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# The role of valproic acid, a histone deacetylase inhibitor, in reducing anxiety levels in rats: an epigenetics study

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# The role of valproic acid, a histone deacetylase inhibitor, in reducing anxiety levels in rats: An epigenetics study

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**Abstract**

Interestingly, rats, like humans, show personality traits: they are born with different anxiety levels (i.e., high (HAn) or low anxiety (LAn) levels). In this study, we investigated (1) if treatment with valproic acid (VPA) can lower anxiety level in trait anxiety rats and (2) how VPA may interact with environment (e.g., enriched (EE), standard (SE) and isolated environments (IE)). VPA is a deacetylase inhibitor that increases Histone 3 acetylation, a known epigenetic mechanism that interacts with stress response proteins, and treats epilepsy and mood disorders. Since rats reared in IE exhibit heightened anxiety levels as compared to those in EE, we suspected that there would be an additive effect of VPA treatment and EE – that this combined treatment would lower the anxiety levels of HAn rats. In order to verify that VPA improved performance on anxiety tests by interacting with the stress response system, Long Evan Rats were perfused with 4% paraformaldehyde for brain tissue analysis using immunohistochemistry to measure corticotropin releasing factor (CRF), a protein implicated in stress. We found that Long Evan rats housed in EE showed less anxiety-like behavior on the EPM compared to other housing settings, and surprising, this was *reversed with VPA*. VPA did lower anxiety-like behavior in IE reared animals. There was a decrease in the CRF levels in LANEE, and an increase CRF in the HANEE housed animals, suggesting no additive effect of combining VPA and EE treatment for anxiety-like behavior, but a benefit for reversing anxiety due to isolation rearing.

## Introduction

### *Background*

#### *Anxiety Disorders are Complex*

Behavior is a complex response to stimuli mediated both by genes and by the environment. For example, pathological anxiety, a complex psychological disorder, involves an *exaggerated* response to stimuli that is a consequence of genetic and environmental influences. Many of the psychological and physiological responses to the class of anxiety disorders (e.g., Panic Disorder, Agoraphobia, Social Phobia) overlap in that they are characterized by tension, excessive worry, stress, irritability, restlessness, to mention but a few. These responses are mediated by genes, neurotransmitters and neurohormones and their receptors as well as by environmental factors and/or interactions between genes, chemicals and the environment. Phenomenologically, anxiety disorders seem to have in common an increased reactivity to the environment, driven by uncertainty and fear of perceived threats (Le-Niculescu et al. 2011). Recent studies of mental health indicate a high frequency of occurrence for anxiety disorders in the general population. In fact, anxiety disorders are the most prevalent (28.8%) of all classes of disorders (Kessler et al. 2005). According to Center for Disease Control, the estimated lifetime prevalence of any anxiety disorder is over 15%, while the 12-month prevalence is 10%. Moreover, the annual cost of anxiety disorders in the US is estimated to be approximately $42.3 billion in the 1990s, and $300 billion in 2002. This increase calls for more studies on mental health, particularly anxiety disorders, focused on understanding the contribution of genes, the impact on different parts of the brain, neurotransmitters and their receptors with the hope of manufacturing new drug targets and treatment options for this disorder that has reached epidemic proportions. Investigations of

anxiety-like behavior in rodents have revealed significant neural, endocrine and endocrine responses associated with anxiety levels in animals (Ravenelle et al. 2013).

*Neurotransmitters Implicated in Anxiety Disorders*

Several brain neurotransmitters including serotonin, gamma-amino butyric acid (GABA), and glutamate have been implicated in anxiety disorders because of the important role each plays in the balance of physiological processes such as cognition and mood. For example, GABA in the dorsal raphe nucleus (DRN) has been implicated in regulating sleep/wake states and influencing anxiety and aggression. As a primary inhibitory neurotransmitter in the central nervous system, GABA counterbalances the excitatory activity of the neurotransmitter glutamate, the most prevalent excitatory neurotransmitter in the central nervous system (CNS). GABA also interacts with serotonin released from the DRN that modulates forebrain circuits involved in emotional states, sleep, motivation and aggression (Soiza-Reilly et al. 2013). In addition, GABA plays an equally critical role in the hippocampus and amygdala again acting as an inhibitory transmitter via ligand gated $GABA_A$ and G protein coupled $GABA_B$ receptors where it may facilitate emotional learning. Each of these receptor types mediates inhibitory postsynaptic transmission in the hippocampus (Isaacson et al. 1993).

Given its crucial role in inhibition and emotion regulation, GABA-containing neural pathways are a primary focus for the treatment of anxiety disorders. Moreover, dysregulation of the DRN has been implicated in the pathophysiology of affective disorders including anxiety and depression (Soiza-Reilly et al. 2013). As in the hippocampus, GABA and serotonin also target the excitatory signals in the amygdala hence reducing the physiological and psychological markers of stress and anxiety. Since the activity of inhibitory neurotransmitters is increased by anti-epileptic drugs (AEDs) it is believed that valproic acid, an AED will increase the inhibitory work of GABA

and serotonin, and decrease the excitatory work of glutamate in the DRN, hippocampus, and amygdala, regions of the brain highly involved in emotional processing in the CNS.

*Adrenocortical Response in Anxiety Disorders*

Of note, it is well known that psychological threats initiate dramatic changes in the endocrine system of the body as another method of cellular communication besides neurotransmitter stimulation. With the onset of anxiety disorders, aberrant stress responses are led by a network of neuroendocrine systems that are found both in the CNS and the periphery. The adrenocortical system originates in the hypothalamic (H) neuroendocrine cells, projecting to the neurohypophysis in the pituitary (P) and signaling the adrenal (A) cortex to release stress hormones. This HPA axis appears to perform three major functions in stress resistance. First, along with other hormones and systems, it participates in the mobilization of energy resources that are necessary for fight or flight. Second, cortisol serves a homeostatic function in regulating the activity of other sensitive systems. Third, cortisol, adrenocortical hormone (ACTH) and corticotrophin-releasing hormone (CRH or factor (CRF)) act in the brain to affect memory, learning and emotions (Stansbury et al. 2008). Recent research has shown that these hormones are secreted in increased amounts for individuals presenting with anxiety disorders (Tringali et al. 2004).

CRH is a 41-amino acid protein that is believed to play a crucial role in stress, anxiety, Alzheimer's and major depression (Dunn et al.1999, Lowry et al.2006, Stout et al.2002). CRH is a highly conserved neuropeptide hormone (Huising et al. 2004, Lovejoy et al. 2014) that is secreted in response to psychological threats. It is a metabotropic neuropeptide (Lovejoy et al. 2014) with two receptors $CRH_1$ (Gilmor et al. 2003, Künzel et al. 2003, Lovejoy et al. 2014 and Mazon et al. 2006, Varman et al. 2012) and $CRH_2$ (Gilmor et al. 2003, Lovejoy et al. 2014, Mazon et al. 2006,

Varman et al. 2012) found in the central nervous system and peripheral tissues (Gilmor et al. 2003, Lovejoy et al. 2014), with $CRH_1$ having higher selectivity for its ligand (Künzel et al. 2003, Lovejoy et al. 2014), which explains its coordination of the HPA stress response (Lovejoy et al. 2014, Pariante et al. 2008, Varman et al. 2012).

Anxiety can be a healthy or unhealthy response to stress, and it leads to enhanced CRF release from the hypothalamus and this, in turn, provokes ACTH release from the anterior pituitary. Under stressful conditions, the paraventricular nucleus of the hypothalamus secrete corticotropin-Releasing hormone (CRH), which, in turn, stimulates the efflux of adrenocorticotropic hormone (ACTH) that controls the production and release of corticosteroid hormones from the adrenal cortex (Dautzenberg, et al. 2001, Pariante et al. 2008, and Holsboer et al. 2008). These hormones are secreted in a series of steps with one initiating the secretion of the other, hence working together to cause numerous neuronal and hormonal changes (Pariante et al. 2008).

CRH is also believed to function as a neurotransmitter as it stimulates the brain and spinal cord to activate the sympathetic nervous system. It also functions as a mediator of stress-related behaviors as stress may lead to elevated secretions of CRH operates via a feedback look to alter future release; as such, altered functioning of this axis in response to stress may underlie anxiety disorder (Grammatopoulos et al. 2002, Lovejoy et al. 2014, and Pariante et al. 2008). Mutations (Dautzenberg et al. 2001). Sustained stress may lead to long term abnormal elevation of CRH with subsequent HPA axis hyperactivity, hence increasing the odds of promoting a heightened stress and high anxiety-like behavior profile (Grammatopoulos et al. 2002, Lovejoy et al. 2014, Pariante et al. 2008, Künzel et al 2003 and Dautzenberg et al. 2001).

In addition, the elevated secretions of CRH and glucocorticoids such as cortisol, their feedback and regulation are believed to be a classic test of HPA axis functioning in those presenting with anxiety disorders. Research has shown that anxiety disorders are associated with increased CRH (Tringali et al.2004) and decreased glucocorticoid receptors (Keck et al. 2005). Given that these hormones are secreted in a series of steps, changes along any region can influence an organism's capacity for managing future stress-provoking events. Stress leads to enhanced CRH release from the hypothalamus which provokes ACTH release from the anterior pituitary. The ACTH then travels through the blood stream to the adrenal cortex where it stimulates the synthesis and secretion of glucocorticoids. Along these lines, long term elevation of central CRH with subsequent HPA axis hyperactivity (Keck et al. 2005), increases anxiety susceptibility and promotes a heightened stress profile and/or instances of high anxiety-like behavior in animals (Ravenelle et al. 2013). In the current thesis, we investigated how changes in CRH protein levels may correspond to behavioral responses to anxiogenic stimuli as other research has implicated that CRH is affected by valproic acid (VPA) in regulating anxiety (Gilmor et al. 2003, Stout et al.2001, Tringali et al. 2004). For example, VPA alters CRH neuronal systems by increasing the molecular concentrations in the endocrine system. Furthermore, repeated administration of VPA has been reported to decrease receptor binding of CRH/$CRF_1$ in the amygdala (Varman et al. 2012), and CRH/$CRF_{2A}$ mRNA in the paraventricular nucleus of the hypothalamus (Gilmor et al. 2003, Stout et al. 2001, Tringali et al. 2004).

*Valproic Acid as a Novel Treatment for Anxiety*

Few studies have examined the combined effects of environmental enrichment and valproic acid treatment in combating anxiety and drug sensitivity. A variety of drug groups have been shown to be effective in treating many of the anxiety disorders, with selective serotonin reuptake

inhibitors (SSRIs) being considered first-line agents for virtually all anxiety disorders (Ameringen et al. 2004). Due to a clinical need for alternative drug treatments since not all patients achieve a complete response with SSRIs (Ameringen et al. 2004), other drugs such as valproic acid have been suggested. Valproic acid (VPA), the drug of focus for the current study, has been suggested to affect anxiety through its epigenetic action on histone deacetylase, thereby disrupting the balance between excitatory and inhibitory neuronal activities (Fukuchi et al. 2009). VPA, a deacetylase inhibitor, increases Histone 3 and Histone 4 acetylation, a known epigenetic mechanism that interacts with stress response proteins. Chronic stress affects the brain and influences the outcome of numerous diseases (i.e., neurodegenerative disorders) and contributes to pathological anxiety due to its ability to increase the secretion of excitatory neurotransmitters, but HDAC inhibitors may provide neuroprotection for these anxious animals. Recent studies have shown that HDAC inhibitors are effective for treating neurodegenerative disorders (Fukuchi et al. 2009, Machado-Vieira et al. 2011) and modulating stress and anxiety as they increase the balance between excitatory and inhibitory neural pathways in the brain. In addition to inhibiting HDAC, VPA has been suggested to increase the brain levels of GABA (Ameringen et al. 2004), and/or inhibit its catabolism in the CNS (Balfour et al. 1994) resulting in anxiolytic effect on behavior. Therefore, the ability of HDAC inhibitors to reverse dysfunctional excitatory-inhibitory regulation (Machado-Vieira et al. 2011), implicates VPA as a suitable therapeutic for the treatment of anxiety disorders. In an effort to explore the therapeutic actions of VPA on anxiety-like behavior, a second aim in this study is to investigate any cumulative advantage of VPA and environmental enrichment on the regulation of the expression of CRH protein.

*The Role of the Environment in Modulating Behavior*

Chronic treatment with anxiety drugs produces long lasting changes in the brain and on behavior. As such, the treatment of anxiety disorders should not only focus on drugs but also explore the impact of the environment and dynamic changes associated with re-training and other cognitive-behavioral interventions. A large number of studies have shown significant differences in animals' behavior associated with environmental enrichment living conditions. Environmental enrichment – the addition of tubes, ladders and running wheels in an enlarged home environment – enhances cognitive, motor and sensory function (Schloesser et al. 2010, Ravenelle et al 2013). This enrichment leads to better performance in various learning tasks, enhanced social play behavior (Morley-Fletcher et al. 2003), and lowers anxiety. Moreover, the enriched environment shows advantages over just social rearing and isolated conditions in reducing anxiety and psychostimulant locomotor sensitivity (Ravenelle et al. 2013) because animals are given a chance to interact, play and engage in sensorimotor enrichment activities in a social setting at all times in EE as opposed to those that are secluded or only have social contact. In addition, a study by Schneider et al. 2005 shows that the interaction with the toys by the animals reared in EE cages compared to SE reversed the harmful effects of prenatal VPA exposure (i.e., reversed pain sensitivity, lowered locomotor activity, enhanced exploratory activity, decreased anxiety and increased social behavior).

*Significance*

Anxiety disorders are among the most prevalent class of disorders contributing to loss of work, emotional challenges and significant comorbidity with substance abuse disorders (Kesler et al. 2005). Understanding the mechanisms that may successfully contribute to environmental modulation of anxiety disorders and/or comorbidity with substance abuse is of great importance.

While epigenetic mechanisms are indicated in mediating environmental enrichment effects such as modifying anxiety profiles, and implicated in modifying changes associated with stimulant drugs of abuse, no research has yet been done to establish the role of histone deacetylase alterations in the benefits of environmental enrichment in reversing anxiety and drug sensitivity. Since rats housed in extreme conditions (isolation) exhibit heightened anxiety levels as compared to those in EE, it was suspected that there would be an additive effect of VPA treatment and EE housing on reducing anxiety and stimulant drug sensitivity.

Experiments have also shown that improvements in behavioral performances were accompanied by a significant increase in cell survival in the limbic system (Schloesser et al. 2010). An investigation of "some brain structures implicated in anxiety such as the hippocampus, amygdala, and the paraventricular nucleus of the hypothalamus, and how these brain structures can be altered by different rearing conditions" (Chapillon et al. 1998) was carried out by measuring the level of stress-related hormone,CRH. It has been shown that stressful stimuli cause a release of CRH in the paraventricular nucleus of the hypothalamus, hippocampus and amygdala (Gilmor et al. 2003, Stout et al. 2001, Tringali et al. 2004). Interestingly, in the absence of stressful living conditioning, such as that created by enriching the environment, adding toys to cages, can similarly reduce anxiety-like behavior en par with antidepressant treatment. Moreover, both types of "dosing" reduce levels of CRF mRNA along the same brain regions implicated in stress. In the present study, we investigated if treatment with valproic acid (VPA) can lower anxiety level in trait anxiety rats, and how VPA may interact with environment (e.g., enriched (EE), standard (SE) and isolated environments (IE)). Since rats reared in IE exhibit heightened anxiety levels as compared to those in EE, we hypothesized that the combined treatment of enriching the environment (EE) and VPA treatment would lower the anxiety levels of HAn rats.

## Methods

*Animals and Vivarium Care*

Long Evans rats from filial 6 (generational line established at UMass Boston from selectively bred parental lines (F0) purchased from Charles River Breeding Labs, Wilmington, MA) were mated overnight and the morning when spermatozoa were found was designated as day one of gestation. All females were allowed to raise their own litters until postnatal day (PND) 21. The offspring were weaned on PND21, and housed separately according to sex. All of the current experiments were carried out on male offspring. All animals were housed in the vivarium of the University of Massachusetts Boston under a controlled temperature environment of $21\pm1^{\circ}C$ on a 12-h light cycle (on at 07:00h and off at 19:00h). The subjects had free access to food (standard laboratory pellets) and water, except when experiencing the 2wk valproic acid treatment in drinking water. All cages were changed weekly with minimum handling. All protocols followed federal guidelines for the use of live animals in research and were approved by the Institutional Animal Care and Use Committee.

*Selective Housing Conditions*

At weaning, a total of 57 male animals were separated into three different environments. 18 rats (9 from High anxiety, and 9 from Low Anxiety – see below for description of phenotyping protocol) were housed in two separate large cages under Enriched Environment (EE), 20 rats (10 from High anxiety, and 10 from Low Anxiety) were housed under Standard Environment (SE) with two rats per cage, 19 rats (10 from High anxiety, and 9 from Low Anxiety) were housed individually per cage under Isolated Environment (IE).

*Behavioral Testing Timeline*

An initial screen was completed using the elevated plus maze (EPM) that was performed on PND50 from which the animals were grouped into extreme trait anxiety level groups. We used the percent open arm (%OA) time and %OA entries on the EPM to measure the level of anxiety in Long Evans rats. We selectively bred non-sibling pairs of low (above median split/lower quartile, greater %OA time) and high (below median split/lower quartile) then randomly housed them in different enrichment environments according to anxiety level (as reported in detail in Ravenelle et al. 2013). Animals showing few %OA time and %OA entries (lower quartile) were designated as *high anxiety* (HAn) and those showing more percent open arm time and entries (upper quartile) were designated as *low anxiety* (LAn).

We used an AB design, getting pre-housing data on all behavioral assays and again after the valproic acid treatment (a new round of behavioral testing on the same measures). Each of the protocols listed below was repeated after the two-week VPA treatment regimen.

*Elevated Plus Maze*

The EPM apparatus used in this experiment was purchased from MED Associates (St. Alban, VT). It consisted of two opposite arms open arms and two opposite closed arms with the middle as the stage. The maze was elevated 70 cm above the floor in a room with 70-lux illumination. Each rat was placed on the junction of the maze alternating the direction the animal was facing, and given a chance to move about the maze in a closed room. The experimental procedure was similar to that described by Ravenelle et al. (2013). Rats were habituated for 15 minutes prior to EPM testing, and the cages were thoroughly cleaned with warm soapy (mild detergent) water. This testing was repeated after a two-week valproic acid in drinking water protocol (see below).

*Open Field Activity*

On PND80, a re-test on EPM was completed and PND239 through PND240 the animals underwent Open Field Testing (OFT) to measure their exploratory behavior. The OFT apparatus used in this experiment was made of four square boxes, elevated 70cm above the floor. We collected data on distance traveled (cm), time spent moving and rearing, and change in activity over 60 minutes were recorded.

*Amphetamine-Induced Locomotor Activity*

Three weeks later, the rats were tested for locomotor response to low dose-amphetamine in locomotor activity cages equipped with photocells and purchased from MED Associates (St. Alban, VT). It was made of four transparent open Plexiglas boxes and automated to a PC to collect whole-body movement and rearing behavior. On a testing day, four rats were placed in room adjacent to the LMA apparatus, then after, they were habituation for 30 min on the test day prior to amphetamine injection (0.5mg/kg, IP). They were then placed back in the LMA boxes for 60 minutes to measure their activity in response to drug treatment

*Valproic Acid (VPA) in Drinking Water and Post-VPA Testing*

Three weeks after amphetamine-induced locomotor activity testing, subjects were given valproic acid (VPA). VPA (4mg/ml) was dissolved in water and administered to the test subjects through drinking water, which was changed on a weekly basis. This treatment went on for 14 days. After 14 days, post-treatment anxiety-like behavior was measured again on all behavioral tests: EPM for 5 min, after which animals were tested in the OFA for 5 min. On another test day, animals received injection of amphetamine (0.5 mg/kg) for locomotor activity testing. Prior to testing, the rats were given 15 min of habituation in a secluded and quiet environment. After the culmination of all behavioral tests, the animals were sacrificed using an overdose of Fatal Plus (sodium

pentobarbital mixture) and transcardially perfused with isotonic saline (0.9%) followed by 4% paraformaldehyde.

*Immunohistochemistry*

Following the perfusions, brains were extracted and post-fixed further in 4% paraformaldehyde and later cryoprotected in 10% sucrose, 4% paraformaldehyde followed by 20% sucrose, 4% paraformaldehyde. After blocking, brain tissue was then frozen micro sectioned using an automated cryostat; tissue was stored until time of immunohistochemical protocol, then it was incubated in CRH antibody (polyclonal (p)AB) that was purchased from Phoenix Pharmaceuticals. The dilution factor used in this research was 1:1000 The IHC protocol took about two days with the first day including thoroughly washing the brain tissue in 6 flushes of 0.05M sodium phosphate buffered saline (NaPBS) for 10 min each. This was followed by 1 h incubation of the brain tissue in the primary pAB in NaPBS + 0.4% Triton-X at room temperature. Tissue was then stored in for 48 h under $4^0$C, then the pAB was washed out thoroughly using 0.05 NaPBS in 6 flushes for 10 min each. This was followed by incubation for 1 h in secondary AB, and then flushed five times in NaPBS for 10 min each. Tissue was then incubated in A/B solution for 1 h, followed by 3 washes for 5 min in NaPBS first then rinsed 3 times in Tris-buffered saline (TBS) for 5 min each. Brain tissue was then incubated in diaminobenzidine solution, rinsed three times in TBS for 5 min each, followed by a 3 rinses in NaPBS for 5 min each. After IHC, the tissue sections were mounted on gelatin-coated slides and cover-slipped and examined under the microscope for final analysis. Scion image analysis was used to capture the region of interest and then tissue was analyzed using NIH ImageJ software.

*Data Analysis*

We analyzed the data using group means ± S.E.M of the average response of subjects tested. Statistical analyses were carried out with Graph Pad Prism (v. 12.0, San Diego, CA). Behavioral measures of both trait anxiety levels (Han and LAn) were examined on the EPM on the basis of the number of entries in the open (OA) and closed arms (CA). From these measurements, the %time spent on OA was calculated as follows: OA time spent/OA time spent + CA time spent. For the LMA and OFA novel tests, the average distance traveled was measured at two time-points, once active a minimum of 40 days in select housing, designated post-housing, and again after post-valproic acid treatment. These measurements evaluated the effects of environmental enrichment alone, valproic acid treatment and the interaction with the environment. We set the alpha at $p<0.05$for all analyses throughout the study.

**Results**

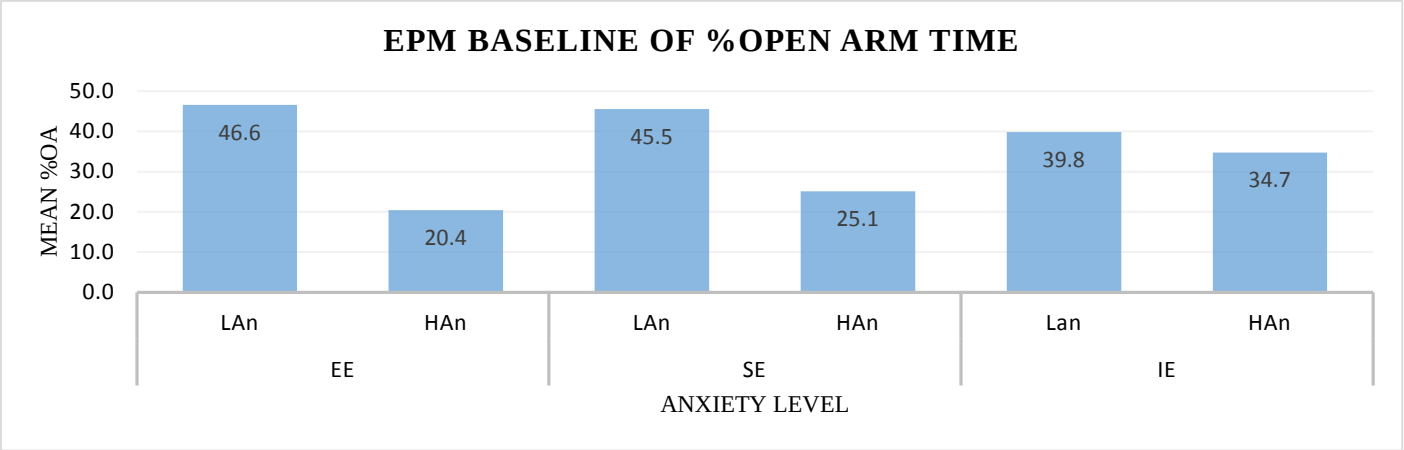



***Figure 1A.*** The graph represents anxiety-like behavior (ALB) of F8 male rats before select housing. The LAn animals consistently showed greater percent time on the open arms (%OA), lower ALB.

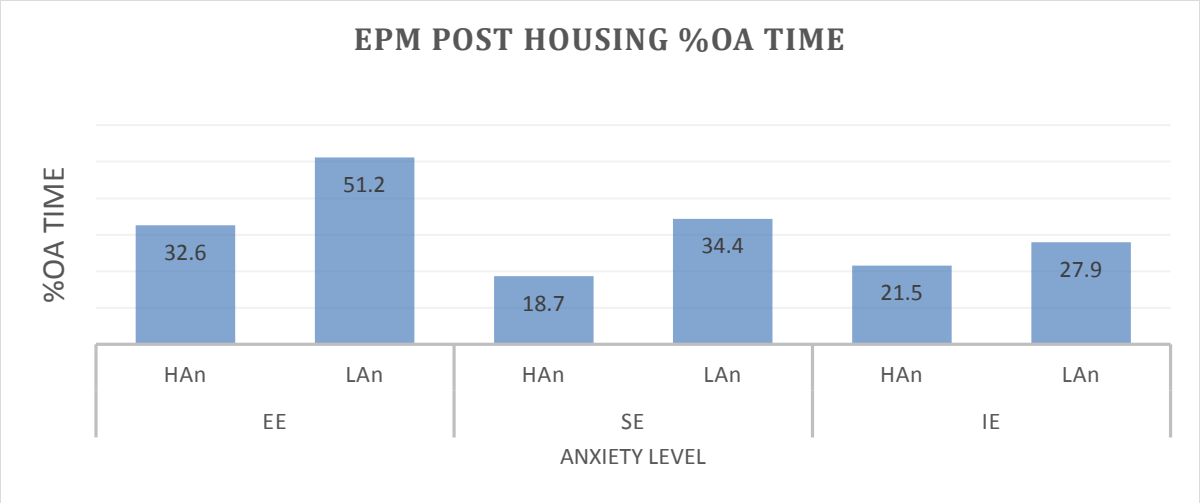



***Figure 1B***. A bar graph of anxiety-like behavior (ALB) of F8 male rats after housing conditions, illustrating that environmental enrichment (EE)-only decreased ALB, i.e. greater %OA time.

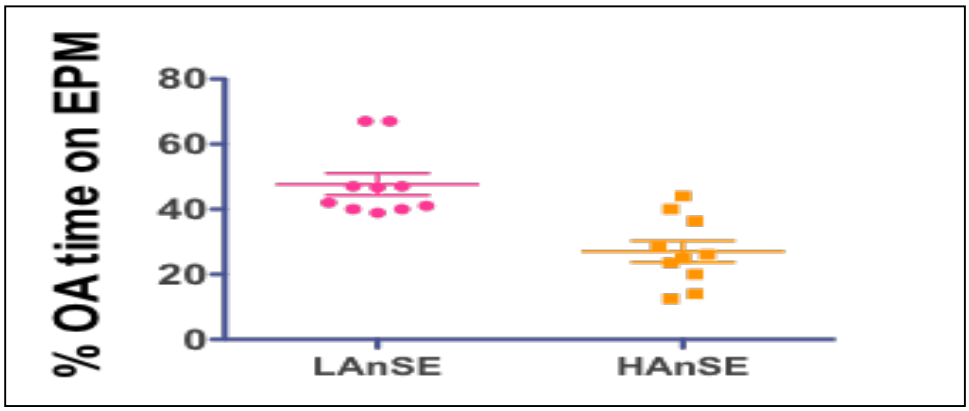
*Elevated Plus Maze – Baseline*

Before housing conditions (Figure 1A), rats were tested on the EPM to allocate them according to their anxiety level. High anxiety rats showed the least amount of time and activity on the open arms. High anxiety rats spent 35% or less in the open arm as shown on the graph, while Low Anxiety rats spent 35% and more on the open arm. After housing, we examined the behavior of the same rats on the same EPM apparatus. Bar Graph B shows that post housing, the anxiety trait was expressed at higher levels in the IE housing as compared to the other housing conditions. In SE, although not as high as IE, the anxiety level is expressed at a less % time than in EE. EE rats spent in total ≈ 16% more time on the OA after housing than baseline. IE rats spent in total spent ≈ 25% less time on the OA after Isolation housing conditions as opposed to baseline. SE rats spent ≈ 18% less time on the OA after housing conditions.

To evaluate the effects of rearing environment only on trait anxiety, we analyzed activity on the EPM, and found that Han SE rats spent slightly more time on the OA than LAn IE, suggesting that social housing improves anxiety levels (Figs. 1A and 1B above). In addition, a

significant difference in the Trait effect was observed with LAn IE spending more %time on the OA than HAn IE. High anxiety rats reared in SE and EE were not very different in the %time spent on the OA. As expected, LAn EE subjects spent significantly more % time on the OA than the rest of the test subjects under different housing conditions. Overall, EE reared rats spent more %time on the OA than rats from other housing environments (Fig 1B, 2B).

**A.**




**B.**

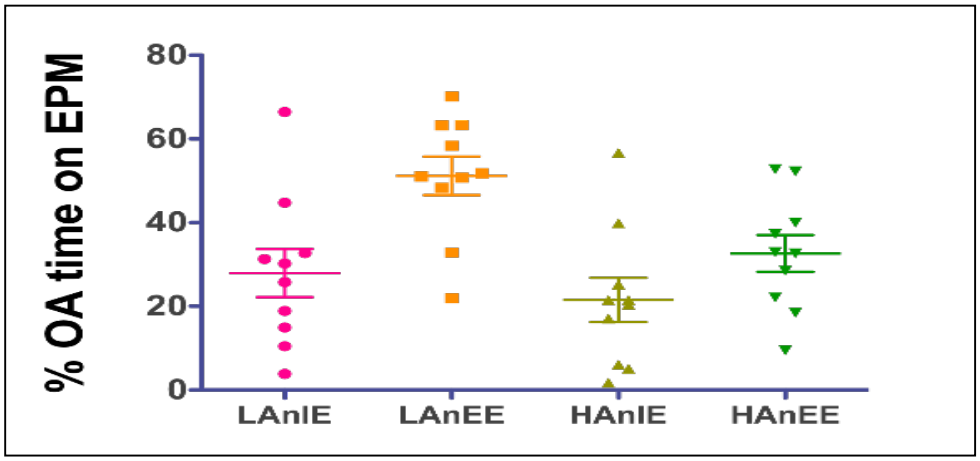
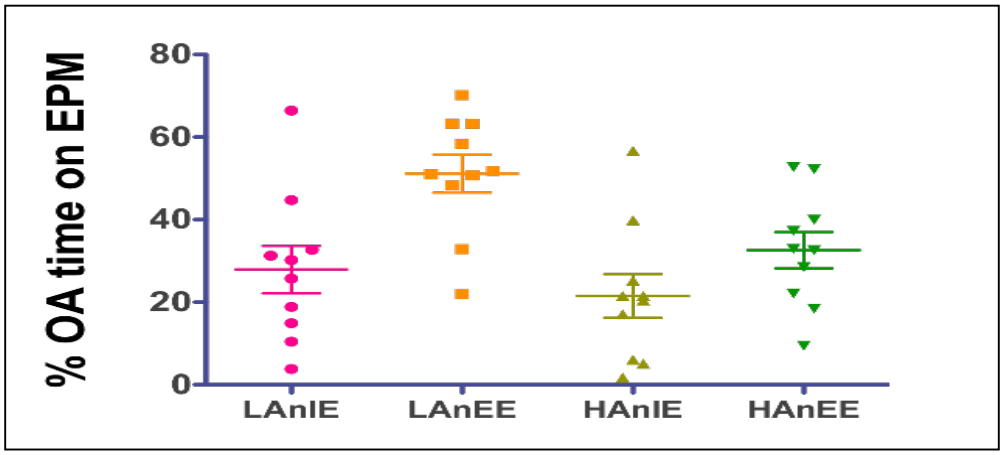



***Fig. 2A-B*** *Scatterplot of anxiety-like behavior (ALB) of F8 male rats following select housing conditions, pre-VPA.* LAn SE (A) rats spent ~42%, LAn IE (B) rats spent ~25%, and LAn EE (B) rats spent ~50% of their time on the OA. HAn EE (B) rats spent ~36%, HAn IE spent ~20%, and HAn SE spent ~35% of their time on the OA.

**A.**




**B.**

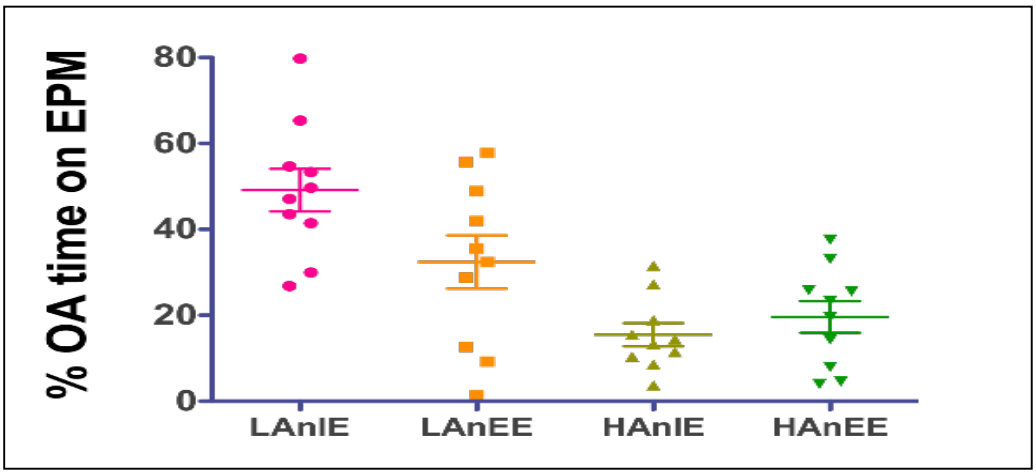


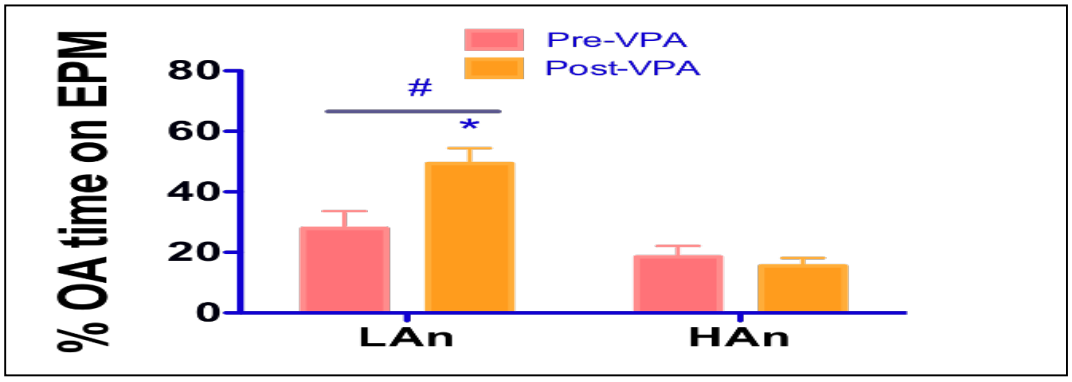

***Fig. 3A-.B*** Scatterplot of F8 ALB in rats from LAn/HAn lines and housed in IE and EE pre-VPA **(A)** and post-VPA **(B)**. VPA reversed ALB for LAn groups and increased ALB for HAn.

*Elevated Plus Maze – Post-Valproic Acid Treatment*

We measured the effect of VPA treatment on the behavior of the test subjects using the EPM, comparing this to pre-VPA responses. Data from Figure 3A demonstrate that LAn EE and HAn EE subjects had a significant increase in %OA time *before* VPA treatment, suggesting EE decreased anxiety-like behavior. However, after VPA treatment, these patterns were reversed; LAn EE and HAn EE showed more anxiety-like behavior (i.e., had less %OA time). By contrast, post-

VPA, a substantial increase in %OA time was observed in the LAn IE (Fig. 3B). Although there was a decreasing trend in %OA time of the HAn rats, HAn EE rats explored the OA more than the HAn IE post-VPA. Figure 3A-B below shows the bar graphs comparing average %OA time for animals pre (salmon-colored bar) and post (orange) VPA in animals reared in IE (Fig. 3A) and EE (Fig. 3B). Rats in IE spent less % time on the OA before treatment, as opposed to rats in EE. LAn animals of both housing conditions had extreme differences in their behavior on the OA before and after treatment. Before exposure to VPA, animals in the EE (Fig. 4B) spent more time on the open arms, after exposure, the same animals (both LAn and Han) become less active on the OA. IE animals (Fig. 4A), however, spent more time on the OA after valproic acid treatment in drinking water.

**A.**




**B.**

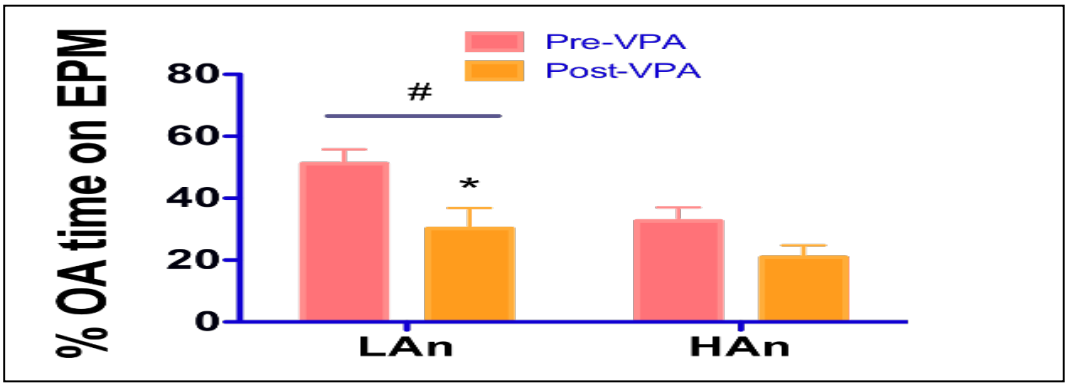
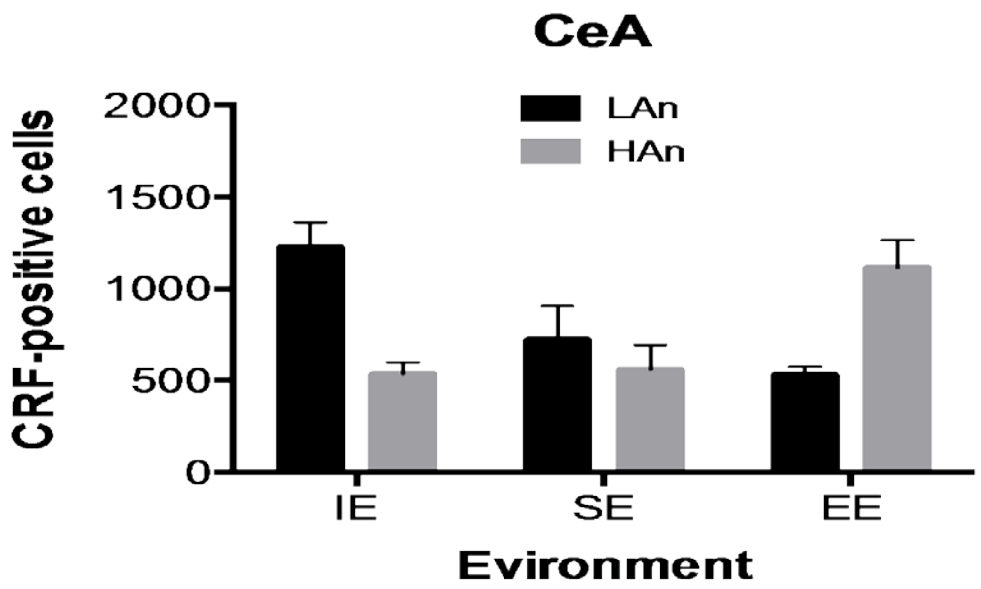


***Fig. 4A-B*** *Bar graph of %OA in LAn/HAn lines for pre- and post-VPA after IE-housing* ***(A)*** *and after EE-housing* ***(B)***. We found a significant effect of housing and treatment (*p<0.05, compared to pre-VPA, same trait; #p<0.05, LAn v. HAn).

**A.**




**B.**

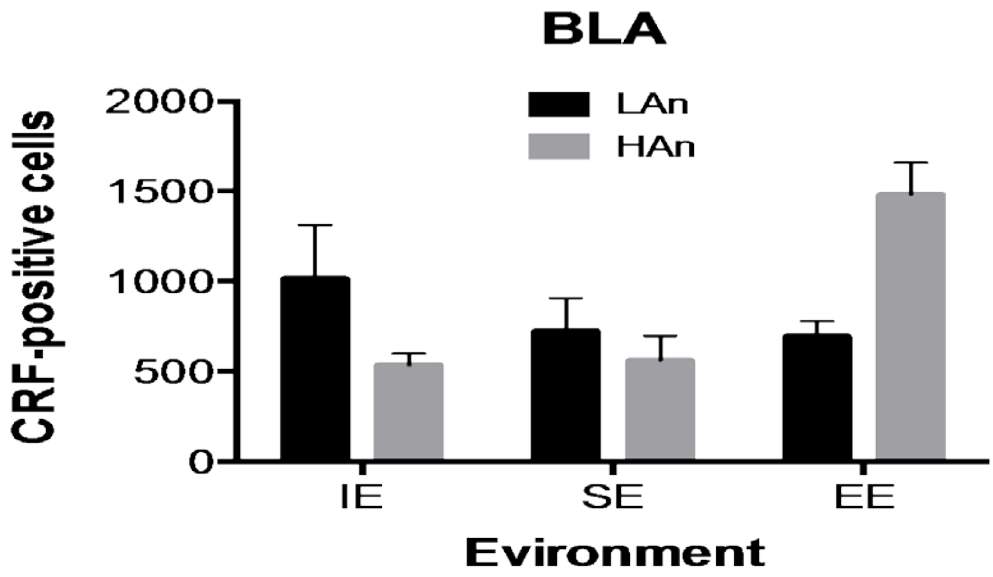


***Fig. 5A-B.*** *Bar graph of immunohistochemistry results for the central amygdala (3A) and basolateral amygdala (3B). A significant Trait* x *Environment effect indicated greater CRH-ir in the LAn IE and HAn EE rats for CeA and BLA ($p<0.01$).*

*Effect of Valproic Acid on Corticotropin Releasing Hormone.*

To analyze the effects of valproic acid on stress hormones, we measured the level of CRH in the different brain regions including the central amygdala (CeA) and basolateral amygdala (BLA). As shown in Fig. 5A, there was a noticeable difference in the expression of CRH hormone in the amygdala across the different housing settings. Our analysis revealed that the level of CRH-positive cells was significantly decreased in LAn EE and increased in HAn EE housed Long Evan rats. Within the IE housed rats, there were more CRH-positive cells in the LAn IE group as opposed to HAn IE. In both brain regions, SE housed Long Evan rats did not show a significant change in CRH-positive cell counts, with LAn SE having slightly more CRH cells than HAn SE. Overall, comparisons between the housing conditions showed that there were more CRH-positive cell counts in LAn IE housed rats in the CeA, as compared to BLA, which indicated more CRH-positive cells in HAn EE. SE housed rats did not shift much in the CRH cell counts in either brain region.

In the hypothalamus, CRH protein levels were significantly increased in HAn EE Long Evan Rats as opposed to other housing groups. In contrast, there was a very noticeable difference between LAn EE and Han EE, as the former had significantly less CRH levels measured. As with the amygdala, SE housed Long Evan rats did not show much difference in the level of CRH across anxiety levels in the hypothalamus. LAn SE had slightly more levels of CRH measured in the hypothalamus than HAn SE. In addition, LAn IE had more CRF-positive cells than HAn IE in the hypothalamus. CRH-positive cells observed from the hippocampus were not as diverse as the

results from the amygdala and hypothalamus across housing groups, but a significant difference was observed when anxiety levels were compared within their distinct housing environments. Almost all HAn trait Long Evan Rats had lower levels of CRH-positive cells measured compared to their colleagues with Low Anxiety trait within the same housing environment. An exception from the above statement was observed in SE housed Long Evan rats, which showed more CRH-positive cells in High Anxiety Long Evan Rats. In comparison to IE, LAn EE had significantly increased CRH-positive cell counts as opposed to LAn IE and HAn IE housed Long Evan rats. In the hippocampus, SE housed Long Evan Rats showed a significant change in CRH-positive cell counts, with HAn SE having more CRH-positive cells measured, than in LAn SE Long Evan Rats.

## Discussion

Results from our experiment demonstrated that environmental enrichment has a positive effect on 9$^{th}$ generation, Long Evans male rats selectively bred for trait anxiety. We demonstrated that the enriched environment reversed the high anxiety behavioral and neural responses to anxiogenic stimuli (Ravenelle et al. 2013). In addition, our results show that treatment with VPA, presumably acting as a histone deacetylase (HDAC) inhibitor, conferred similar benefits for the animals reared in the isolated environment. Finally, given the coordinating role of the CRH in mammalian stress response, our investigations suggest that brain changes in CRH protein expression following VPA treatment may underlie the benefits to anxiety of this treatment. VPA has been shown to induce epigenetic action that includes interactions with brain CRH neuronal systems. Our findings further implicate this phenomenon as correlative CRH protein level changes in hypothalamic and extra-hypothalamic systems accompanied the modification(s) in VPA-treated rats reared in isolated environment as opposed to those that are already enriched (i.e., housed in EE)

*Environmental conditions effect elevated plus maze activity*

Prior to environmental housing, the test subjects were selectively separated based on a median split separating lower quartile %OA (HAn) and higher quartile % OA (LAn). In general, HAn subjects showed significantly less activity on the open arm as opposed to LAn subjects before housing, after housing a reverse was observed. It was also observed, however, that both LAn and HAn of IE decreased in activity tremendously after housing indicating an increase in anxiety level. High Anxiety EE animals showed significantly less activity on the OA of EPM before housing averaging 20 %; after housing, we observe that the trend significantly increases from 20% to 51.2% OA time. In addition, SE Han animals showed a significant increase in % OA after housing from 25% to 35%. This trend demonstrates the significant impact of environment to the anxiety trait. In summary, rearing conditions in this experiment exerted an influence on behavior on the EPM. Our results were consistent with those of other studies when we examined the locomotor activity on the elevated plus maze following environmental enrichment since Ravenelle et al. (2013) found that EE conditions increased the percent open arm exploration as well as SE conditions, as opposed to IE.

Enriching the environment with toys, ladders and socializing the test subjects provided them an opportunity to interact and exercise all of which have been shown to improve the anxiety and stress levels in affected subjects. This exposure to numerous activities works as a medium of eliminating stressful anticipations hence motivating the animals to explore potentially stressful environments such as OA on the EPM. The lack of sensorimotor, visual and social exposure in isolated environments may explain the observed tremendous decrease in both LAn and Han exploration of OA after housing. The rats in the IE did not have toys, ladders or fellow rats to interact with and keep them busy hence increasing their stress and anxiety levels. This is observed

when the baseline EPM %OA time decreases from 40% in LAn and 35% HAn to 22% and 28% respectively.

*Valproic acid interaction with the environment and resulting effects on trait anxiety*

In this research, we also examined the combined effect of environmental enrichment and drug treatment in combating anxiety-like behavior in rats. Our findings from this research were incompatible with other studies suggesting an increase in locomotor activity in anxiety trait rats after treatment with VPA. Given that VPA is an HDAC inhibitor, we speculated that Valproic acid would interact with the environment and affect the stress response system by altering the CRH systems for the anxious Long Evans rats reared in isolation. There is supporting evidence in VPA rats (rats treated prenatally with VPA on gestation day 12) are more anxious after environmental enrichment have lower locomotor activity, enhanced exploratory activity and decreased anxiety (Schneider et al. 2006; 2007; Ravenelle et al. 2013).

Behavioral results from our research, however, show that HDAC alterations do not benefit environmental enrichment in reversing anxiety-like behavior. Before VPA treatment, EE rats had a significant increase in %OA time, suggesting the successful effect of the enrichment on decreasing anxiety-like behavior. However after VPA treatment, these patterns reversed as both LAn EE and HAn EE decreased in %OA time, demonstrating an increase in anxiety level in the Long Evans rats used in this experiment. It is, however, interesting to note that the rats that were not enriched or reared in a social environment (IE) benefitted from VPA treatment. IE animals spent more time on the OA after exposure to VPA, hence showing that the anxiety-like behavior of the rats was reduced, but at a very low rate.

*Valproic acid interaction with the environment and resulting effects on CRH proteins*

Our suspicion that the combined treatment of VPA and EE would lower anxiety levels of HAn rats was challenged. However, we did speculate that VPA and EE likely impacted protein expression of CRH in brain areas important for anxiety. Analysis of post-mortem brains after VPA treatment showed increased levels of CRH protein concentrations in multiple brain regions of HAn EE housed animals, an indication of increased anxiety as was observed in our behavioral tests. CRH is a neuropeptide mediator of the stress axis and physiological responses in the central nervous system, and it is secreted by the neurons in the paraventricular nucleus of the hypothalamus, amygdala and hippocampus. Clinically, CRH protein levels are increased in individuals presenting with behavioral disorders including anxiety, and associated symptoms can be reversed with treatment such as valproic acid. Valproic acid treatment, commonly used to treat epileptic patients, and a novel treatment for anxiety and depression, was also successful in improving anxiety in our LAn lines, which are also extreme anxiety animals since low anxiety is abnormal and can adversely impact survival of animals. VPA was not successful in ameliorating anxiety responses in the other extreme, our HAn lines, though it was successful for isolated environment (IE) Long Evans rats in this study. From these findings, it would be safe to report that both environmental enrichment and valproic acid are good treatments for anxiety disorders, but not when used as co-treatments, particularly for those suffering from high anxiety. Based on the brain analysis, it would be safe to add that if the combined treatment is considered, it might be beneficial in treating low or moderate anxiety levels at a small scale. It is also important to report that our findings may differ from other research results, further highlighting an important therapeutic role for measuring *pre-existing anxiety levels* prior to treatment. For example, Schneider et al. (2007) findings show that the combination of VPA and enriched environment combat anxiety like behavior, while our results show the opposite. This difference may be due to

the fact that our research deals with postnatal treatment administered through drinking water, while in the study by Schneider et al. (2007) the authors administered VPA during embryogenesis, and that the authors did not determine pre-existing anxiety levels before treatment.

## Conclusion

In summary, we found that Environment Enrichment may serve as a novel therapy and treatment for anxiety-like behavior. These findings are in support of several recent studies that reported an increase in EPM activity of HAn EE long Evan Rats (Ravenelle et al. 2013,) signifying reduced indices of anxiety (Varman et al. 2012). Findings in the present study provide evidence that administration of Valproic Acid may serve as a probable treatment for Lowered anxiety-like behavior in IE animals. This effect was however not observed when Valproic acid and EE were combined-there was no additive effect of combining VPA and EE treatment. The CRF/CRH-mRNA levels in the in the LAn IE measured on the low end, while high levels of CRF/CRH-mRNA expression was observed in HAn EE housed Long Evan rats after the combination. It is possible that the mode of administration of VPA may have contributed to discrepancy observed. It was administered through oral administration in solution (dissolved in water), which is prone to Fast-past effect. Although pain-free, only part of the drug may have been absorbed hence explain the trend in EPM behavior, and CRF/CRH-mRNA observed. May be if the drug was administered through intracranial mode of administration, or if the concentration of the drug was different, our hypothesis of seeing additive effect of combining the two treatments may have been supported, but this is entirely a different procedure all together which was not the focus of this research, but would make a great further study.

From the present study the combination of Valproic acid treatment and environment enrichment, and how they affected CRH-mRNA expression in the examined brain regions shows

that combining VPA and EE treatment for anxiety-like behavior may not be of additive effect for therapeutic purposes, but it may be beneficial in cases of low or moderate anxiety-like behavior. A better understanding of effective mechanisms of administration, and time of treatment on how VPA may alter anxiety-like behavior, will be required to achieve this goal.

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