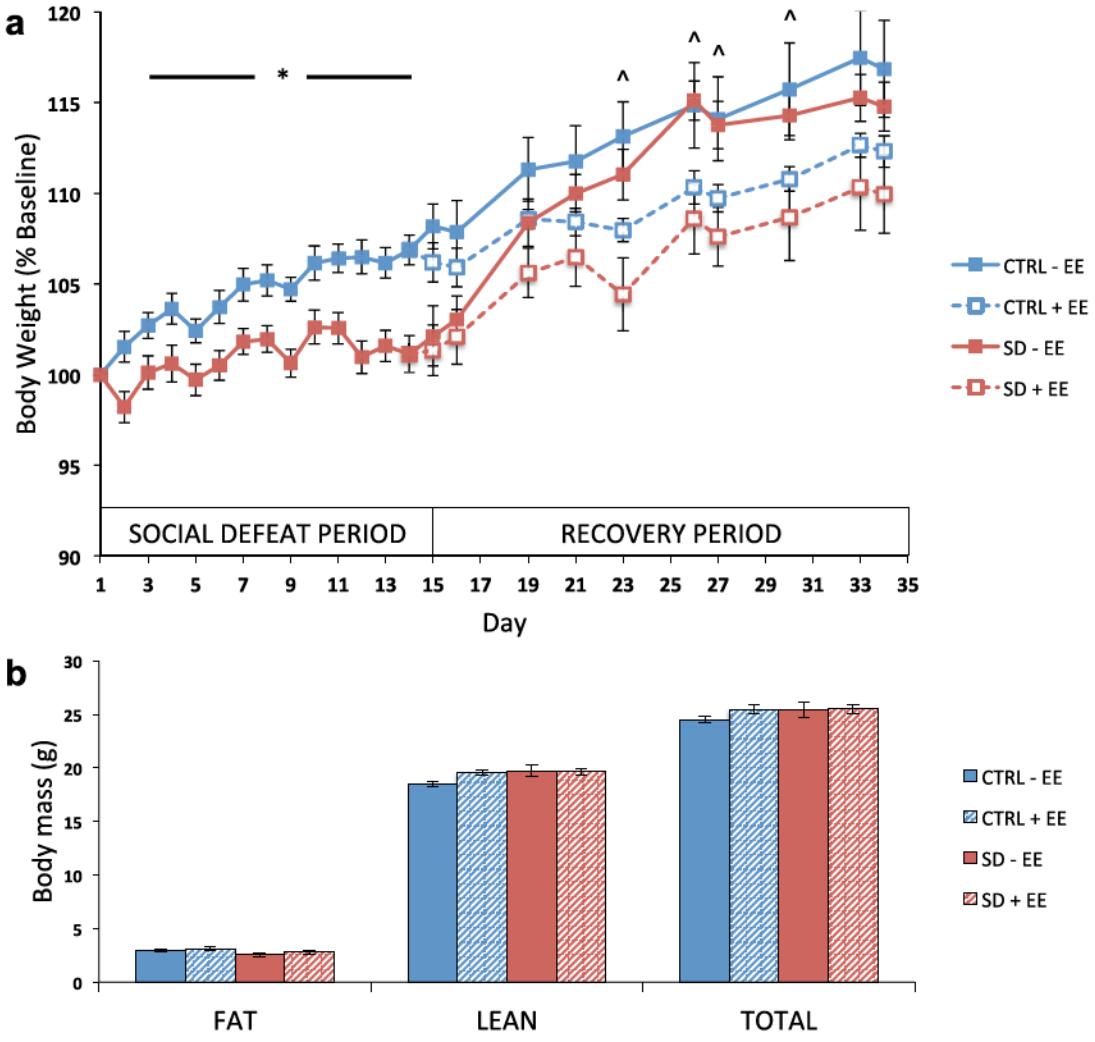


Figure 20. Social defeat stress impairs body weight gain. Recovery from social defeat results in rapid weight gain that is moderated by environmental enrichment. (**a**) Mice exposed to SD gained weight more slowly than unstressed controls throughout the 14 day social defeat period. During the recovery period, mice that had been exposed to social defeat gained weight more rapidly than unstressed controls. This effect was moderated by exposure to environmental enrichment, though there were no significant differences between the groups after 3 weeks of recovery. (**b**) At the end of the experiment, there were no differences in body composition. Data represent mean ± SEM. For post hoc analysis on individual days, *p<0.05 SD v. CTRL; ^p<0.05 +EE v. -EE.

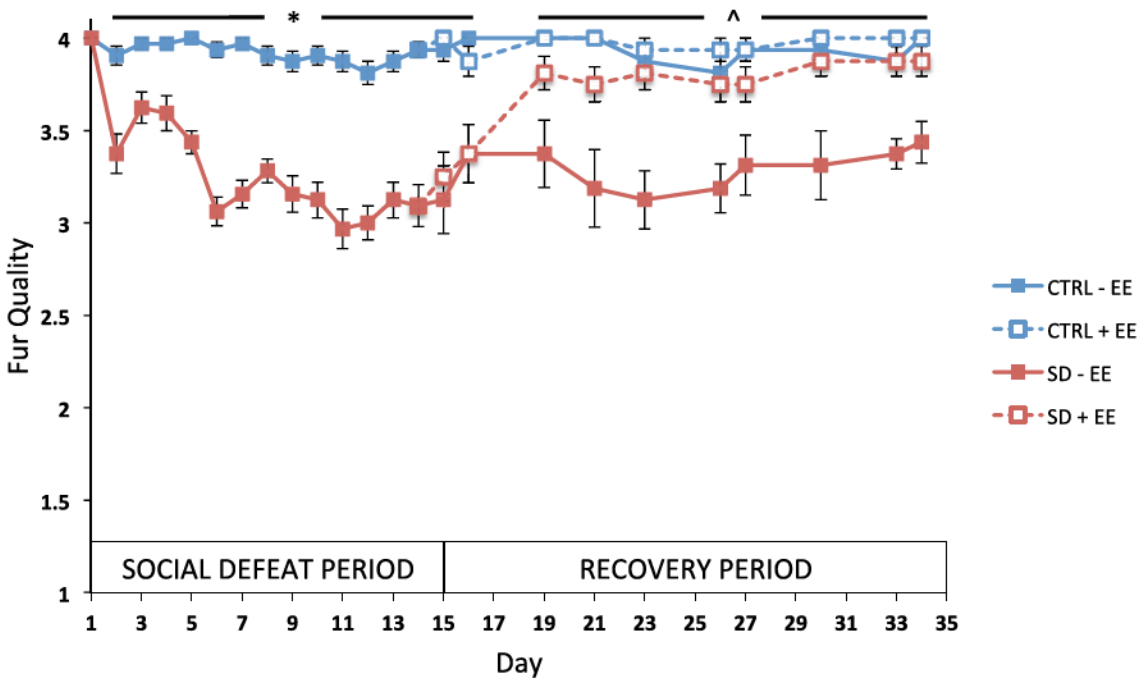


Figure 21. Social defeat results in long-term impairment in grooming that is reversed by environmental enrichment.

Mice exposed to SD had decreased fur quality throughout the 14 day social defeat period. During the 3 week recovery period, mice that had been exposed to social defeat continued to have impaired fur quality. This effect was reversed by exposure to environmental enrichment. Data represent mean ± SEM. For post hoc analysis on individual days, *$p<0.05$ SD v. CTRL; ^$p<0.05$ SD-EE v. all other groups.

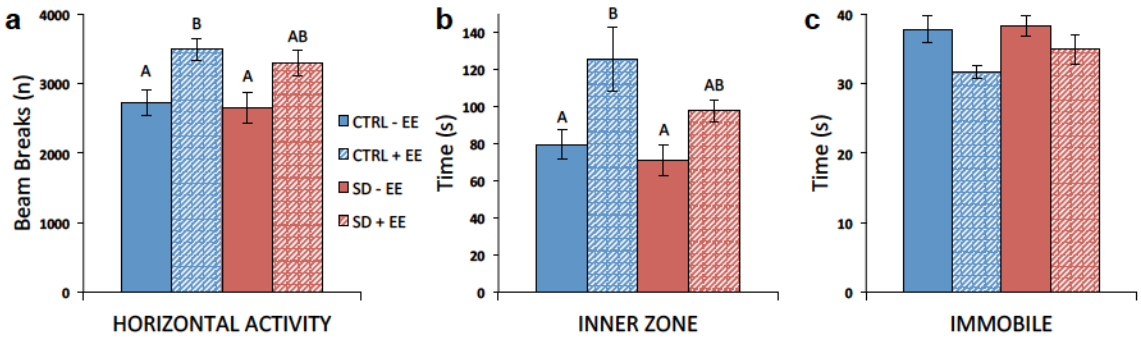


Figure 22. Environmental enrichment may increase exploratory behavior independent of past stress past exposure.

(**a**,**b**) In the open field, mice with access to EE displayed greater total activity and spent more time in the inner zone. (**c**) There were no effects of stress or enrichment on time spent immobile in the open field. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

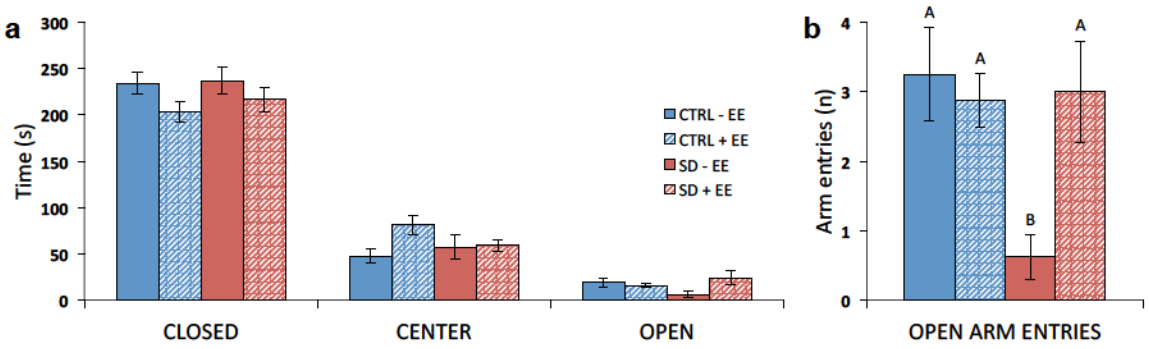


Figure 23. Social defeat results in long-term anxiety-like behavior that is reversed by environmental enrichment.

(**a**) There were no significant effects of stress or EE on time spent in the closed, center, or open zones of the elevated plus maze. (**b**) However, SD-EE mice made fewer entries into the open arm while SD+EE mice were indistinguishable from unstressed controls. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

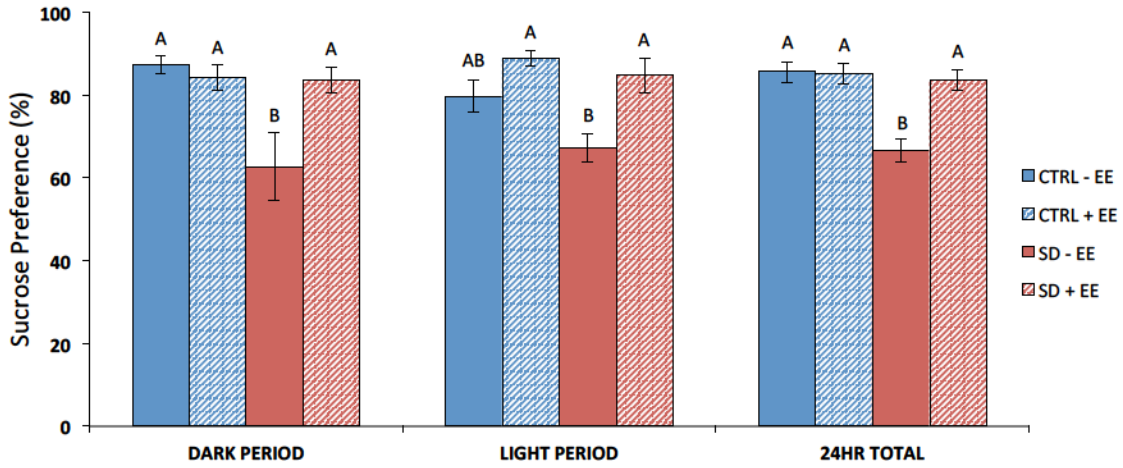


Figure 24. Social defeat stress results in long-term anhedonic-like behavior that is reversed by environmental enrichment.

After a 3 week recovery period, SD-EE mice continued to displayed a decreased preference for a sucrose solution while SD+EE mice were indistinguishable from unstressed controls. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

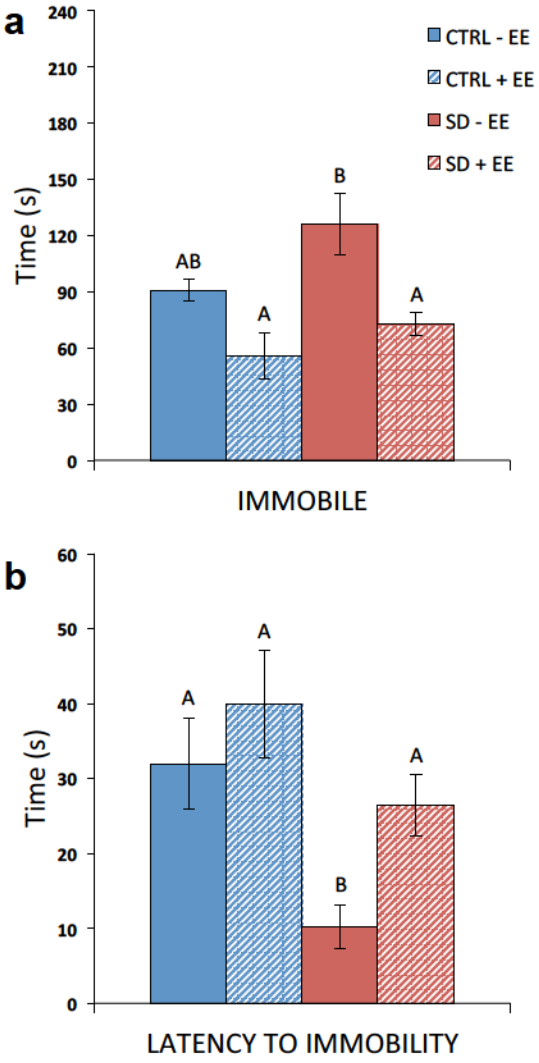


Figure 25. Social defeat stress results in long-term learned helplessness-like behavior that is reversed by environmental enrichment.

(**a**,**b**) After a 3 week recovery period, SD-EE mice continued to displayed increased immobility time and decreased latency to immobility in the forced swim test while SD+EE mice were indistinguishable from unstressed controls. Data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).

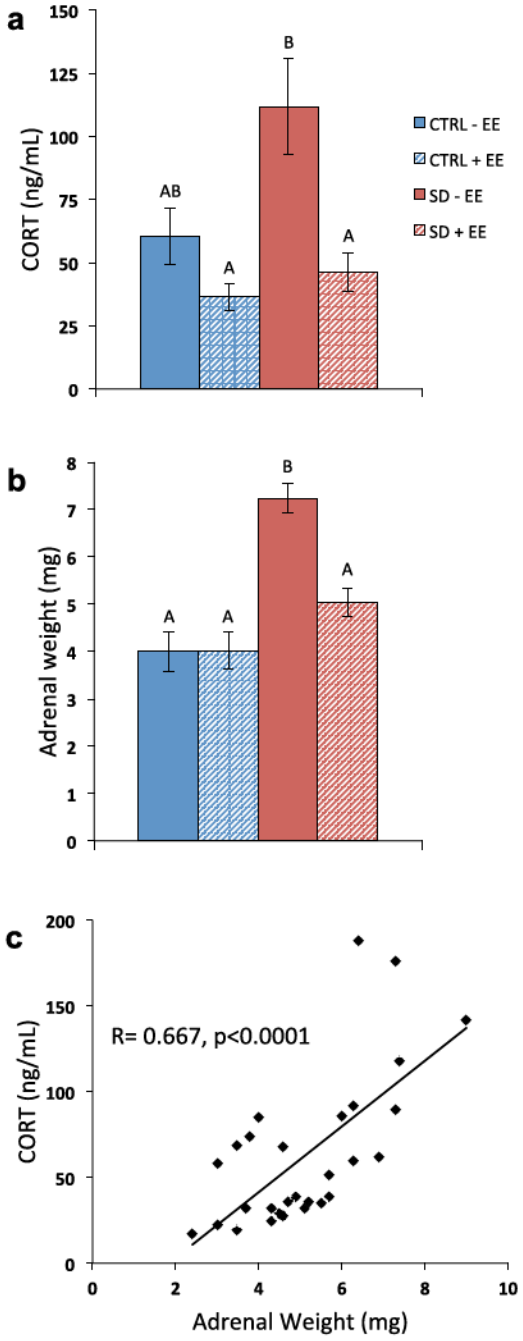
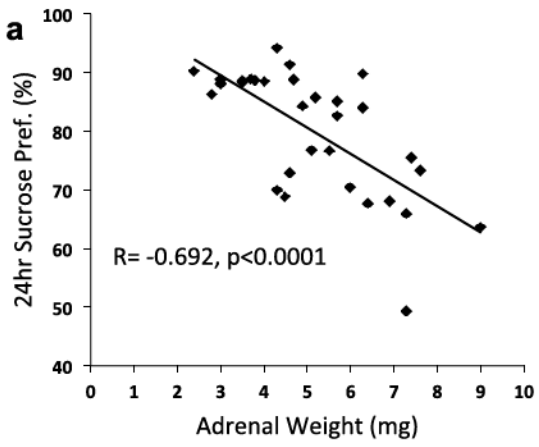
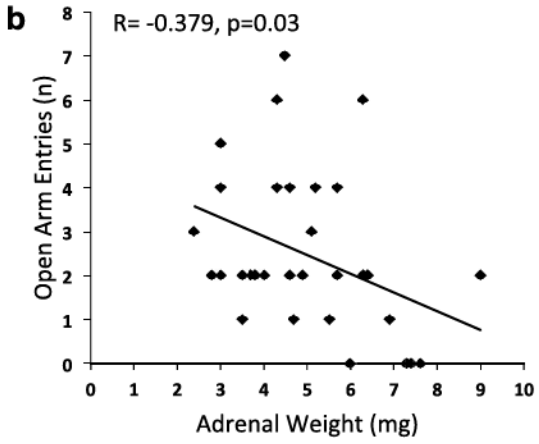

a
CORT (ng/mL)
150
125
100
75
50
25
0
AB
A
B
A
CTRL - EE
CTRL + EE
SD - EE
SD + EE
b
Adrenal weight (mg)
8
7
6
5
4
3
2
1
0
A
A
B
A
c
CORT (ng/mL)
200
150
100
50
0
R= 0.667, p<0.0001
0
2
4
6
8
10
Adrenal Weight (mg)

Figure 26. Social defeat stress results in a long-lasting increase in baseline CORT and adrenal hypertrophy that is reversed by environmental enrichment.

(**a**) After a 3 week recovery period, baseline CORT levels remained elevated among SD-EE mice while SD+EE mice were indistinguishable from unstressed controls. (**b**) Chronic overproduction of stress hormones in response to SD is reflected by adrenal hypertrophy among SD-EE at the conclusion of the experiment. Again, SD+EE mice were indistinguishable from unstressed controls. (**c**) Across all groups, adrenal weight was positively correlated with baseline CORT. In (**a**,**b**) data represent mean ± SEM. For post hoc analysis, groups that do not share letters are significantly different ($p<0.05$).


a
24hr Sucrose Pref. (%)
R= -0.692, p<0.0001
Adrenal Weight (mg)


b
R= -0.379, p=0.03
Open Arm Entries (n)
Adrenal Weight (mg)

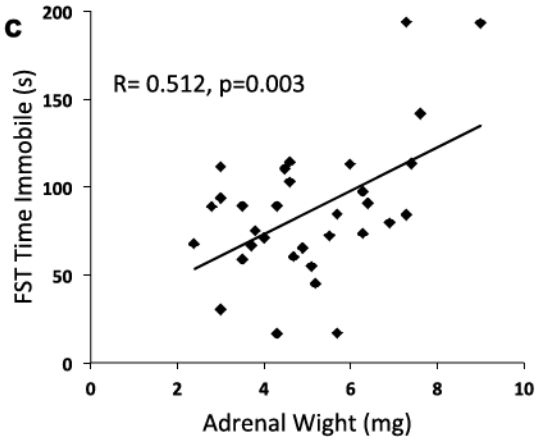

c
R= 0.512, p=0.003
FST Time Immobile (s)
Adrenal Wight (mg)

Figure 27. Depression-like and anxiety-like behaviors correlate with adrenal weight. (**a**) Across all groups, adrenal weight negatively correlated with sucrose preference. (**b**) Adrenal weight was also negatively correlated with entries into the open arm of the elevated plus maze. (**c**) Adrenal weight positively correlated with immobility in the forced swim test.

CHAPTER 4:

CONCLUSIONS, IMPLICATIONS & FUTURE DIRECTIONS

In the nearly 80 years since Selye first described the Generalized Adaptation Syndrome and 70 years since the first reports on the psychological effects glucocorticoids, there has been a growing consensus, backed by a vast literature of clinical epidemiology, animal model research, and neurobiology, that exposure to prolonged or severe stressors increases the risk of virtually all psychiatric disorders. However, a great deal remains unknown about the mechanisms underlying these associations and how we should approach the treatment or prevention of stress-related psychiatric disorders. In this report, we set out to provide a small, incremental addition to our understanding of these issues by using mice to model the effects of stress on the brain and behavior.

In the first study, we were able to demonstrate that relatively mild chronic variable stress induced cognitive impairment but that aged mice are particularly susceptible. Interestingly, stress exposure was also associated with a dramatic increase in the expression of *Bace1*, which plays a critical role in the pathogenesis of Alzheimer's disease, in the hippocampus of young adult mice and in the hippocampus, prefrontal cortex, and amygdala of aged mice. Additionally, we found that the expression of *Bace1* appears to be regulated by promoter region DNA methylation and that stress exposure decreases the methylation of several CpGs. Together, these data reaffirm the role the HPA axis in the Alzheimer's disease β-amyloid pathway and emphasize the need to carefully monitor cognitive function among patients (especially elderly patients) who have been exposed to high levels of stress or treated with glucocorticoids. Moving forward, we hope to explore in greater depth the mechanisms that directly link stress

exposure to cognitive impairment as well as changes in the methylation and expression of *Bace1* with a particular focus on the role of glucocorticoids.

In the second series of studies, we found that chronic social stress results in anxiety-like behaviors, depression-like behaviors, and increased baseline stress hormone levels, which can persist for weeks after the withdrawal of social stressors. Together these data confirm previously reported effects of chronic social stress on mood, anxiety, and HPA-axis function and further suggest that the social defeat paradigm may be useful as a model of chronic, unremitting mental illness. In future studies we aim to understand the effects of social stress on gene expression and DNA methylation in the brain as a means of uncovering mechanisms that may be involved in affective disorders. By comparing samples collected immediately following social defeat to samples collected after a prolonged recovery period, we may begin to understand the extent to which the pathophysiology underlying chronic, unremitting affective disorders is dynamic over the course of the illness.

Finally, in both studies, we demonstrated that the stress-induced phenotypes could be completely rescued by exposure to environmental enrichment, thus providing support for non-pharmaceutical, behavioral interventions as treatments for both cognitive and affective disorders. Moving forward, understanding the mechanisms by which environmental enrichment effectively prevented the observed effects of stress on cognition, mood, anxiety, HPA-axis activity, gene expression, and DNA methylation will be a critically important area of future study that may ultimately provide insights into

novel therapeutic targets for the treatment of Alzheimer's disease, major depression, and other stress-related psychiatric disorders.

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