Role of Glutamate Transporter EAAT2 in Stress-Induced Depression

Undergraduate Research Thesis

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by

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Abstract:

To date, drug treatments for Major Depressive Disorder (MDD) have focused mainly on monoamine neurotransmitters such as noradrenaline and serotonin. However, monoaminergic antidepressants require long therapeutic latency and do not work for some subpopulations of patients. Therefore there is a pressing need to develop more effective means for treating MDD. Several lines of evidence indicate that dysfunction of the glutamatergic system plays a critical role in the pathogenesis of depression. Repeated stress results in an increase in extracellular glutamate and the overall dyshomeostasis of the glutamatergic system, which may lead to MDD. Glutamate transporter EAAT2 is primarily expressed in astrocytes and is mainly responsible for glutamate homeostasis in the forebrain. This study seeks to determine if increased EAAT2 expression could reduce susceptibility to depression due to unpredictable chronic mild stress. EAAT2 transgenic mice, which express ~ 2 fold more EAAT2 protein, were used in this study. EAAT2 mice and their wild-type littermates undergo a month of unpredictable chronic stress. During this month, weekly behavioral analyses including sucrose preference tests were performed to analyze the extent of depression-like symptoms. After one month of chronic stress, other behavioral tests including open field, novelty-suppressed feeding, and forced swim test were conducted. Following behavioral tests, brains and adrenal glands were harvested to determine the weights of the adrenal glands and EAAT2 protein expression in the prefrontal cortex by Western blot analysis. Results show no significant differences in all behavioral tests except for coat state assessment. There were also no significant differences between testing conditions in the weight of adrenal glands. EAAT2 expression was not significantly different between conditions within each mouse strain. This evidence suggests that we were not successful

in producing depression-like symptomology with the unpredictable chronic mild stress model. Proposed future studies would use a modified unpredictable mild stress model with different stressors for increased durations as well as a different mouse strain that would be treated with a small molecule compound, LDN/OSU-0212320, which our lab has shown in previous experiments to increase the expression of EAAT2.

Introduction:

An estimated 12.8~16.6% of people in the United States and in Europe will experience at least one Major Depressive Episode (MDE) within their lifetime (Alonso et al. 2004; Kessler et al. 2005). Major Depressive Disorder (MDD) is characterized by the occurrence of at least one MDE with the presence of at least five symptoms listed in Fig 1 (page 12) for at least two weeks (American Psychiatric Association). Common symptoms include 1) depressed mood for most of the day, nearly every day, 2) markedly diminished interest or pleasure in all or almost all activities, and 3) significant weight loss or weight gain. MDD is less likely to be seen until early teens and the overall prevalence of the disease is about 16.6%, with younger adults having the steepest prevalence curve (Kessler et al. 2005; Kessler et al. 2003). Currently, the pathogenesis of MDD is not completely understood and the development of MDD is variable. Some people develop depression when exposed to chronic stressful situations while others do not. This difference is due to many other vulnerability factors that are not well understood. To date, research and drug treatment have focused on monoamine neurotransmitters, such as noradrenaline and serotonin. However, these drug treatments exhibit long therapeutic latency and do not work for all patient subpopulations, as a reported 30% of patients given current antidepressant treatments do not respond to treatment (Rush et al. 2006; Paul and Skolnick 2003).

While we do not currently understand the pathogenesis of depression, past studies provide an insight on how the glutamate system may be involved. Glutamate is the major excitatory neurotransmitter and is found in nearly all parts of the brain. In brain image studies, MDD patients show decreased activity in the prefrontal cortex (PFC), an executive brain center,

and increased activity in the amygdala and the anterior cingulate cortex, which are involved in mood modulation (Hamilton et al. 2012; Siegle et al. 2007). These changes in activity at the systems level correspond to changes in neuronal architecture at the single cell level, including altered dendritic arborization and spine morphology/density. Along with neuronal changes, postmortem studies have shown decreased astrocyte number in those diagnosed with mood disorders (Rajkowska and Stockmeier 2013; Sanacora and Banasr 2013). These changes may be due to the effect of glucocorticoids on the glutamatergic system. Stressful events trigger the release of glucocorticoids from the adrenal glands that then bind to glucocorticoid and mineralcorticoid receptors on presynaptic glutamate neurons all over the brain including in the prefrontal cortex, hippocampus, and amygdala (Popoli et al. 2012; Musazzi et al. 2013). Binding of these glucocorticoids to receptors causes depolarization-evoked release of glutamate (Popoli et al. 2012; Moghaddam 1993). However, repeated unpredictable stress causes not only glutamate release but also decreased extracellular glutamate uptake due to reduced expression of Excitatory Amino Acid Transporters (EAATs), which regulate the homeostasis of extracellular glutamate levels (Popoli 2012 et al.; Zink et al. 2010). These together (increased glutamate release and decreased glutamate uptake) result in an increase in extracellular glutamate level. Excess glutamate can cause excitotoxicity which may lead to the neuronal architecture changes seen in the post-mortem tissue of MDD patients (Musazzi et al. 2013). Similarly, the glutamate system is the primary regulator of the stress hormone, corticosterone. High levels of corticosterone are indicators of stress and anxiety. Therefore, chronic unpredictable stress or depression results in decreased EAAT2 expression, high levels of corticosterone, and decreased

synaptic density and astrocyte number (Chen et al. 2014; Choudary et al. 2005; Liu et al. 2014; Miguel-Hidalgo et al. 2010).

Due to these changes in the brain and the possible implication of glutamate, researchers have begun to look at different glutamate receptors as potential targets for therapeutics. One of these targets includes N-methyl-D-aspartate receptor (NMDA receptor) antagonists such as MK-801 and CGP 37849. These compounds had antidepressant effects; however, they can lead to other problems with learning and memory over time (Papp and Moryl 1994; Kovacic and Somanathan 2010). Recently, ketamine, a NMDA receptor antagonist that is usually used as an anesthetic agent, has been shown to decrease symptoms of depression with almost no therapeutic latency and could help those unaffected by other treatments (Serafini et al. 2014; Zarate et al. 2006). However, ketamine has been shown to have transient effects immediately following treatment including changes in blood pressure, changes in heart rate, sensory disturbances, decreased mental capacity, dizziness, blurred vision, and headaches as well as unknown long-term effects (Zarate et al. 2006; Rot et al. 2009). Other studied targets include glutamate metabotropic receptors (mGluRs). Both Group I (mGluR1 and mGluR5) and Group II (mGluR2 and mGluR3) glutamate metabotropic receptors have been targets for therapeutics; antagonists to both groups have had positive results in terms of decreasing immobility in rodent forced swim and tail suspension tests (Pytka et al. 2016). Basimglurant, an mGlu5 receptor antagonist, has been shown to have antidepressant effect in rats, and was recently studied in a clinical trial that showed increased antidepressant results when in conjunction with other antidepressant treatments such as selective serotonin reuptake inhibitors (Palucha et al. 2005; Quiroz et al. 2015). While all this research has shown the impact that the glutamate system may have on the

pathogenesis of depression, there is still much to be done in this field in terms of finding the best treatment for all patients. Rather than treating patients with antagonists that completely turn off parts of the glutamate system, it may be beneficial to look at the maintenance system of glutamate as a potential target.

Glutamate transporter EAAT2 (Excitatory Amino Acid Transporter 2) is primarily expressed in astrocytes and is mainly responsible for glutamate homeostasis in the forebrain (Bjørnsen et al. 2014; Rothstein et al. 1996; Tanaka et al. 1997). The dysfunction of a single astrocyte could impair glutamate removal at thousands of synapses. As mentioned above, excess glutamate in the synaptic cleft can diffuse extra-synaptically and activate other glutamate receptors, which can cause over-stimulation and lead to neuronal death. Prior experiments have shown that the loss or the blocking of EAAT2 function results in anhedonia (loss of pleasure in usually enjoyed experiences), anxiety behaviors, and rapid eye movement (REM) sleep deficits, which are all symptoms of depression (Bechtholt-Gompf et al. 2010; Cui et al. 2014; John et al. 2012; Lee et al. 2007). In addition, past studies have shown that chronic antidepressant treatment induces higher EAAT2 expression, which may be part of its therapeutic effects (Chen et al. 2014; Zink et al. 2011). These findings indicate that there is a negative correlation between EAAT2 expression and depressive symptoms and suggest that the loss of EAAT2 may contribute to depressive symptoms.

Therefore, it may be possible that enhanced EAAT2 expression could prevent the development of depressive symptoms and/or ameliorate depressive symptoms. A study has demonstrated that ceftriaxone, which can increase EAAT2 protein levels via transcriptional activation, reduces helplessness behavior in the forced swimming and tail suspension tests in

mice (Mineur et al. 2007). Although other features of depression were not addressed—specifically anhedonic behavior, a core symptom of depression—this study suggests that enhanced EAAT2 expression could be a potential therapeutic target for depression. Therefore, further detailed investigations are needed. The objective of this study is to investigate whether increased EAAT2 expression could prevent the development of chronic stress-induced depression. In this study, I used an EAAT2 transgenic mouse line that was previously generated in our laboratory to address this question (Guo et al. 2003). These EAAT2 transgenic mice express ~1.5-2 fold more EAAT2 (Guo et al. 2003). If this study is successful, we will use a novel brain-penetrant small molecule LDN/OSU-0212320—recently developed by our laboratory (Kong et al. 2014)–to investigate if drug-induced EAAT2 expression can ameliorate depressive symptoms when depression is already present and whether increased EAAT2 can restore pathological changes caused by chronic stress.

Modeling MDD and MDE symptomology in rodents can be done in several ways. The most used animal models of depression based on experimental manipulations are the repeated maternal separation, learned helplessness, social defeat, unpredictable chronic mild stress, and chronic corticosterone models (Belzung 2014). There are also behavioral analyses that are designed to measure specific symptoms that are seen in depression such as resignation (helplessness), lack of motivation, anhedonia, anxiety, and irritability (Belzung 2014). For this study, we determined that the unpredictable chronic mild stress model was most useful for our purposes. This model was first created in the early 1980s and uses several mild stressors (food and water deprivation, changes in light cycle) in an unpredictable manner over a significant duration to view the anhedonic symptomology seen in depressed human patients (Slattery and

Cryan 2014). Likewise, this model, as compared to other animal models of depression, is more realistic in terms of modeling how chronic stress (a multitude of small events rather than one large stressful event) can lead to MDD (Wilner et al 1992). The results seen from this model are a decrease in sucrose preference—a test that measures anhedonia—and increased immobility for the forced swim test—a measure of resignation or helplessness (Streklova et al., 2004). Therefore, we hypothesize that this model will allow us to reproduce depression-like symptoms in wild-type stressed mice while our EAAT2 transgenic mice will develop little or no such symptoms as a result of the neuroprotectiveness of EAAT2.

Diagnostic Criteria

A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.
   - **Note:** Do not include symptoms that are clearly attributable to another medical condition.
   1. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad, empty, hopeless) or observation made by others (e.g., appears tearful). (**Note:** In children and adolescents, can be irritable mood.)
   2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).
   3. Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day. (**Note:** In children, consider failure to make expected weight gain.)
   4. Insomnia or hypersomnia nearly every day.
   5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).
   6. Fatigue or loss of energy nearly every day.
   7. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).
   8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).
   9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Fig 1: Above is the list of possible symptoms for diagnosis of Major Depressive Disorder as seen in the Diagnostic and Statistical Manual for Mental Disorders, Fifth Edition (DSM V). (American Psychiatric Association).

Research Overview:

Preliminary Studies:

Prior to beginning the unpredictable chronic mild stress study, we tested EAAT2 transgenic mice and their wild-type littermates (FVB/N strain) under forced swim test conditions to assess if increased EAAT2 could reduce helplessness behavior. As shown in Fig 2A, we found that EAAT2 mice had significantly lower scores of immobility and higher scores in swimming as compared to their wild-type counterparts (n=7; Two-way ANOVA: $F(2,36)=11.25$, $p=0.0002$; Sidak's multiple comparisons test: $p=0.0054$ for both swimming and immobility). In addition, we also performed a forced swim test using wild-type mice after daily treatment with 40 mg/kg of LDN/OSU-0212320 for three days as well as with vehicle-treated littermates (n=14). Again, we found that there was a significant increase in the immobility scores of the compound treated mice as compared to vehicle controls (Fig 2B; Two-way ANOVA: $F(2,78)=6.550$, $p=0.0023$; Sidak's multiple comparisons test: $p=0.0147$). These results suggested that increased EAAT2 reduced helplessness behavior and encouraged us to investigate the effects of increased EAAT2 after chronic stress.

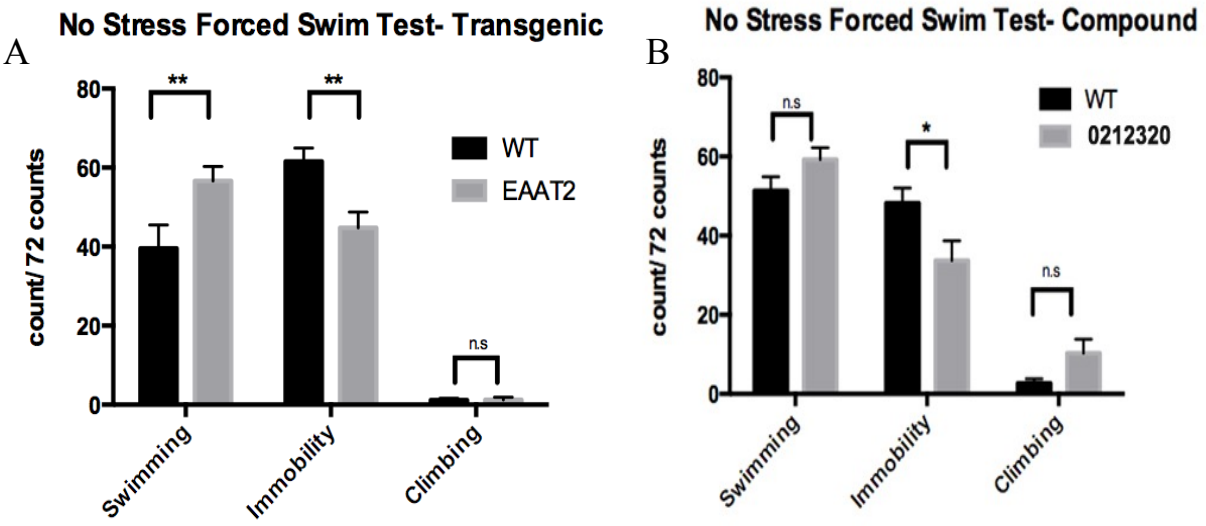


Fig 2: Preliminary results show significant decreases in immobility during the forced swim test in both EAAT2 transgenic mice and mice treated with LDN/OSU-0212320. EAAT2 transgenic mice also show significant increases in swimming behavior. *=$p<0.05$ **=$p<0.01$

Hypothesis:

I hypothesize that increased expression of EAAT2 provides neuroprotection against the development of depressive-like pathology due to chronic stress.

Purpose:

To test this hypothesis, in the present study, I investigated if EAAT2 transgenic mice exhibit significant less depressive symptoms than their wild-type littermates after one month of unpredictable chronic mild stress. If successful, we will later investigate if drug-induced EAAT2 expression through the use of one of our compounds could ameliorate depressive symptoms when depression is already present and whether increased EAAT2 could restore pathological changes caused by chronic stress. If this latter study is also successful, it would suggest that

increased EAAT2 is a potential therapeutic strategy for MDD and would also suggest that our compounds have potential for treating MDD. This study is important for examining a different route for therapeutic development in the treatment of MDD. With such a high subpopulation (up to 30%) not responding to normal MDD treatments, it is imperative that we look at all possible targets (Rush et al. 2006).

Methods:

Experimental Subjects:

An EAAT2 transgenic mouse line (FVB/N strain), which was previously generated by our laboratory (Guo et al. 2003), was used in this experiment. A total of 38 male mice (22 EAAT2 mice and 16 wild-type mice) were used over three different cohorts (cohort 1=15 mice, cohort 2=10 mice, cohort 3=13 mice) based on date of birth. Mice were subjected to stress conditions at the age of 3-4 months. All mice were male and were split into four testing conditions: wild-type non-stressed (n=7), wild-type stressed (n=9), EAAT2 non-stressed (n=11), and EAAT2 stressed (n=11).

Transgenic mice were determined by PCR using extracted DNA from tail biopsies using the AccuStart II Mouse Genotyping Kit by Quantum Biosciences with EAAT2 transgene-specific primers (5'-GGCAACTGGGGATGTACA-3' and 5'-ACGCTGGGGAGTTTATTCAAGAAT-3'). PCR conditions were 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, 72°C for 2 min for 30 cycles, followed by a 10-min extension at 72°C, and a 6% agarose gel was used in gel electrophoresis to view DNA bands.

The splitting of wild-type and EAAT2 transgenic mice into stressed and non-stressed conditions was based on littermate controls and controlling for weight. All mice had access to food and water *ad libitum*, except for the 18 hours before sucrose preference testing and the 24 hours before the novelty-suppressed feeding test. Animals were on a 12:12 light:dark cycle (6 am:6 pm) when not exposed to light-based stressors. Cohort 1 mice (n=15) were originally group caged by littermate and condition, however, stressed mice became too aggressive and were single

caged to avoid injuries. Cohorts 2 and 3 (total n=28) mice were all single caged to avoid bodily harm.

Unpredictable Chronic Mild Stress Model:

Before beginning the unpredictable chronic mild stress model, mice were habituated to handling conditions, weight measurement procedures, and the sucrose preference tests over a minimum of 2 weeks. After this habituation period, mice were split into conditions according to littermates and weight prior to stress. Mice of similar weights were balanced between different testing conditions as much as allowed. All mice had their body weight measured one to three times a week throughout the experiment. Weight was not measured on the morning of a sucrose preference test, as mice were water deprived the night before and this could affect weight measurements. All mice underwent weekly sucrose preference tests during the month of stressed conditions, followed by one additional test during the behavioral analysis period. All mice underwent behavioral analysis after the month of stressed conditions. Mice in the stress conditions were exposed to two stressors every day (one in the morning, one in the afternoon) for one month, followed by behavioral tests during which they received one stress (Fig 3). Stressors during this month were chosen so that no same stress was repeated as the next stress (at least one different stressor in between). A chart of possible stressors is shown below (Table 1) along with a sample calendar (Table 2). No water related stressors were done on the day of sucrose testing as they were undergoing water deprivation. All mice stressors and behavioral analyses were done prior to 6 pm to return mice to normal dark period.

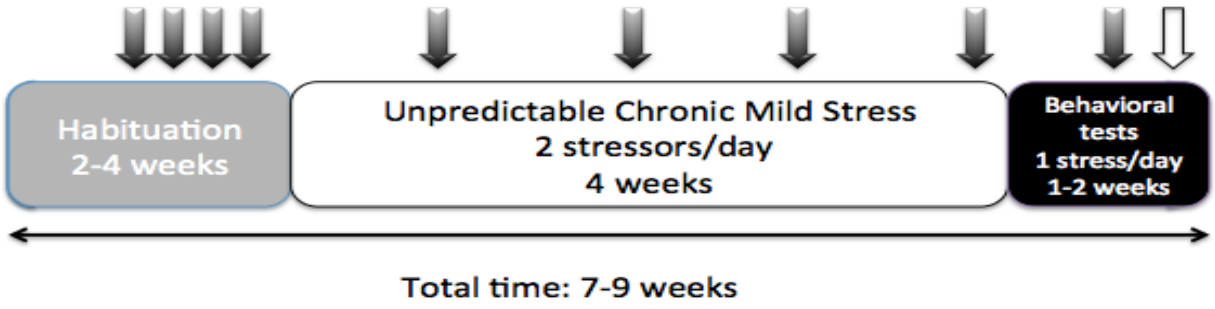


Figure 3: Unpredictable Chronic Mild Stress model timeline. Black arrows represent sucrose preference tests. The white arrow represents the coat state assessment and nesting behavior data used for analysis. During the full duration of the experiment, body weight was measured two to three times a week (not shown here).

Stressor	Duration	Description	Literature
Rat Odor	3 hours	Mice were placed in new cages that had bedding mixed with used rat bedding (which included rat feces). Enrichment was removed.	Bergner et al. 2010
Dark during Light cycle	3 hours	Mice cages were placed in a dark testing room during normal light hours.	Ibarguen-Vargas et al. 2008
Wet Bedding	3 hours	Bedding was moistened with the addition of approximately ~100 mL of water. Excess water was drained prior to testing. Enrichment was removed.	Bergner et al. 2010 Wilner et al. 1992 Ibarguen-Vargas et al. 2008 Surget et al. 2008
No Bedding	3 hours	All bedding and enrichment were removed from home cages.	Ibarguen-Vargas et al. 2008 Surget et al. 2008
Cage Rotation	15 minutes	Mice cages were placed on an orbital shaker on gentle rotation (~40 rpm). Food, water, and enrichment were removed.	Monteiro et al. 2015
Strobe Light	1 hour	Mice cages were placed in a dark testing room and exposed to a medium intensity strobe light.	Reich et al. 2009 Grippo et al. 2005
Cage Tilt	8 hours	Mice cages were placed at a 45° tilt.	Wilner et al. 1992 Ibarguen-Vargas et al. 2008 Surget et al. 2008
Predator sounds	15 minutes	Mice were exposed to a soundtrack containing a mixture of predator sounds including the sounds of: cats, dogs, hawks, and snakes.	Ibarguen-Vargas et al. 2008 Surget et al. 2008

Table 1: Chart represents the stressors used in this experiment. Other experiments that used the same or similar stressors are listed.

	SUN	MON	TUES	WEDS	THURS	FRI	SAT
STRESS		BW Dark Strobe	Rotation Tilt Water dep	Dark Rotation Sucrose	BW Tilt Strobe	Dark Wet	Rotation Predator
STRESS	Strobe Rotation	BW Wet No bed	Dark No bed Water dep	Wet Predator Sucrose	BW Dark Wet	Strobe No bed	Rotation Predator
STRESS	Strobe Rotation	BW No bed Rat	Tilt Predator Water dep	Dark Rotation Sucrose	BW Tilt Strobe	Dark Rotation	Strobe Rotation
STRESS	Predator Strobe	BW Wet No bed	Dark Strobe Water dep	Rat Predator Sucrose	BW Wet Strobe	BW Rotation No bed	Predator Strobe
BEHAVIOR	Rotation Predator	BW OF Rat	Y-maze Strobe Water dep	Dark Rat Sucrose Nest overnight	BW analyze nest and coat No bed Food dep	BW NSF Rotation	FST Predator
BEHAVIOR	FST Strobe	BW Dark Sacrifice	Sacrifice				

Table 2: A sample calendar for stressed mice under our unpredictable chronic mild stress paradigm. BW= Body Weight. Dep= deprivation. Sucrose= Sucrose Preference. OF=Open Field. NSF=Novelty-Suppressed Feeding. FST= Forced Swim Test.

Behavioral Tests:

Sucrose Preference:

Sucrose preference was done in order to get a quantitative measure of anhedonia in mice. Anhedonia, or the loss of pleasure in normally pleasurable objects or actions, is measured by the loss or lack of preference for sucrose water. Normally, mice would prefer sucrose water to normal water. We deemed that preference was demonstrated when 70% of the total fluid intake during a testing period was sucrose water.

During the habituation period prior to stress conditions, mice were first exposed to two water bottles filled with distilled water. These two bottles were then replaced with 1% sucrose water, followed by one bottle of distilled water and one bottle of 1% sucrose. When two different liquids were given, the bottles were alternated daily so that the sucrose water was not continually on the same side. Also during this time, sucrose preference tests were done every three days to acclimatize mice to the different testing cage conditions (single-caged conditions) and handling. After a 2 to 4 week habituation period (determined by age and consistency of sucrose preference results), stress conditions began. During this time, sucrose preference tests were done once a week and once more during behavioral analysis.

Testing procedure began by filling numbered water bottles either with distilled water or 1% sucrose water. Tests began with all odd bottles being filled with 1% sucrose water and all even bottles being filled with distilled water; however, this was alternated with each test. Each bottle was then weighed and weight was recorded. Once all bottles were prepared, each mouse was placed into a cage separate from its home cage by itself. Mice then received one bottle filled with water and one bottle filled with 1% sucrose water with the odd labeled bottles always being

on the left side of the cage. This along with alternating what filled even and odd bottles allowed us to view if there were any side preferences. No side preferences were seen after the habituation period. Mice had access to these bottles for 1 hour before bottles were removed and reweighed. Total amount of water consumed was determined. The amount of sucrose water consumed was divided by the total and then multiplied by 100 to determine the percent of sucrose preference. In cohorts with a high number of mice, testing occurred in 2-3 rounds in order to minimize time between the first and final mouse receiving the water bottles in any given round. Mice had the tests done in the same order in order to maximize the likelihood of consistent results between tests. The final sucrose preference test (done during the week of behavioral analyses) was used in the data results analysis.

Open Field:

Open field test was done in order to compare levels of anxiety between mice as determined by the amount of time spent in the center of a novel testing arena. More anxious mice are expected to spend less time in the center of the arena and more time on the outer edges as walking into exposed open space can cause more anxiety (as it may, for example, allow for more opportunities for predation if any predators were around).

Prior to testing, each mouse had a blue or a black circle painted on his back with non-toxic paint. This is later used to track movement during analysis. Each mouse was individually placed in a 30 cm x 27 cm x 30 cm plastic box that obscured excess light for 3 hours (this extended time was to collect data for locomotor activity as well). A camera recorded the movement of each mouse during this time. After testing, videos were divided into 10 minute segments starting when the scientist's hand was completely out of screen (to avoid its movement

from affecting the analysis). The first 10 minute video was then analyzed using Image J to track the movement of each mouse within a square with specific pixel lengths depending on the camera angle. These pixel lengths and the specific x and y-axis coordinates of the corners of the analysis square were recorded for each mouse. From this analysis, we cut the data in half using the slice count (the number of image frames), and scored each of those slides as a 0 or a 1. A score of 1 indicated that a mouse was located within the coordinates of the center of the square. The center of the square was determined by taking the x and y-coordinates of the corners of the whole analysis area and determining the points that divide the outer sixths of the full square from the inner 4/6. The scores are then added to determine the number of frames that the mouse was located in the center of the box and this was either multiplied by 300 (to get the total time spent in the center as there are 300 seconds in a 5 minutes interval) or divided by the total number of frames in five minutes multiplied by 100 (to determine the percent of time spent in the center). Between mice, the testing arenas were sanitized with 70% ethanol.

Y-maze:

Y-maze testing was done in order to measure the short-term memory deficits and motor activity of mice. Using the unpredictable chronic mild stress model we would not expect to see any difference in either percent of spontaneous alternations nor number of arm entries, which shows motor activity (Pothion 2004).

For this test, a gray plastic apparatus with three identical arms measuring 35 cm × 5 cm x 10 cm at 120° angles from one another was used in a darkened room where each arm was exposed to between 29-31 lux. Each arm was labeled A, B, and C and a camera was set up looking toward the center of the maze from arm A. Prior to testing, mice were single caged and

brought into the testing room but kept completely in the dark. Before an individual mouse was tested, it was allowed to acclimate to the room's light settings for five minutes. The mouse was then placed in arm A and allowed to explore the maze for 10 minutes. Each arm entrance was marked and the mouse was considered to have entered an arm of the maze when all four feet had crossed into an arm different from the one it had just come from. Motor activity was determined by the number of arms entered in the 10 minute testing period. Percent of alternations was determined by the number of spontaneous (non-repeating) alternations made within the last three successive arm entrances divided by total arm entrances, multiplied by 100.

Novelty-Suppressed Feeding:

The Novelty-Suppressed Feeding test is a behavioral analysis that is said to reflect the anxiety levels of mice. In this test, a mouse is deprived of food for 24 hours then placed in a foreign arena in a brightly light room where a piece of food has been placed in the center. The longer the time it takes the mouse to enter the anxiety causing conditions (a foreign open area in a bright room) to eat the food indicates that the mouse is more anxious (Samuels and Hen 2011).

The day before the test, all mice were weighed and food was removed from cages. This was done at least 24 hours prior to the planned experiment time the next day. On experiment day, mice were brought into the testing room to acclimate (approximately 1 hour before testing began). During acclimation, fresh food pellets that are approximately the same size were chosen so that there was one for each mouse being tested. Pellets were then weighed and weights were recorded. A litter box (30 cm x 45 cm x 17 cm) was filled 3 cm deep with standard bedding. A small white circle (approximately 12 cm in diameter) was cut from filter paper and placed in the center of the box. A camera was set up to record the test. Mice prior to testing were then moved

to a clean waiting cage while water and enrichment were removed from their home cages. The pre-weighed pellet was then placed in the current mouse's home cage, ready for after the testing process. When ready for testing, a fresh, unweighed, pellet was placed in the center of the circle and the mouse was placed in the arena always starting in the bottom left corner. Upon release, a stop-watch was started. Only when the mouse had grabbed the pellet with both forepaws and begun eating was the timer stopped (sniffing or moving the pellet did not warrant stopping the clock). Immediately, the mouse was then removed from the arena and placed into its home cage with the pre-weighed pellet. A 5 minute timer was then set while the mouse was allowed to eat the pellet. Meanwhile, the next mouse and testing arena were prepared for testing. After 5 minutes, the pre-weighed pellet was removed and reweighed. Once all mice were tested, they too were weighed. Food was then returned to the mice.

Coat State Assessment:

Coat state assessment was done several times throughout the course of the experiment with the final assessment taking place during behavioral testing. This was the data used for analysis. Mice were scored from 0-7 where a mouse earned 1 point for each of the following areas if they looked well-groomed (normal): head, neck, forepaws, dorsal coat, ventral coat, hind legs, and tail region (Bergner et al. 2010). Therefore high scores indicate better grooming behaviors. This score has been seen to decrease with increasing depression-like symptomology and is thought to similar to depressive patients who display poor hygiene (Bergner et al. 2010).

Nesting Analysis:

Lack of nest building in mice is thought to be indicative of lack of motivation. In this test, mice were single caged (if not already) overnight in a new cage with a new Nestlet

(approximately 5 square cm of cotton bedding). The next morning, nests were rated on a scale of 0-5 based on a previous protocol (Deacon 2006). Higher scores indicated a better, well-formed nest and 0 indicated that the Nestlet was still whole. Therefore, we expect depressed mice to have a lower average score.

Forced Swim Test:

The forced swim test is one of the most widely used tests for rodent models of depression (Petit-Demouliere et al. 2005). It is used to model resignation or helplessness and is specifically used to view the effects of antidepressants on immobility time.

The day before performing the test, tap water was set aside in two large containers to equilibrate to room temperature overnight (23-25° C). The day of testing, water temperature is measured and two 4000 mL circular containers were filled with ~2000 mL (about 13 cm deep). A camera was set to record both containers at the same time. Dividers were used to block the view between the containers and around each side, except for the side facing the camera. When ready for testing, two mice were placed into the water feet-first and a timer was set for 10 minutes. After the time was completed, the mice were removed and dried before being returned to their home cages. The water was then removed, the container was wiped clean with 70% ethanol, and new water was added. This same procedure was then repeated the following day and day 2 data was used for final analysis.

To analyze this data, the videos were watched starting at minute four (so that only 6 minutes were actually analyzed) and that time was broken into 5 second intervals using an interval timer. Instead of measuring total immobility time, a score of 1 was given in one or more of the following categories—swimming, immobility, and climbing—as determined by whether

that action occurred during that 5 second interval (Cryan et al. 2005). Swimming was defined as any movement other than the movements required to keep the mouse afloat that cause propelled movement in a horizontal fashion. Immobility was defined by the lack of any movement other than those required to keep the mouse afloat. Climbing was defined as the use of two limbs on the wall of the container in any attempt to make vertical movement. As the total analysis time was 6 minutes, the highest score in any one category that could be earned was 72.

Tissue collection:

Mice were euthanized and brains were removed. Forebrains were split in half and the left hemisphere was fixed in 4% paraformaldehyde (PFA). For the right hemisphere, the cortex, subcortex, hippocampus, and the prefrontal cortex were dissected and frozen to -80° C. Blood serum was also isolated and kept stored at -80° C. The adrenal glands were removed in cohorts 2 and 3, dried, and weighed.

Western Blot Protein Analysis:

EAAT2:

Tissue samples were homogenized in 300 uL of 1x cOmplete, a proteinase cocktail inhibitor. Homogenization was done using sonification at least 40 times at a power of three using a Fisher Scientific 60 Sonic Dismembrator until there were no large pieces of tissue. Protein density was determined by performing an optical density (OD) reading on each sample. This was done by reading 1:20 sample dilutions in 96-well plates with a Coomassie Plus (Bradford) Assay. This was measured using a spectrophotometer plate reader at 595 nm along with a standardization ladder of bovine serum albumin (BSA). Based on protein readouts, 1 λ and 0.1 λ solutions were made with 1x SDS running buffer with added β-mercaptol. This was done for all

prefrontal cortex samples and the hippocampal samples from cohorts 1 and 3 (n=28). These samples were stored at -20° C if being used soon or -80° C if stored for longer periods of time.

The next steps to quantify EAAT2 were to separate proteins based on size and transfer them onto a nitrocellulose membrane. Samples were first removed from the freezer and allowed to warm to room temperature. Samples were then activated at 95° C for 5 minutes, briefly placed on ice, then quickly spun at top speed. On an 8% acrylamide gel, 5 µL of ladder and 10 µL of the 0.1 λ samples were loaded (for a total of 1 µg of protein sample per well). 100 V of electricity was used to separate proteins based on size over approximately 2 hours. This gel was then placed onto a nitrocellulose membrane inside of a transferring apparatus and more electricity was used to transfer protein from gel to membrane (100 V, two cycles of 50 minutes with a replaced ice pack in between cycles to keep transfer buffer cool). This membrane was then stored until ready for development (usually done the next day).

To develop the membranes, 5% milk was first added to block non-specific binding. This milk was then rinsed off with PBST (phosphate buffered saline with 0.1% Tween-20), and then primary antibodies specific to the protein of interest were added and the membrane was on a shaker for an hour. If multiple proteins were being studied, the membrane was first cut into sections according to the size of the protein being studied (for example, just above the marker for 52 kB if looking at both GFAP and EAAT2) and each piece of membrane would be treated with the appropriate primary in separate containers. Afterwards, the membranes were rinsed with three washes of PBST for 10 minutes each. Following washes, secondary antibody (mouse or rabbit in 1% milk depending on the primary) was added and again allowed to shake for 1 hour. This was followed by 3 more washes for 10 minutes each. The membrane was then treated with

Advansta WesternBright ECL for 2 minutes. Film was then exposed by the light released by the membrane and developed.

Protein concentrations were quantified using Image J and normalized by dividing the results of EAAT2 by the results for GAPDH. This was done to reduce the differences in loading between samples. Only samples that seemed to have similar loading based on GAPDH were compared.

PSD95:

In order to view if any synaptic loss occurred due to stress, synaptic and extrasynaptic membranes were separated in hippocampal tissues from cohort 2 (n=8, two samples not used). To do so, hippocampal samples were homogenized using a dunce 30 times and a 1 mL syringe 15 times. Sample was then spun at 1Kxg for 10 minutes and the supernatant was isolated from the pellet. For higher yield, the pellet containing the nuclear fraction could be further homogenized and spun again to remove the supernatant once more. The supernatant was then adjusted to a concentration of 500 µg/500 µl and spun again at 10 Kxg for 15 minutes. The supernatant of this spun sample was then removed and stored at -80° C. The pellet was then homogenized again with the addition of an extraction buffer containing 0.5% Triton X. The synaptic density is unaffected by this detergent, thus only the extrasynaptic membrane is broken down into the solution. This was then spun at 32 Kxg for 20 minutes. The supernatant (containing the extra synaptic membrane) was removed (additional spins may be necessary). The pellet (containing the synaptic density) was then resuspended in a Tris-HCl/proteinase inhibitor/phosphatase inhibitor solution.

From there, OD and Western were done similarly as described in EAAT2 protein reading using EAAT2 and PSD95 primary antibodies. Quantification was also done using Image J; however, GAPDH was too low in the synaptic density membrane, therefore it was not used to normalize readings.

Statistical Analysis:

All data was analyzed for significance using one and two-way analysis of variance (ANOVA) using the statistical program Prism 6 (V6.0h, GraphPad Software Inc.). Post-hoc tests (when necessary) was done using Tukey's test and Sidak's multiple comparisons test on the same program. Significance was deemed as p-values <0.05. Data that was 2 standard deviations away from the mean were excluded. Standard Error of the Mean (SEM) error bars were placed on each graph.

Results:

Behavioral Analyses:

Y-Maze

Motor activity of all mice ranged between 38-75 arm entrances with EAAT2 stressed and wild-type stressed mice having mean values around 51 as compared to non-stressed conditions having means around 56 and 57. The differences in means between testing conditions were found to be insignificant through a one-way ANOVA (Fig 4A; $F(3,34)=1.621$, $p=0.2027$). The percent of correct alterations for these mice ranged from 52-86%, and all groups had means around 70%. Thus the differences between wild-type, EAAT2, and stressed conditions were found to be insignificant with a one-way ANOVA (Fig 4B; $F(3,34)=0.2022$, $p=0.8942$).

Novelty-Suppressed Feeding

The results of the Novelty-Suppressed Feeding test are represented in Fig 4C and show that wild-type non-stress, EAAT2 non-stress, and EAAT2 stress mice took a mean time to bite the pellet around 260-270 seconds, while wild-type stressed took a mean time of 215 seconds. However, the actual times per mouse ranged between 46.76 seconds and 1550 seconds (nearly 26 minutes). Three mice, one in all conditions except wild-type non-stress, took over 20 minutes to bite the pellet. Thus, there was a wide individual variation within each condition that resulted in there being no statistical differences between groups (Fig. 4C; $F(3,34)=0.03826$, $p=0.9898$).

Open Field

The percent of time spent in the center of the open field chamber ranged from 12% to 50% for each individual mouse with the largest range occurring in the EAAT2 non-stressed and stressed conditions. The mean percent of time spent in the center was lowest in wild-type non-

stressed mice (22.8%) and nearly equal in all other conditions (EAAT2 non-stress spent 28.5%, wild-type stressed spent 29.0%, and EAAT2 stressed spent 27.9% of the time in the center). One wild-type non-stressed mouse was excluded for being two standard deviations outside the range of the other mice. The differences between testing conditions as determined insignificant by a one-way ANOVA test (Fig 4D; $F(3,33)=0.7137$, $p=0.5508$).

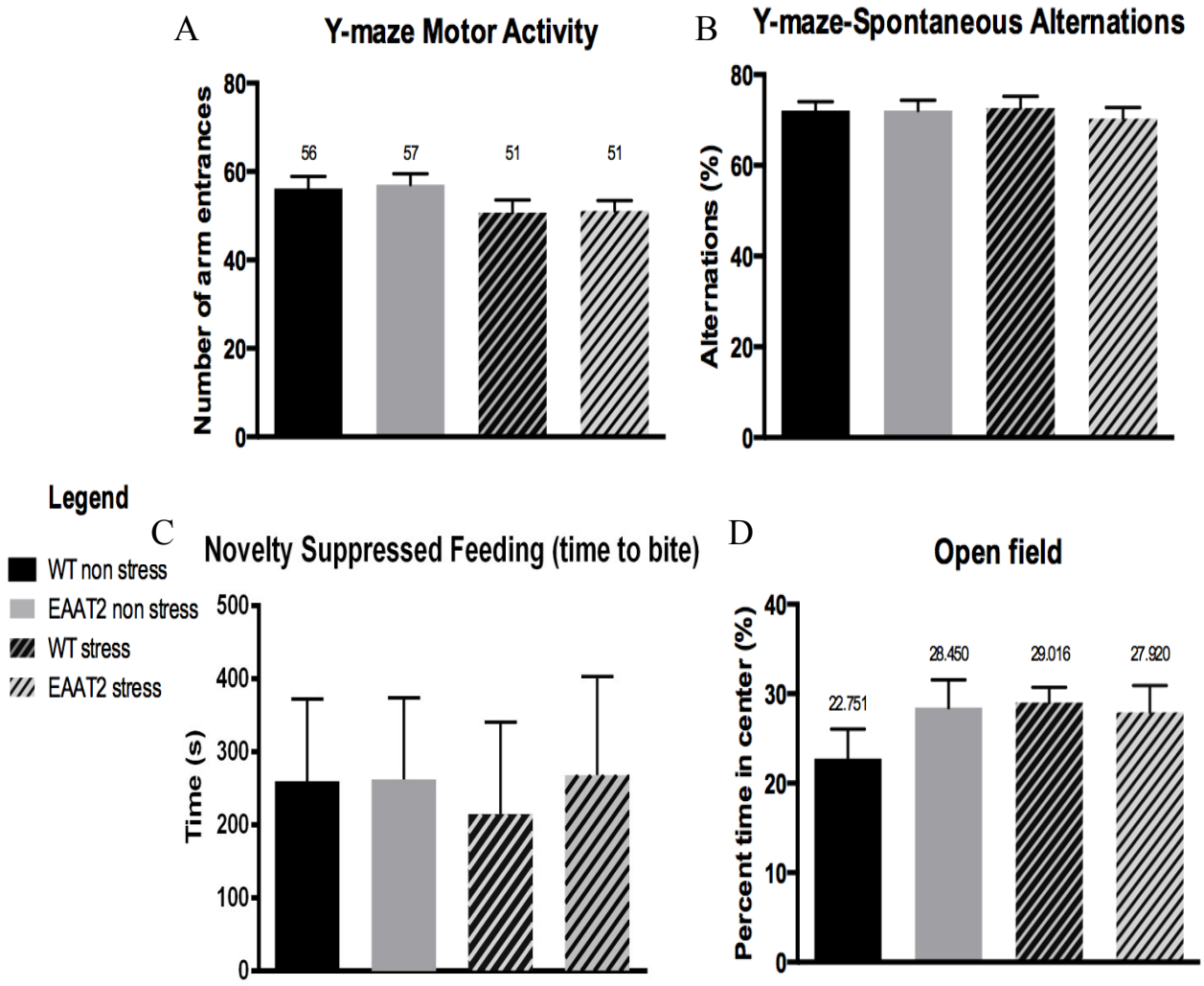


Fig. 4: Analysis of behavioral tests: No significant differences were seen in motor activity (A) or spontaneous alternations (B) in the Y-maze test. There were also no significant differences in the Novelty-Suppressed Feeding test (C) and the Open Field test (D). Numbers show mean values as points of reference.

Sucrose Preference:

The percent of preference for sucrose ranged between 47% and 90% for each individual mouse. The mean sucrose preference for each condition was: 68.4% for wild-type non-stressed, 73.0% for EAAT2 non-stressed, 73.9% for wild-type stressed, and 80% for EAAT2 stressed. Five mice were excluded for being two standard deviations away from the mean (2 wild-type stressed, 2 EAAT2 stressed, and 1 EAAT2 non-stressed). We would think that the stressed conditions would have lower percents of sucrose preference, but we saw the opposite results. However, statistical analysis showed that there were no significant differences between test groups therefore they were actually all about the same (Fig 5A; $F(3,29)=1.493$, $p=0.2371$).

Nesting Scores:

EAAT2 mice (both stressed and non-stressed) presented with slightly higher nest making scores (3.5) as compared to their wild-type counterparts. Wild-type non-stressed had a mean score of 3, while the stressed conditions had a slightly lower score of 2.5. With statistical analysis, however, this decrease was found to be insignificant (Fig 5B; $F(3,35)=1.299$, $p=0.2901$).

Coat State Assessment:

Differences in coat state between experimental conditions were deemed highly significant with a one-way ANOVA (Fig 5C $F(3,35)=6.571$, $p=0.0012$). Post-hoc tests (using Tukey's multiple comparisons tests) revealed that the differences seen between EAAT2 non-stressed and Wild-type stressed, as well as Wild-type non-stressed and EAAT2 stressed were both deemed significant ($p=0.0476$; $p=0.0118$). EAAT2 non-stress and EAAT2 stress were found to be extra significant ($p= 0.0039$). However, there were no significant differences between Wild-type non-

stress and wild-type stressed despite there being a decrease in score from a mean of 6.3 to 4.7, respectively (p=0.0898).

Forced Swim Test:

With mean swimming scores of approximately 59-63 counts, all mice displayed similar swimming behaviors with wild-type non-stress spending the most time swimming at 63 counts. Immobility was also not very variable with wild-type stressed spending the most time immobile at 44 counts, followed by 42 counts by EAAT2 stressed and wild-type non-stressed, and EAAT2 non-stressed spending the least amount of time immobile at 39 counts. EAAT2 stressed mice spent the most time climbing with a count of about 10, while all other conditions were between 5-7 counts. Despite small differences, swimming, floating, and climbing were all deemed insignificantly different between test groups (Fig 5D; two-way ANOVA: $F(6,102)=0.2948$, p=0.9382).

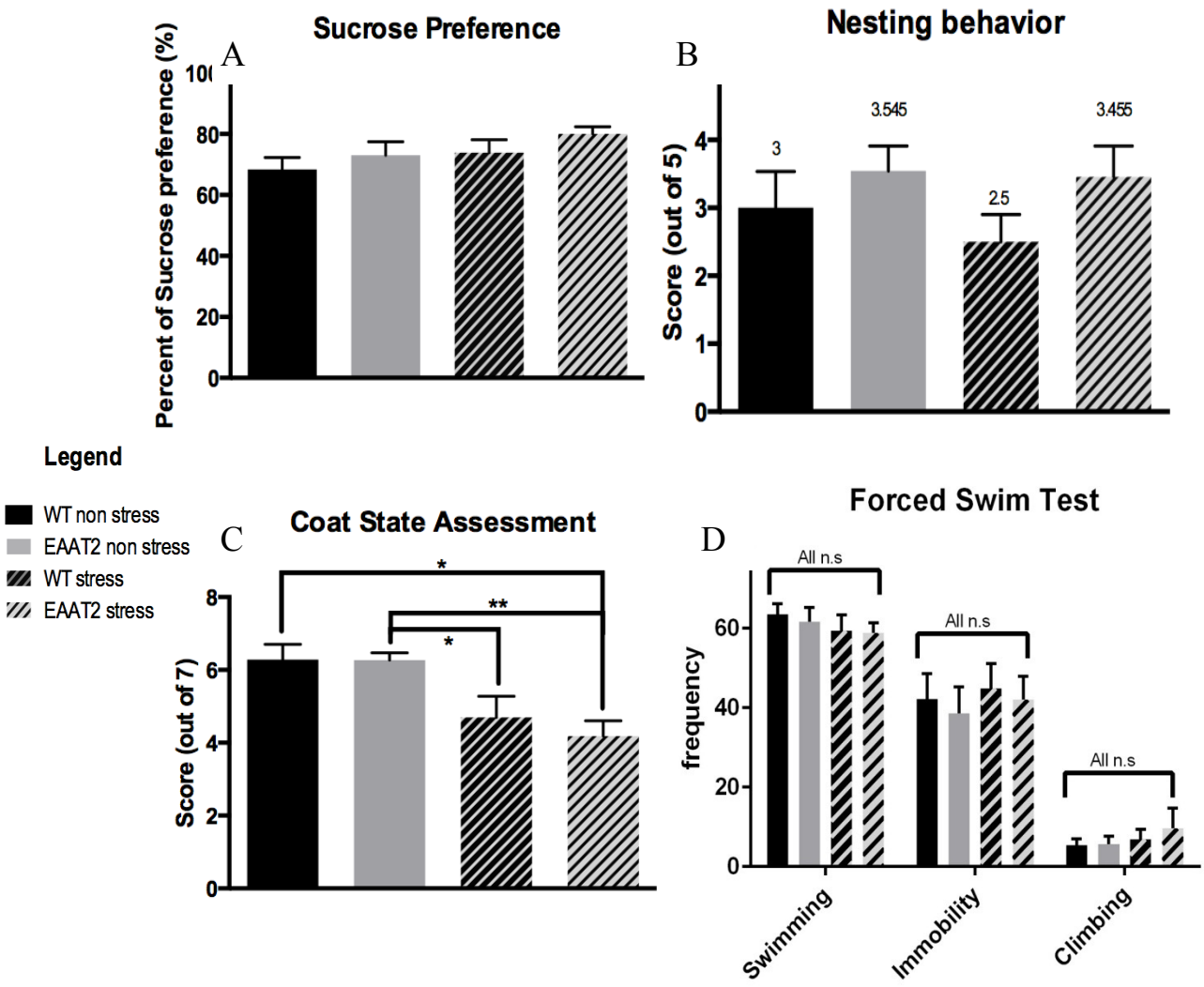


Fig. 5: Analysis of behavioral tests: no significant differences were seen in sucrose preference (A), nesting behavior (B), or forced swim test (D). Significant results were seen between wild-type non-stressed vs. EAAT2 transgenic stressed and between EAAT2 transgenic non-stressed vs. wild-type stressed and EAAT2 transgenic stressed. *=$p<0.05$; **=$p<0.01$; numbers show mean values as points of reference.

Tissue Analyses:

Body Weight:

Over the course of the experiment, body weight change was very cohort and individual dependent. Cohort 1, as a whole, gained weight (between 4%-17% change normalized for final body weight). Cohorts 2 and 3, on the other hand, ranged from losing approximately 8% to

gaining approximately 4% weight. This caused great variation in the graphs so that each had large SEM error bars. Both non-stressed conditions showed means of approximately 6% change while wild-type stressed had a mean change of 4% and EAAT2 stressed only changed about 2%. Due to such variability, the differences in the percent of body weight change were deemed insignificantly different through a one-way ANOVA (Fig 6A; $F(3,34)=0.3527$, $p=0.78740$).

Adrenal Gland Weights:

The mean weight of the right adrenal glands were approximately less than or about 1% higher in both stressed conditions (6-7%) as compared their non-stressed counterparts (both about 5.7%). In the left adrenal gland, the mean percent of body weight of the left gland was around 7-7.5% for stressed conditions, while wild-type non-stressed had a mean of 6.7% and EAAT2 non-stressed had a mean of 5.9%. The weights of both the right and left adrenal glands were therefore deemed to be insignificantly different between test groups (Figs 7B and 7C); ($F(3,19)=1.294$, $p=0.3054$; $F(3,19)=1.316$, $p=0.2985$). Higher adrenal gland weight would have been expected in stressed mice due to the increased creation of corticosterone due to stress.

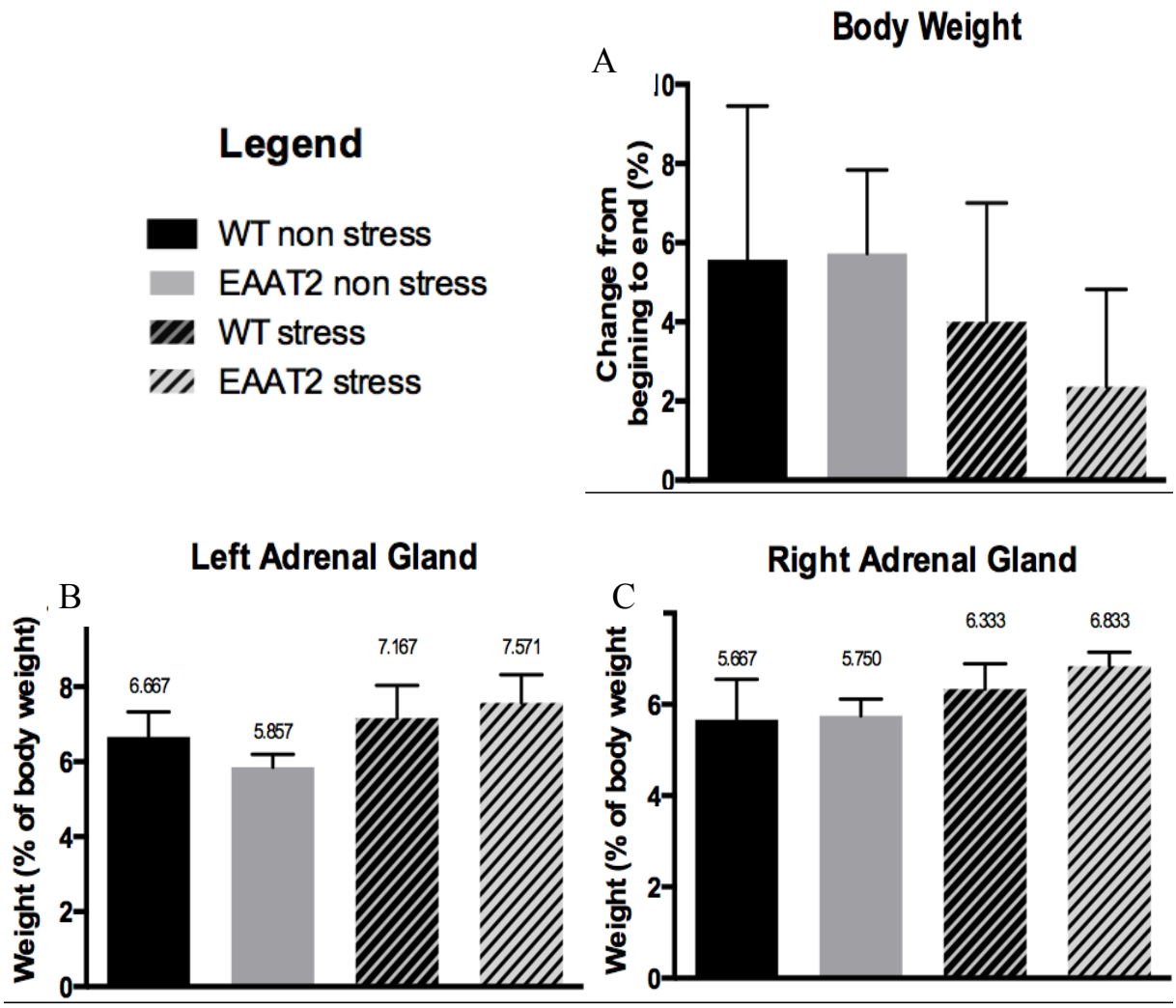


Fig. 6: No significant differences between conditions were seen in body weight (A), left adrenal gland weight (B), or right adrenal gland weight (C). Numbers show mean values as points of reference.

Western Blot:

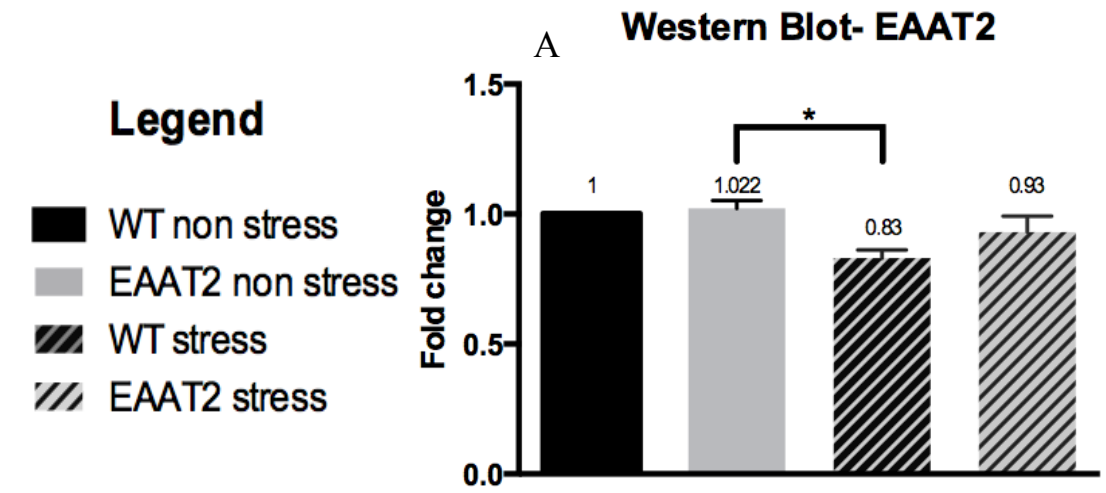
EAAT2:

Samples of the prefrontal cortex of all mice from over all three cohorts were analyzed for EAAT2 distribution. Three sets (each containing a wild-type non-stress/stress and EAAT2 non-stress/stress mouse) were chosen for being most similar in terms of loading and lack of multimers. Analysis of these three sets of mice showed significance through a one-way ANOVA

(Fig 7A; $F(3,8)=5.277$, $p=0.0267$). Post-hoc analysis using Tukey's multiple comparisons tests, however, revealed only a significant difference between EAAT2 non-stress and wild-type stress fold changes ($p=0.0283$). However, the lack of significance between the fold change of wild-type non-stressed and stressed and EAAT2 non-stressed and stressed indicate that there was no significant change in EAAT2 distribution due to the stress done in this experiment and suggest stress paradigm was not successful.

PSD95:

Only samples from mice in cohort 2 (n=8) were available for analysis of synaptic density. Fig 7B shows the Western blots of the two sets of samples (equal amounts of protein (15 ⌈g) were loaded for each lane). Quantitative analysis without normalization shows a significant decrease in PSD-95 immunointensity of the wild-type stressed mice as compared to non-stressed (47-57%), but there are no obvious differences between wild-type non-stress, EAAT2 non-stress, or EAAT2 stressed conditions. These results suggest that there is a marked decrease in post-synaptic density in wild-type stressed mice, but not in EAAT2 stressed mice.



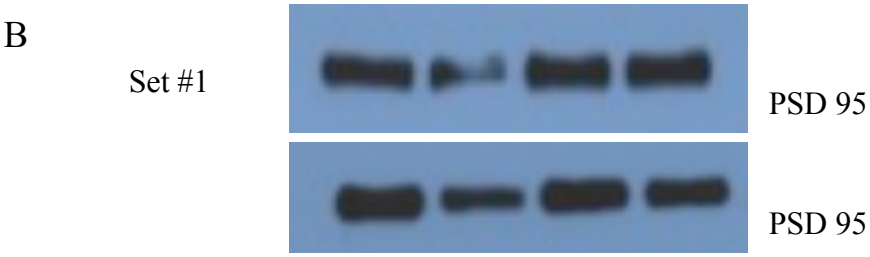


Fig. 7: Quantification of EAAT2 protein levels using Western Blot revealed only a significant difference between EAAT2 non-stress mice and wild-type stressed mice in three comparable sample sets. GAPDH expression levels were used to normalize the results. (A) The synaptic density was isolated in two sets of samples and shown here. No GAPDH was present in order to normalize for quantification, however, non-normalized data shows about a 50% decrease in expression of PSD95. (B) WT=Wild-type; EAAT2= EAAT2 transgenic mice; N.S.=Non-stressed; S=Stressed. Numbers shown are mean values as points of reference. *=$p<0.05$

Discussion:

The results of this experiment suggest that the mice were not sufficiently stressed to display a depression-like phenotype. This conclusion is drawn from the results of both behavioral data (which showed nearly no significant differences between conditions, except for the coat state) and quantification of EAAT2 in the prefrontal cortex (which only showed a significant difference between EAAT2 non-stressed and wild-type stressed mice). According to literature, we would have expected to see significant differences in sucrose preference between stressed and non-stressed conditions (Wilner 1997) as well as differences in protein expression of EAAT2 in the prefrontal cortex (Choudary PV et al. 2005; Sanacora G and Banasr M 2013).

These results could have been due to a number of factors. First, we will explore the experimental set-up. While stressors of the unpredictable chronic mild stress model are meant to create an environment that is just that (filled with unpredictable but mild stressors), it is possible that the stressors we chose from the scientific literature were not stressful enough. Our application of stressors may have been different enough from literature that, after 4 weeks, there was still not enough to cause a measurable effect. Likewise, it could have been that our mice adapted to the stress due to inert resilience. Therefore, it may be better to do stressors that may be categorized as more stressful such as confined space/restraint stress, social defeat stress, or temperature related stress (Jung et al. 2014; Monteiro et al 2015). If the types of stressors used remain unchanged, varying the duration of the original stressors might prevent habituation (Bergner et al. 2010). In addition to adding one or multiple stressors, it may also be beneficial to increase the span of the stress from 4 weeks to 8 weeks (Monteiro et al 2015).

Another possible explanation for these results may be due to strain differences between the FVB/N mouse phenotype for some of the tests. For example, papers have shown that FVB/N mice have showed significant difference between stressed and non-stressed mice when looking at coat state, but have not seen significant differences in the Novelty-Suppressed Feeding test (Ibarguen-Vargas et al. 2008). Similarly, we do not know if our transgenic EAAT2 mice strain would have strain specific responses to these tests. That said, it may prove better to work with another mouse model that is used more often in the unpredictable chronic mild stress literature, such as the C57BL/6 mouse strain, which has had positive results (Jung et al. 2014; Monteiro et al. 2015). We originally used the FVB/N mouse strain as our wild-type as that is the strain from which our EAAT2 transgenic mouse line was originally derived. Therefore, in switching the mouse strain, we would also need to switch to treating our mice with our experimental compound as a means of increasing EAAT2 levels. It is important to note that strain difference would still not account for the lack of differences in the other behavioral assays, such as the Forced Swim Test which was done in the preliminary studies with wild-type and EAAT2 transgenic mice that had significant differences (Fig. 2A).

The largest concern for this study is the individual variation among mice. The variability within condition groups (best seen in the range of bite times in the Novelty-Suppressed Feeding test) was just as great if not sometimes larger than between conditions. The reproducibility of the unpredictable chronic mild stress model has been called into question before (Slattery and Cryan 2014; Wilner 1997). This begs the question of whether the variability seen here is due to the low number of total mice used in the study (n=7-11 per condition), or if this is a limitation within the model itself.

If I were to propose a future study, I would test if increased EAAT2 can provide neuroprotection against depression-like symptoms such as anhedonia using C57BL/6 treated with our compound LDN/OSU-0212320, which has been shown to increase expression of EAAT2 in previous experiments (Kong Q et al. 2014). I would also adjust the experimental set-up by adding one or more of the stressors mentioned earlier (restraint, social defeat, or temperature variable stress) and by increasing the duration of the stressors in this experiment. By doing so, I would expect to see clear differences between stressed and non-stressed mice in some of our behavioral assays along with potential differences due to treatment with our small molecule compound as compared to vehicle treated mice. I would also include female mice, which were not included in this study, to view the potential sex differences in treatment with our compound. While there are some sex differences between male and females during behavioral analysis (such as increased immobility during Forced Swim Test and more erratic changes in sucrose preference for non-stressed females), I feel that it is important to accurately portray the population who could benefit from this treatment by including the sex that is more at risk for the disease (Konkras and Dalla 2014; Dalla et al. 2009). This proposed experiment would be crucial in determining another potential route of therapeutics for those suffering from Major Depressive Disorder as a means of protecting the affected from future Major Depressive Episodes. If compound-treated mice did show remarkable differences from vehicle-treated mice, it would also show that LDN/OSU-0212320 may be a potential drug candidate for treating those with MDD.

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