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BIOCHEMICAL AND BEHAVIORAL EFFECTS OF ENVIRONMENTAL ENRICHMENT ON STRAIN-DEPENDENT VULNERABILITY TO ANXIETY AND DEPRESSION IN THE CHICK SEPARATION STRESS PARADIGM

By:
Sydney Marie Anchor

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford, Mississippi
December 2016

Approved by:

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ABSTRACT

Increased attention has been directed towards determining how environment interacts with genetics on the manifestation of stress-related disorders. This study investigates the differential effects of an enriched versus impoverished environment on behavioral and biochemical endpoints of depression between stress-vulnerable and stress-resilient strains in the chick anxiety-depression model. Black Australorp and Production Red strains were housed in either enriched or impoverished conditions for 4 days and then socially isolated for 90 min. Rate of distress vocalizations (DVocs) were recorded throughout the isolation period and latency to behavioral despair was calculated. Immediately following testing, bilateral hippocampal tissue was harvested and brain-derived neurotrophic factor (BDNF) levels were analyzed via an ELISA assay. A no-test group of chicks removed directly from the home cage was used as a control. Regardless of housing conditions, stress-vulnerable Black Australorps entered behavioral despair more quickly than the Production Reds. Significant decreases in BDNF were seen as a result of an isolation stressor, but were dependent on the complex interaction of genetic line and housing stress conditions. Decreases were only detected in Black Australorps housed in impoverished conditions and the Production Red housed in enriched conditions. These findings may be relevant to understanding the importance of an individual's environment when treating anxiety and depression in stress-vulnerable populations.

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1. INTRODUCTION

Major depressive and generalized anxiety disorders are incapacitating behavioral disorders affecting a large fraction of the population, with conservative estimates predicting 46.4% occurrence of either disorder at some point in a person's lifetime.[1] At its core, major depressive disorder (MDD) is a complex disorder with diagnostic criteria ranging across a wide variety of behavioral and pathophysiological attributes.[1-3] Symptom-based classification by the Diagnostic and Statistical Manual of Mental Disorders—Fifth Edition (DSM-V), as well as previous editions, defines depression as the simultaneous presentation of multiple diagnostic criteria within an explicit time frame.[3] However, none of these diagnostic criteria are exclusive to major depressive disorder. This vague definition leads to significant overlap between diagnoses of other mood and bipolar disorders as well as enables frequent misdiagnoses, both falsely contributing behavior to depression as well as overlooking an alternative physiological cause for the behavior.[2] Furthermore, MDD is rarely a singular diagnosis and is often accompanied by later diagnosis of another anxiety or mood disorder, the most common being a generalized anxiety disorder.[3] This inability to exclusively diagnose MDD and other stress-related disorders has led researchers to begin to characterize anxiety and depression as two extremes of a spectrum of abnormal behavior. One example of this re-characterization is the recent restructuring of the DSM-V in terms of diagnostic criteria for anxiety and depression. While the two still represent distinct categories, a new specification of "with anxious distress" has extended the definition of an MDD diagnosis, allowing for a better description of a depressed individual with anxious tendencies. While this may be a trend towards a

more fluid continuum between the two conditions, further work must be encouraged in order to reduce this stark distinction between these two related syndromes.

While etiological contributions to the development of stress-related disorders have long been debated, few conclusions have been agreed upon. Several studies over the past ten years have begun to detect distinct genetic discrepancies between individuals vulnerable to stress-related disorders and those who are not. Studies of prevalence between twins have determined an overall heritability of stress-related disorders to be about 32 – 38% .[7] Furthermore, studies have revealed a 34% greater risk of developing a depressive disorder in children of parents with some type of stress-related disorder.[7-9] Specific genes have also been found to be associated with stress-related disorders, especially those involved in regulation of the sympathetic nervous system, neurogenesis, and the serotonin system. Additionally, a recent meta-analysis of genomes of patients with and without certain psychiatric disorders revealed 15 regions of at least 17 short-nucleotide polymorphisms correlated with a clinical diagnosis of MDD along with several other mood disorders.[8,9] While the roles of these genes in the development of depression may still be widely unknown, this connection further supports the importance of genetics in the development of and predisposition for stress-related disorders.

To further complicate the matter, while genetic vulnerability may play an important role in predicting responses to stressors, it is not the sole determinant of the onset of stress-related disorders. Studies of environmental factors associated with stress-related disorders continue to reveal correlations between early stressful experiences and later adult vulnerability to depression. Interestingly, recent research

has produced results which provide evidence for both the protective and debilitating effects of early childhood stress. Distinctions between these outcomes have been found to be largely reliant upon the intensity of the stressor and the environment of childhood care. Mild stress during childhood can produce a protective resilience against the later development of depressive symptoms in response to stress during adulthood.[10-12] However, in seeming opposition to this, extreme childhood mistreatment and chronic stressors can produce a sense of learned helplessness in individuals which has been associated with later vulnerability to the development of MDD. An enriched environment can provide exposure to novel surroundings which can also produce protective effects against stress-related disorders. Furthermore, socially and environmentally isolated children have been found to be more prone to the development of stress-related disorders later in life.[10] While the effect of childhood stress may be nuanced, it is clear that it plays a definitive role in the development of stress-related disorders.

Neurogenesis and increased dendritic branching during both periods of mild stress and high maternal care may contribute to later protection against stress-related disorders. Reduction of neurogenesis has been associated with the initiation of depressive disorders. Several studies have shown that overall decreased hippocampal volume in both hemispheres, detected using magnetic resonance imaging (MRI), has been identified in individuals suffering from MDD.[13] While this relationship between loss of synaptic integrity as a result of neurogenesis may be well established, the events which initiate this change are widely debated and this is likely not a mere causal relationship between a specific set of circumstances producing antidepressant

effects. Instead, a variety of environmental conditions may be associated with changes in neurogenesis and the ultimate effect of these changes. Increases in dendritic branching and antidepressant effects have been detected during both periods of predictable mild stress and exposure to enriched environments.[14,15] This suggests that both may provide protective neurogenesis and the development of coping mechanisms which alleviate the depressive response to acute stressors later in life. However, these results vary greatly in individuals in the clinical setting and the reason for these discrepancies is largely unknown. In addition to this, stress hormones and glucocorticoids produced during periods of extreme chronic stress have been seen to inhibit these mechanisms of neuroplasticity and produce a subsequent vulnerability to depressive behavior.[16]

This modulation of structural integrity by neurogenesis has long been associated with the activity of brain-derived neurotrophic factor (BDNF). Not only has reduced concentrations of BDNF in the dentate gyrus of the hippocampus been found in patients with major depressive disorder, but direct infusion of BDNF into the hippocampus has also produced immediate, relatively long-lasting antidepressant effects.[17] Furthermore, several classes of effective antidepressants have been seen to increase BDNF concentrations.[18] Many researchers believe that current antidepressants may be indirectly treating the physiological mechanisms resulting in depression. Also pointing to the importance of BDNF and neurogenesis is the association of a single nucleotide polymorphism of the BDNF gene, Val66Met, with response to antidepressant treatment[19]. The Val66Met allele has been associated with differences in the bioavailability and overall activity of the BDNF protein and recent

studies have shown that the heterozygous val/met genotype may contribute to vulnerability to depression.[20] Subtle differences in environment and levels of stress seem to have massive impacts on the effect of neurogenesis and BDNF activity on vulnerability to depression.

Such discrepancies in the role of environment and genetics on the development of stress-related disorders may in part be due to the unknown interaction between these two etiological contributions. While results remain disputed, several genes have been found to be associated with this interaction. Currently, certain alleles associated with low serotonin reuptake from synaptic clefts have been associated with an increased vulnerability to the development of depression following childhood stress.[21,] However, more recent developments have pointed away from the importance of serotonin signaling pathways and towards a neuroplastic foundation for the development of depression with indirect alterations to serotonin.[22] Stress can impair the effects of BDNF on neuronal plasticity in a way similar to when wild type BDNF is replaced with its functional variant, Val66Met. It would not be surprising if newly elucidated relationships between genetic makeup and stress vulnerability play a role in the relationship between the maintenance of neuroplasticity with the development of stress-related disorders. However, further research is necessary to determine this complex relationship. Overall, major depression and anxiety can best be explained by a combination of environmental and gene modifiers that seem to affect stress-induced depressive behavior.

A better understanding of symptomology, pathophysiology, etiology and treatments for any clinical syndrome relies on the development and utilization of

representative animal models.[23] Currently, researchers utilize several animal models of depression that aim to simulate different behavioral aspects of depression, such as behavioral despair and learned helplessness, as the result of an acute stressor.[24,25] These models are typically rodent models that can be made up of either one test investigating one specific trait or multiple tests demonstrating a variety of behaviors. A single test can illustrate one specific aspect of behavior, such as the forced swim used to determine onset of behavioral despair. Multiple aspects of behavior can be demonstrated through either the use of several tests together or one test with multiple levels of analysis, such as the elevated-plus maze which investigates anxiety based upon on the animals' levels of exploration, total activity throughout the maze, and assessment of potential risks.[24,25] While these tests may provide an accurate model of some of the aspects of depression, they offer only a one-dimensional analysis of behavior, failing to investigate symptoms of anxiety and other mood disorders frequently associated with depression. Only limited success has been seen in using these models for drug screenings or further investigation of the neurobiology and biochemistry of the overall disorder.

To better address the clinical picture of depression, current animal models have worked towards addressing additional endophenotypes of depression and similar stress-related disorders. This endophenotypic mapping is a strategy developed by Josef van der Staay to better simulate human syndromes in animal models by addressing the symptomology, pathology, etiology (both environmental and genetic), and effective treatments of a specific disorder.[23] One area of interest is a better representation of the genetic heterogeneity presented as part of the clinical syndrome.

Animal models are specifically designed to eliminate this diversity and utilize subjects of identical genetic background.[2] However, this is far from an accurate depiction of the many different individuals suffering from varying degrees of depression in a clinical scenario. It is evident that genetic makeup can modulate an individuals' predisposition towards developing MDD in response to a stressful event; however, animal models have only recently begun to address this interaction. Furthermore, all attempts by researchers to develop transgenic rodent models targeting genes associated with stress-related disorders fail to address the effects of chronic stressors on depressive behaviors, a key aspect of stress-related disorders, and none have shown significant success. Some new research has investigated the effect of genetic manipulation of BDNF expression in regards to a stress response. One study of $BDNF_{Met}$ knock-in mice (a genotype similar to the BDNF Val66Met polymorphism associated with depression in humans) demonstrated a vulnerability to depressive- and anxious- behavior when mice were exposed to a stressor.[26] However, results from similar experiments investigating effects of BDNF knock-out seem to contradict these findings and fail to show that inhibition of BDNF expression directly produces depressive behavior.[26-28] This may largely be due to an inability to specifically knock-out BDNF in targeted areas of the brain, such as the dentate gyrus of the hippocampus. These studies may become more relevant as researchers begin to develop ways to do so. Currently, no rodent models of depression have been able to fully represent a similar vulnerability to stressful events due to genetic background as has been seen in humans.

Animal research regarding the complex interaction between genetically-mediated stress sensitivity and the effects of an impoverished or enriched environment is only in its very early stages. Some researchers have attempted to initiate this by investigating the differential effects of changes to housing environment in rats bred specifically to display high or low anxiety.[30,31] Results of these studies indicate that high anxiety rats were less anxious when housed in an enriched or standard environment. Only the enriched conditions produced effects on BDNF signaling in comparison to those raised in an impoverished environment (standard housing in social isolation). However, like other early research in stress-related disorders, this study focuses only on anxiety, thereby excluding one end of the spectrum of behavior consistent with the diagnosis of anxiety and depression. Similarly, when neuropeptide Y (NPY), a peptide believed to play a role in anxiety and specifically in conjunction with BDNF, is knocked-out in mice, depressive behavior is produced.[32] Exposure to environmental enrichment, however, impairs spatial memory and learning with little to no effect on patterns of behavioral despair in all test subjects. Interestingly, environmental enrichment did decrease hippocampal BDNF levels; however, this was only detected in the NPY-knockout mice.

A major concern associated with many of these animal models of depression is their basis on old diagnostic criteria which inherently exclude comorbidity between anxiety and depressive disorders. Earlier editions of the DSM (DSM I-III) included a hierarchical structure of exclusion principles, placing specific disorders in a ranking system which diagnosis of a disorder of higher rank excluded the diagnosis of another disorder, even though a patient's symptoms may qualify them for a diagnosis of

both.[7] This system of diagnosis was especially detrimental to the study of anxiety disorders that, under newer diagnostic methods (DSM III-R, IV, and V), are associated with the diagnosis of other mood disorders, especially major depressive disorder. Thus, many animal models developed before this adjustment fail to address symptoms of anxiety often associated with MDD. Furthermore, as previously discussed, research has begun to suggest that anxiety and depression disorders make up a spectrum of abnormal behavior, thus making hybrid animal models of depression and anxiety increasingly relevant to the translational application of animal research.[33] Currently, very few hybrid models of anxiety and depression exist and the few that do fail to study a stress-induced form of depressive or anxious behavior. This failure of animal models to replicate human behavior is further demonstrated by the low efficacy of drugs discovered using current animal models. Many of the drugs deemed effective using animal models are ultimately unsuccessful in the treatment of about 40-60% of the population suffering from MDD and GAD.[34] For the small fraction of patients who do see results, effects are delayed by about 4-6 weeks and are ultimately transient for about half of this group. Refining current animal models of anxiety and depression is essential to develop more effective forms of treatment, as well as to better understand these complicated disorders.

In an effort to address this failure by the scientific community, Sufka et al. has developed a hybrid model of anxiety and depression using domestic chick fowl exposed to an acute but ongoing stressor, social isolation.[36] Social isolation of chicks elicits distress vocalizations (dVocs) which follow a pattern of response characterizing both an anxiety and depression phase. Upon initial exposure, dVoc

rates begin at a very high rate and illustrate a state of heightened stress, the anxiety phase. Over time, these dVoc rates decline by about 40-60 percent and, after about 30 minutes of social isolation, reach a decreased, steady rate as the chick enters a state of behavioral despair, the depression phase. This transition from the anxiety to depression phases better illustrate the comorbidity of anxiety and depression frequently identified in a clinical diagnosis. Initial validation of the paradigm indicated that the model illustrated the effects of all four major classes of antidepressants as well as several anxiolytics. Socially raised chicks were treated with the respective drug 15 minutes preceding social isolation. Effective antidepressants were seen to elevate average dVoc rate during the depression phase, thereby alleviating the behavioral despair response.[37] Effective anxiolytics were seen to decrease the dVoc rate during the anxiety phase, thereby reducing the initial stress response and demonstrating anxiolytic effects.[38] Furthermore, these studies identified a lack of antidepressant effects for novel antidepressants that had been screened as effective in rodent models of depression but later failed to produce results in early clinical trials. Thus, these studies were able to validate the model as clinically relevant for a pharmacological endpoint of analysis.

The model has been further refined to address several other relevant endophenotypes of anxiety and depression. Subsequent studies have demonstrated vulnerabilities to stress at both the genetic and environment level. By quantifying the entry into behavioral despair between the anxiety and depression phases, two strains of chicks were identified as behavioral outliers in their response to stressful events as compared to seven other genetic lines that were homologous in their stress response.

These two strains displayed contrasting stress responses in opposition to each other thereby highlighting two extremes of the spectrum of stress vulnerability. A stress resilient strain, Production Red, was found to enter the depression phase at a slower rate (increased latency) as compared to a stress-vulnerable strain, Black Australorp.[40] This distinction between the two allows for the study of the response to a stressor for a more diverse sample of genetic backgrounds, therefore better mirroring the diverse population of patients suffering from stress-related disorders. In addition to these behavioral differences, strain-dependent biophysiological alterations in response to an acute stressor have been measured.[41] Drastic changes to hippocampal BDNF levels throughout the isolation period were measured in the stress-vulnerable Black Australorp strain, but not in the stress-resilient Production Red strain. This lack of homeostatic regulation of BDNF remains consistent with a decrease in BDNF levels seen in depressed patients, therefore paralleling a pathology seen in the clinical setting.

This paradigm has been further validated to address the role of environmental effects on stress- induced behavior. White leghorn chicks (genetically neutral in their vulnerability to stress) raised in an enriched environment were seen to display an increased time to enter behavioral despair as compared to those raised in a standard impoverished environment.[39] Therefore, in this genetic line, environmental enrichment was seen to mediate protective effects against depressive behavior in response to an acute stressor. However, the effects of an enriched versus impoverished environment have not been studied in the aforementioned genetic lines vulnerable or resilient to stressful events.

To our knowledge, the effects of an enriched or impoverished environment have not been studied in two genetic strains presenting a resiliency or vulnerability to stress. Furthermore, changes in BDNF have not been investigated in direct response to an acute but ongoing social separation stressor. The chick separation stress paradigm allows an investigation of these two contrasting responses to stressful events on both behavioral and biochemical endpoints as the result of a continuous isolation stressor. The present research addresses this complex interaction and aims at better understanding the role of genetic heterogeneity in the clinical population and the biochemical mechanisms by which this response occurs.

2. MATERIALS AND METHODS

The following procedures were approved by the University of Mississippi Institutional Animal Care and Use Committee (Protocol #16-014) and were conducted in accordance with the principles of animal care as detailed in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

2.1. Subjects and housing characteristics

Male Black Australorp and Production Red (*Gallus gallus*; Ideal Poultry, Cameron, TX, USA) chicks were obtained 2 days post-hatch and housed in 44 x 61 x 40 cm stainless-steel cages with 12-14 chicks per cage. Food (Purina Start and Grow, St. Louis, MO, USA) and water were provided ad-libitum through one quart gravity-fed feeders and waterers. Room temperature was maintained at 21 ±1 °C and overhead illumination was maintained on a 12 h light-dark cycled schedule. Maintenance was preformed each day during the middle two quarters of the light cycle.

2.2 Enrichment characteristics

Subjects housed in an enriched environment were housed in the same conditions as previously stated with added enrichment features including mirrors (to create the illusion of a social environment), perches, an area with sand and food (to allow for foraging, dust bathing, and exploratory behavior), and white and yellow strings hung from the top of the cage (for additional pecking stimulation). These features were chosen based upon previous enrichment studies.[42-46]

2.3 Isolation apparatus

A six-unit test apparatus containing Plexiglas chambers (25 x 25 x 22 cm) surrounded by sound-attenuating media to reduce the transmission of sound to adjacent units was used to record distress vocalizations (dVocs) as a result of social isolation. Each unit was illuminated by a 25-W light bulb and ventilated by an 8 cm diameter rotary fan (Model FP-10AX S1, Commonwealth Industrial Corp., Taaipei, Taiwan). Chicks were monitored throughout isolation period by miniature video cameras (Model PC60XP, SuperCircuits, Inc., Liberty Hill, Texas, USA) mounted in the corner of the apparatus at floor-level and routed through a multiplexor (Model PC47MC, SuperCircuits). dVocs were recorded via microphones [Radio Shack Omnidirectional Model 33-3013 (modified for AC current)] mounted at the top of each chamber and routed to a computer equipped with custom designed software for data collection (continuous acquisition with sample rates >10/sec and 75% sensitivity). This configuration allowed for the reliable recordings of dVocs but not twitter vocalizations not induced by stress.

2.4 Procedure

Black Australorps and Production Reds were tested at different sessions separated by a month. Chicks were housed for 4 days in either an impoverished housing environment or enriched housing environment (n= 36- 40 chicks per condition). Following 4 days in the appropriate conditions, chicks were tested 7 days post-hatch during the middle three-quarters of the chicks' light cycle. Groups of nine chicks were removed from their home-cage, weighed to identify potential outliers (i.e.

low weight), and color-coded with a felt marker for identification. 6 of these chicks were placed in the isolation apparatus and dVoc measurements were recorded for 90 min (sample size n = 18 chicks per experimental group). Simultaneously, the remaining 3 chicks were euthanized via decapitation to harvest brain tissue as a no test, non-isolated control group. Following the test period, half of the cohorts in the isolation apparatus were returned to their home-cage while the other half were euthanized via decapitation to harvest brain tissue. Brains were quickly harvested and placed on dry ice for 8-9 min. Bilateral hippocampal sections were removed and tissues were stored at -70°C until analysis could be conducted. In total 9 tissue samples were collected per experimental condition.

A sandwich ELISA was performed using BDNF Emax Immunoassay ELISA kit obtained from Promega. A 96-well plate was coated with Anti-BDNF Monoclonol Antibody (mAb) and incubated overnight at 4 °C. Plates were then washed using wash buffer, and the nonspecific binding sites were blocked for a period of 1 hour with 200 µl/well block and sample buffer. Tissue extracts were prepared using lysis buffer. Standard and tissue samples (100 µl/well) were added to the wells after washing and were incubated for a period of 2 hours with shaking. After washing, Anti Human pAb (100 µl/well) was added to each well and was incubated for a period of 2 h. A species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) was added to each well so as to detect the amount of specifically bound pAb. Unbound conjugate was removed by washing, and incubation with chromogenic substrate like TMA/TMB substrate was carried out to determine the color change. The final color intensity was measured using a plate reader at 450 nm.

2.5 Dependent Measures

Average rate of distress vocalizations was calculated for consecutive 3-minute blocks throughout the isolation time period. To compare onset of behavioral despair (latency to depression phase), the difference in dVoc rate from the anxiety phase (0 -1 minute) to the depression phase (30-60 minutes) was calculated and a threshold value at which the dVoc rate had declined by 25%, 50%, 75%, and 95% of this difference was determined. The time point at which the average of three consecutive minutes was equal to or less than this threshold value was then determined. A repeated measures ANOVA was used to determine significance of depression onset values with one- or two-way ANOVAs conducted at specific threshold points in order to determine specific comparisons between groups.

Average BDNF concentration was measured using an ELISA assay as described previously. Two-way ANOVAs were performed separately on test and no test, non-isolated groups with subsequent one-way ANOVAs preformed on Test birds in order to determine the effect of environment and strain.

3. RESULTS

The effect of social isolation on dVoc rates in the Black Australorp and Production Red strains housed in enriched or standard impoverished conditions is presented in **Figure 1**. In all groups, dVoc rates were initially high (0-3 min), characterizing the anxiety phase, and then decreased by about 40-60% until reaching a steady state at about 30 min and remaining stable for the remainder of the test period (30-90 min), characterizing the depression phase. While these results summarize the transition between the anxiety and depression phases, a common measurement used to determine behavioral effects and/or stress vulnerability in behavioral despair is to calculate how quickly animals enter the depression phase. In order to compare these rates of change between the two phases, onset of behavioral despair (the depression phase) was determined for four thresholds of the percent reduction of dVoc rate between the initial rate during the anxiety phase (0-1 minutes) to the baseline depression rate (30-60 min), as previously described. These calculations are summarized in **Figures 2a-b**.

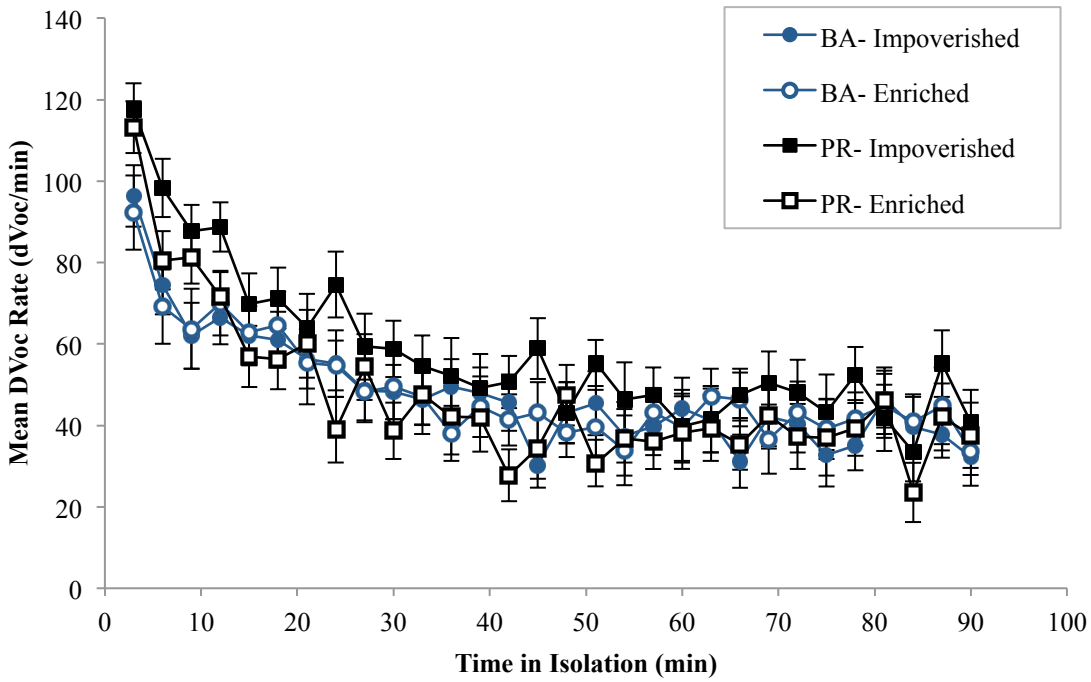


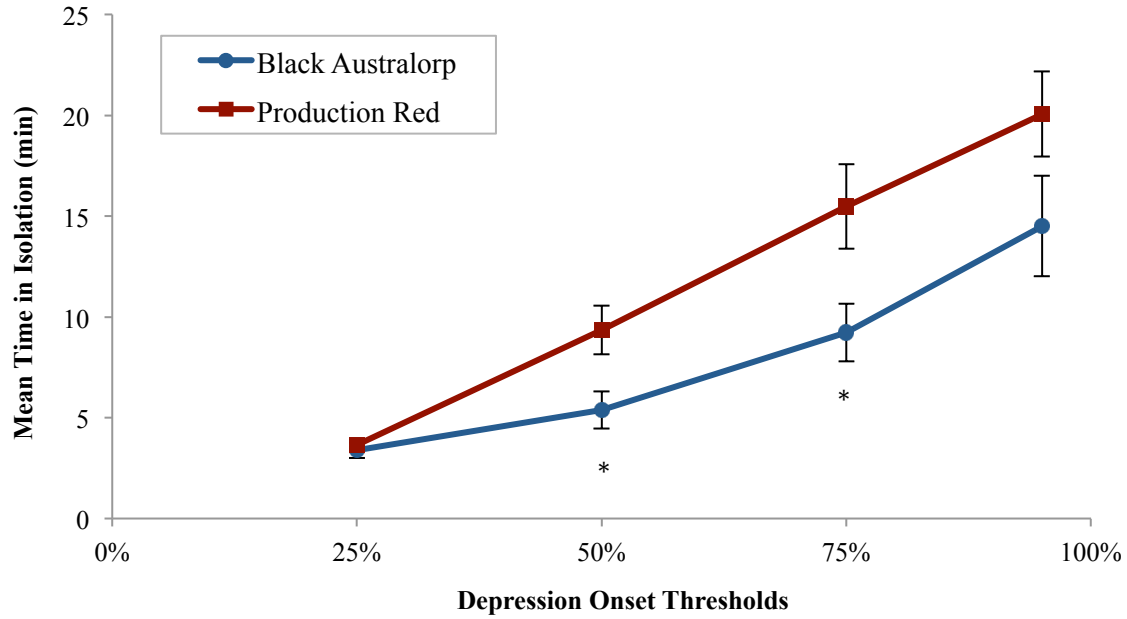
Figure 1. Mean rate of distress vocalizations (dVocs) in three-minute intervals throughout 90-minute isolation period for Black Australorp (stress vulnerable) and Production Red (stress resilient) housed in impoverished or enriched conditions. Values equal mean rate measured in rate/min ± (SEM). BA (Black Australorp) and PR (Production Red). Sample sizes were n = 16- 18.

In order to confirm previous studies establishing a shorter onset of behavioral despair for the Black Australorps in comparison to the Production Reds, onsets of the depression phase in the Black Australorp and Production Red strains housed in only standard impoverished conditions are compared in **Figure 2a**. Initially, dVoc rates in both strains decreased at a similar rate with no significant differences detected at the 25% threshold. However, differences began to emerge at the 50% threshold mark. Production Reds took longer to enter the depression phase in comparison to the Black Australorps at the 50%, 75%, and 95% threshold. A two-way repeated measures ANOVA was conducted and revealed a significant main effect for time $F(3,31) = 25.834$, $p < .000$, significant main effect for strain $F(1,33) = 5.677$, $p < .023$, and significant time x strain interaction $F(3,31) = 2.994$, $p < .046$. Simple effects tests were performed for each threshold using one-way ANOVAS, which revealed a significant main effect for strain at the 50 percent, $F(1,33) = 6.545$, $p < .015$, and 75 percent threshold, $F(1,33) = 6.100$, $p < .019$, and approached significance at the 95 percent threshold, $F(1,33) = 3.149$, $p < .085$.

A summary of the effects of enriched housing conditions on onsets of the depression phase in the Black Australorp and Production Red strains is presented in **Figure 2b**. As previously seen in the standard impoverished conditions, Black Australorps enter the depression phase more quickly than the Production Reds in both environmental conditions. Within each strain, chicks raised in an enriched environment entered the depression phase slightly quicker than chicks raised in standard impoverished conditions of the same strain. A three-way repeated measures ANOVA was conducted and revealed a significant main effect for time $F(3,64) =$

40.636, $p < .000$, a significant main effect for strain, $F(1,66) = 9.086$, $p < .004$, and a significant time x strain interaction $F(3,64) = 4.136$, $p < .010$. There was no significant main effect detected for environment, no significant interaction for the time x environment term, and no significant interaction for the time x environment x strain term. Simple effects tests were performed for each depression onset threshold using two-way ANOVAS. At the 50 percent threshold, the two-way ANOVA revealed a significant main effect for strain, $F(1,66) = 11.538$, $p < .001$, but no significant main effect for environment or significant interaction between strain x environment. At the 75 percent, the two-way ANOVA revealed a significant main effect for strain, $F(1,66) = 7.556$, $p < .008$, but no significant main effect for environment or significant interaction for the strain x environment term. At the 95 percent threshold, the two-way ANOVA revealed a significant main effect for strain, $F(1,66) = 5.059$, $p < .028$ but no significant main effect for environment or a significant interaction for the strain x environment term.

A. Standard Impoverished Conditions



B. Standard Impoverished and Enriched Conditions

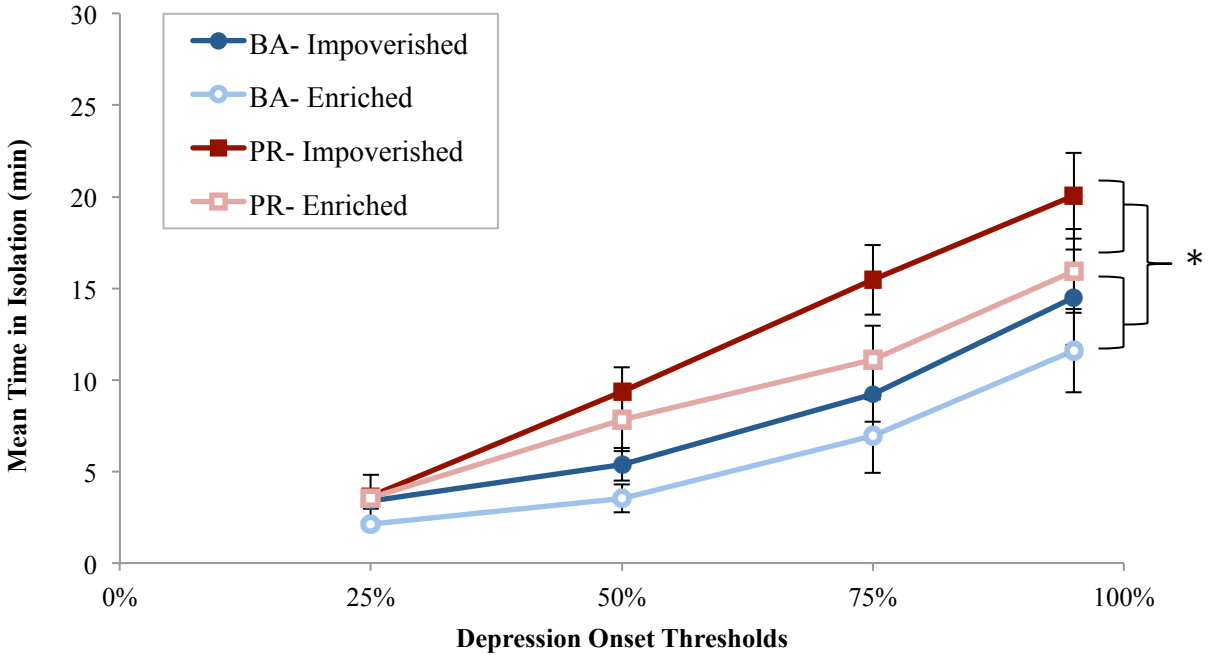


Figure 2. Onset of depression phase for Black Australorp (stress vulnerable) and Production Red (stress resilient) chick strains housed in standard impoverished conditions **(A)** and enriched or impoverished conditions **(B).** Values equal mean time ± (SEM) measured in min to reach four thresholds of depression onset represented by a percent reduction in dVoc rate. Asterisk (*) indicates shorter latency to behavioral despair. BA (Black Australorp) and PR (Production Red). Sample sizes were n = 16-18.

Average hippocampal BDNF concentration in Black Australorp and Production Red strains removed directly from their home cage or exposed to 90 minutes social isolation is summarized in **Figure 3.** Hippocampal BDNF levels were similar between the No Test, non-isolated, birds of all groups. Among the Test birds, there were no differences between the Production Red housed in impoverished or enriched conditions. However the isolated Test Black Australorp birds housed in enriched conditions displayed increased BDNF in comparison to the Test Black Australorp birds housed in impoverished conditions. A three-way ANOVA was performed and revealed a significant main effect for strain, $F(1,38) = 4.972$, $p < .032$, a significant strain x environment x test interaction $F(1,38) = 8.388$, $p < .006$, and a trend towards a significant interaction between strain x environment , $F(1,38) = 4.972$, $p < .052$. No significant main effects for environment or test or interactions between strain x test or environment x test were found. A two-way ANOVA was performed on the Test and No Test birds separately to determine differences in BDNF due to strain and environmental conditions with or without social isolation. No significant main effects due to strain or environment or an interaction between strain x environment for the No Test birds was detected. Because of these findings, no further analyses were performed on the No Test birds. Alternatively, in the Test birds, a trend toward a significant main effect for strain, $F(1,18) = 1$, $p < .062$, and a significant interaction for strain x environment, $F(1,18) = 1$, $p < .004$ was detected. No significant main effect for environment was detected. From here, separate one-way ANOVAs were run on only Test birds in Black Australorp or Production Red in order to determine differences within each strain due to environmental conditions. A

significant main effect of environment was found in the Test birds of the Black Australorp strain, $F(1,22) = 5.565$, $p < .028$, but not in the Test birds of the Production Red strain, $F(1,11) = 2.400$, $p < .152$. Two- way ANOVAs were run on the Production Red and Black Australorp strains to determine differences in BDNF following testing and differential effects due to strain. For the Production Red, a significant interaction between environment x test was detected $F(1,22) = 5.423$, $p < .032$ but no significant main effect for environment or test was detected. For the Black Australorp, a significant main effect was detected for environment, $F(1,20) = 5.893$, $p < .025$, and an interaction between environment x test approached significance, $F(1,20) = 3.293$, $p < .085$, but no significant main effect for test was detected. Finally, simple effects analysis using one-way ANOVAs was performed to determine differences between No Test and Test groups of each housing condition and strain combination. For the Production Red housed in enriched conditions, a significant main effect for test was detected, $F(1,12) = 6.502$, $p < .029$, but no significant main effect for test was detected for the Production Red housed in standard impoverished conditions. For the Black Australorp housed in a standard impoverished conditions, a significant main effect for test was detected, $F(1,12) = 6.502$, $p < .046$, but no significant main effect for test was detected between Black Australorp housed in enriched conditions.

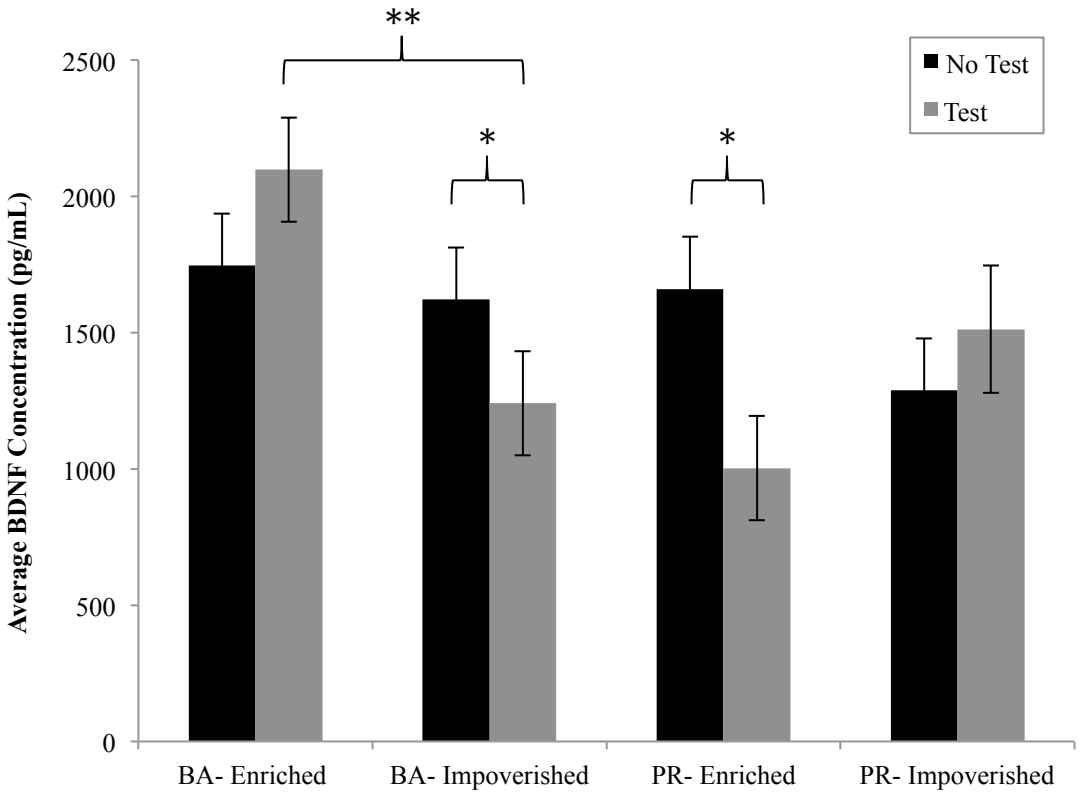


Figure 3. Hippocampal BDNF in the Production Red (stress resilient) and Black Australorp (stress vulnerable) strains housed in standard impoverished or enriched conditions without social isolation or following 90 minutes of social isolation. Value equals average concentration of BDNF in pg/mL ± (SEM). Hippocampal tissue was harvested from no test groups immediately following removal from home cage and from Test groups following 90 minutes in social isolation. BA (Black Australorp) and PR (Production Red). Asterisk (*) indicates significant difference between test and no test group, double asterisk (**) indicates significant difference between environments. Sample size n = 4-6.

4. DISCUSSION

The aim of this research was to investigate the complex interactions between genetic and environmental modifications of the stress response. Chicks of two strains representing outliers of the stress response, the stress-vulnerable Black Australorp and stress-resilient Production Red, were housed in either standard impoverished or enriched conditions for four days. Following this period, they were exposed to an isolation stressor for 90 minutes and dVoc rates were quantified to measure the progression of the stress response. Hippocampal tissue was harvested from chicks following the isolation test and from separate groups of non-isolated chicks representing both strains and environmental conditions to act as a control group. BDNF concentrations in these samples were analyzed using an ELISA assay.

The progressive change in dVoc rates throughout the test session remains consistent with previous literature which shows that social isolation in chicks produces a robust two-phase stress response.[37-39] Independent of strain or housing condition, isolated chicks displayed a similar pattern of stress illustrated by the initial heightened rate of dVocs during the anxiety response (first three minutes of isolation) and a subsequent decrease to a stable rate for the remaining test period (31-90 minutes). This progression from anxiety to behavioral despair as the result of an acute but unresolved stressor models the anxiety-depression continuum which is consistent with the high comorbidity seen between anxiety and depression in the clinical picture.

In the present study, both stress-vulnerable and stress-resilient chicks housed in standard impoverished housing conditions displayed the same differences in their onset of behavioral despair as seen in earlier reports. Black Australorp, stress-

vulnerable birds demonstrated a quicker onset of behavioral despair while the Production Red, stress-resilient birds, demonstrated a slower onset of behavioral despair.[40,41] This distinction highlights the two strains' differences in stress-vulnerability. Other animal models of depression use this measure of onset of behavioral despair or total time spent in behavioral despair as an illustration of stress vulnerability[23,24]. When housed in enriched environments, the two strains of chicks responded to isolation with this same pattern of contrasting stress responses as seen in chicks housed in impoverished environments. Therefore, these results confirm differences in stress vulnerability between the two strains as dictated by genetic predispositions to the stress response in both an enriched and impoverished environment.

In contrast to our predictions based on past studies published by this laboratory, environmental enrichment failed to slow the onset of behavioral despair in both strains[39]. In fact, there was a tendency for housing in an enriched environment to accelerate the decline into behavioral despair in both strains of chicks, however this was not significant. There are several reasons why these results may be inconsistent with past studies. First, it should be noted that previous research reported that environmental enrichment attenuated onset into behavioral despair in chicks of the White Leghorn strain, not the Black Australorp or Production Red strains used in the present study. In studies of vulnerability to depression, this strain displayed a neutral response to a stressor. Alternatively, the strains in this particular project were determined to be outliers in their behavioral response to a stressor. Therefore, differences in the effect of an enriched environment on these two strains may actually

provide insight into how these strains of chicks with differences in their genetic predisposition to stress response to environmental manipulations; however, additional research is necessary to come to any conclusions based on this information.

A second explanation of these inconsistences may be the method of shipping which differed from studies published previously by this laboratory. Rather than almost immediate delivery following hatching (about 12 hours), chicks for this project were not received until about 48 hours after hatching and may have been repeatedly subject to a wide variety of stressors during this shipping period. While this difference in shipping methods was not robust enough to affect differences in genetic vulnerability, there is the possibility that it may have prevented the effects of environmental manipulation on this behavioral response to stress.[31]

While these results may not be consistent with reports produced by this laboratory, they are consistent with the aforementioned study of NPY- knockout mice. In a transgenic mouse model of stress vulnerability, knockout of NPY produces stress-vulnerability and an increase in depressive behavior.[32] However, housing in an enriched environment for a prolonged period did not produce robust behavioral responses in these stress-vulnerable mice. Similarly, in the present study, it does not seem that housing in an enriched environment provided antidepressant effects in the stress-vulnerable strain of chicks. To our knowledge, no studies have been conducted using models of stress resiliency. Our results indicate that short-term exposure to environmental enrichment does not seem to drastically affect this stress-resilient strain of chicks.

Brain-derived neurotrophic factor (BDNF) is understood to help maintain the integrity of synaptic communication and can act as a marker for maintenance of neurogenesis and protection against neural cell loss.[15,16] Previous research has established that such increases in neurogenesis are likely to provide antidepressant properties. In the present study, there were no differences in baseline levels of BDNF across strain and housing conditions in non-isolated control groups. These similarities in baseline BDNF levels between the Black Australorp and Production Red strains are consistent with other studies produced by this laboratory.[41] However, previous reports indicate that environmental enrichment typically produces increases in BDNF levels.[31] Such changes due to an enriched environment were not detected in the present study. This difference could be due to a number of factors. First, the length of housing enrichment tends to be longer in other studies of environmental enrichment, ranging from three months to almost a year of housing. This is dramatically longer than the four days for which chicks in this study are exposed to environmental enrichment. Therefore, this length of time may simply not be enough time to produce robust changes in BDNF. Second, as described previously, the two-day shipping period may entail high levels of stress which might mitigate changes in BDNF in response to environmental enrichment.

The effects of genetic and environmental differences on isolation-induced alterations in BDNF are nuanced. Results from the current study seem to be dependent on the combination between both genetic vulnerability to stress and the environment in which they are housed. In the stress-vulnerable Black Australorp chicks housed in an enriched environment, BDNF concentration following an

isolation stressor was seen to remain the same or be slightly elevated, though not significantly, when compared to non-isolated birds. However, BDNF levels in stress-vulnerable chicks housed in an impoverished environment following the isolation period were seen to be dramatically decreased in comparison to non-isolated birds. Alternatively, BDNF concentrations in stress-resilient Production Red chicks following an isolation stressor revealed a very different pattern in BDNF response. When stress-resilient chicks were housed in enriched conditions and exposed to an acute isolation stressor, a decrease in BDNF in comparison to non-isolated birds was detected. When housed in an impoverished environment, a moderate but not significant increase in BDNF was seen following social isolation as compared to non-isolated chicks.

Because of the varied results reported by researchers investigating BDNF, it is difficult to determine if these conflicting changes remain consistent with current literature. Previous literature in this laboratory has reported dramatic differences in the regulation of BDNF between the stress-vulnerable and stress-resilient strains. These differences have been attributed to differences in homeostatic mechanisms of BDNF expression[41]. However, to our knowledge, this is the first report of the complex interaction between environmental manipulation and gene vulnerability on BDNF expression. While many researchers measure baseline BDNF following unpredictable chronic stress or environmental enrichment, very few record changes to BDNF following an acute stressor or how BDNF changes following a stress response after housing in an enriched or impoverished environment.

Interestingly, BDNF levels following an acute but prolonged stressor may begin to reveal important aspects of this complex relationship between an environmental stressor and gene vulnerabilities to depression. One idea we have developed to better explain these results is the conceptualization of impoverished housing conditions as a predictable mild stressor. Unlike unpredictable chronic stress, research regarding predictable mild stress has revealed very different effects on subsequent responses to later stress vulnerability.[12] Several early studies have identified that predictable mild stress can attenuate later responses to stress and initiate neurogenesis which may provide these protective effects. Some researchers have begun to explain this phenomenon using the mismatch hypothesis of psychiatric disorders. This theory proposes that early mild or moderate stress "matched" with later stressful events in adulthood provides inoculation to stress and protects against depressive behavior in adulthood[35]. However, our results indicate that the differential effects of predictable mild stress remain largely dependent on genetic predisposition to stress vulnerability (Table 1).

Strain	Gene Stress Load	Housing Conditions	Environmental Stress Load	Isolation Stress Level	Change to BDNF
Black Australorp	High	Impoverished	High	High (Chronic stressors)	↓
	High	Enriched	Low	Moderate	⊘
Production Red	Low	Impoverished	High	Moderate	⊘
	Low	Enriched	Low	High (Mismatch hypothesis)	↓

Table 1. Illustration of stress sequence based upon strain and environment differences

Exposure to predictable mild stress in the stress-vulnerable Black Australorps decreases their ability to modulate a later response to stress induced by social isolation and thereby results in a depletion of BDNF. Housing in an enriched environment, however, bolsters their ability to respond to later isolation and does not significantly affect BDNF levels. Alternatively, predictable mild stress provides a protective effect against later isolation in the stress-resilient Production Reds which, therefore, aids in the stabilization of BDNF following a stressor. Finally, stress-resilient chicks housed in an enriched environment are not inoculated to stress with a period of predictable mild stress and, therefore, later exposure to a stressor depletes BDNF levels.

To our knowledge, this is the first report of the complex interaction between environmental modifiers and genetic outliers of the stress response on BDNF following an acute stressor. In the current literature, there is no consistent pattern of the effects on BDNF due to enrichment or varying magnitudes of stressful events. Our results are highly suggestive that these environmental modifiers function in relation to other underlying genetic components and may be a reason for the mixed results currently seen in both animal and clinical research. We urge researchers to begin to develop methods to integrate both differing genetic predispositions to stress as well as differences in environmental modifiers to better understand this complex biophysiological mechanisms underlying these inherent differences.

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