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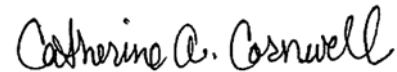
A Comparison of Anxiety-related Behavior on the Elevated Plus Task Following Environmental Enrichment in Two Mouse models for Autism

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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Spring 2019

Honors Capstone Project in Psychology



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Abstract

This study pertained to the effects of environmental enrichment, which is a physical post-weaning treatment that has been shown to induce beneficial changes in behavior, in two mouse models. Autism is a neurodevelopmental disorder that impacts social behavior and communication. It has been found to develop due to both genetic and environmental influences, and to be comorbid with anxiety disorders. The present study assessed anxious behavior, as measured by the elevated plus task, as an indirect symptom of Autism. Maternally separated CD-1 mice were used as a model of environmentally influenced autism and BTBR mice were used as a model of genetically influenced autism. The results indicated that the process of maternal separation did not induce more anxious behavior when compared to controls, but environmental enrichment was found to reduce anxious behavior in both maternally separated animals and controls. They also indicated that there was an effect of strain on behavior when comparing BTBR mice and controls, but no significant effects of enrichment were found.

Executive Summary

Autism spectrum disorder, or autism, is a neurodevelopmental disorder that can affect people of any age. The main symptoms of autism are problems with social situations and communication and repetitive behaviors. These difficulties can negatively impact everyday life for a person that has autism, particularly at work and school. Many people with autism also suffer from other clinical disorders, one of which is anxiety, and this can contribute to the impact of autism.

There is still so much we do not know about autism, so it is the role of researchers to uncover the mysteries of the disorder. Animal models are often used to learn more about autism and how to treat it. These animals are usually rodents because they have some similar behaviors to humans and can reproduce quickly. The following research details work in the Behavioral Neuroscience Lab at Syracuse University and pertains to the use of mice as model animals to learn about autism.

The work investigates anxious behavior on the elevated plus task as an indirect measure of autism due to the comorbidity between the two disorders. Because one of the things that is known about autism is the fact that it is influenced by both genetic and environmental factors, this research used two separate mouse models. Maternally separated CD-1 mice, that were separated from their mothers for three hours a day for the first two weeks of life, were used as an environmental model and BTBR mice, an inbred strain, were used as a genetic model. We then environmentally enriched some of the mice to assess if the enrichment, which entailed providing those animals with more space and stimulating toys, would combat anxious behavior.

The task the mice were tested on consisted of an apparatus raised off the ground that had two open arms and two closed arms. The mice were left undisturbed to explore the enclosure for five minutes. The amount of time spent in the open arms, the number of entries into the open arms, and the percent of time spent in the open arms were recorded. The task is based in the premise that the more

anxious an animal is, the less time they will spend in the open arms and the less times they will enter into the open arms. The average percent of time spent in the open arms for a normal animal is twenty-five percent (Cornwell, 2017).

The results of the experiments showed there was no effect of maternal separation on any of the three measures on the elevated plus; however, there was an effect on enrichment on all three measures of the elevated plus. It was found that enrichment decreased anxious behavior on the elevated plus for both maternally separated mice and controls. This effect of enrichment was not found on any of the elevated plus measures for the BTBR mice, but effects of strain were found for the time in open arms measure and the percent of time in the open arms measure. The enriched BTBR mice exhibited more anxious behavior than the enriched controls.

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This thesis is a culmination of a lot of work from a lot of people. Without the following people, I would not have been able to complete this research.

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Thank you all very much for your assistance and support. I appreciate you all very much.

Advice to Future Honors Students

Dear Future Honors Students,

If you are reading this, then you are probably starting to think about your thesis. If that is the case, then you are nearing the end of your time in honors and I would like to offer you a few pieces of advice.

This first thing is something that you have been told time and time again: **START EARLY!** This may seem daunting, but if you plan ahead and break down the work, it's not so bad. You don't want to be at the end of your senior year and rushing to finish.

The second thing is to use your resources. The honors program provides so many things to help you along. If you need help staying on track, meet with your advisor and make a plan. If you need motivation, go to the writing sessions or boot camp work through things with your peers. If you need money to help your project along, apply for funding. Honors is here to make this experience the easiest possible one for you, so take advantage of that.

Finally, my last piece of advice is to enjoy yourself and your honors experience. Writing a thesis can be challenging at times, but it is also rewarding. You are putting all of this work into studying, understanding, and sharing something that you are passionate about. You should also take interesting classes. Honors classes cover some topics that you can't find anywhere else in the university. Finding something that you don't know much about, but intrigues you, and then learning about it can make college more enjoyable, especially when you have a full semester of challenging classes.

You have all put so much work into getting to this point, but it is paying off. You have all of the capabilities to complete this and accomplish so much more, and in a short while you will be giving advice to future students.

Sincerely,

A handwritten signature in black ink, appearing to read "Khemah Burre". The signature is written in a cursive style with a large, looping initial "K".

A Comparison of Anxiety-related Behavior on the Elevated Plus Task Following Environmental Enrichment in Two Mouse models for Autism.

Introduction

The purpose of this study was to determine if environmental enrichment could influence a measure of anxiety in two animal models of autism. BTBR mice are bred to exhibit autistic-like behaviors. This makes BTBR mice a good proxy for people who develop autism due to genetic factors. Evidence from our past work suggests that maternal separation of the outbred mouse strain, CD-1 mice, during the first two weeks of life, produces autistic-like behaviors that can be prevented by environmental enrichment. This makes maternally separated CD-1 mice a good proxy for people who develop autism due to environmental factors.

Autism spectrum and anxiety are both disorders that affect many people and can be very debilitating. Autism spectrum disorder is a neurodevelopmental disorder that affects approximately 1 in 68 children in varying degrees and is characterized by socio-communication deficits and repetitive thoughts and behaviors (Burrows, Timpano & Uddin, 2017). It has also been found that there is a comorbidity between autism and generalized anxiety disorder—meaning that many people that are diagnosed with autism are also diagnosed with generalized anxiety disorder. Anxiety and other subclinical symptoms, such as depression, are widespread in individuals that have autism spectrum disorder and the combination of these afflictions impair function over time (Burrows et al., 2017). It is because of this impairment of function that autism has become a popular topic in the neuroscience and psychology fields. The fact that the symptoms of autism last over the full span of that person's lifetime drives scientists to want

to learn more about autism and how to treat it. One way to learn more about autism is through the use of model animals which produce autistic symptoms analogous to those seen in humans.

Rodents such as rats and mice, are popular animal models because they are easily housed, their development and behaviors are easily observable and relatively similar to humans, and they have a short enough life cycle that you can test multiple generations (Melina, 2010). However, there are still certain conditions that must be met in order for the rodents to be considered good models for specific disorders, including the factors thought to be responsible for the development of the disorder in humans. In the case of autism, researchers have focused on genetic animal models, since genetic factors are thought to be important contributors to autism in humans (Bailey et al., 1995). An example is the BTBR mouse strain.

A 2008 study by McFarlane et al. entitled “Autism-like behavioral phenotypes in BTBR T+tf/J mice” exhibited the usefulness of BTBR mice as autistic models. The study tested the mice on various tasks directly related to autistic symptoms in humans and then compared them to controls in order to gain understanding of BTBR behavior. The mice displayed “reduced social approach, low reciprocal social interaction, and impaired juvenile play” (McFarlane et al., 2008). The BTBR behavior in regard to social transmission of food preference and high levels of self-grooming indicate social and communication difficulties and repetitive behaviors, respectively (McFarlane et al., 2008). With these both being core symptoms of autism in humans, BTBR mice displaying these characteristics make them strong genetic models for autism.

Autism can also develop due to environmental factors (Homberg et al., 2016; Grabrucker, 2013; Hegarty et al., 2019); however, animal models have not focused on this

possibility in autism. Previous work, however, has investigated the environmental models of other disorders, such as anxiety. Research conducted on rats by Francis, Diorio, Plotsky, and Meaney (2002) has used maternal separation (MS), which is a type of early life adversity model involving removing rodent pups from their mothers for three hours a day during the first two weeks of life. MS is most effective during the first two weeks of life because that period is critical to the pups' development (Fox, 1965). In the study, Francis et al. found that maternal separation induced deficits in behavior on an anxiety task. This can be thought of as being representative of anxiety disorders in humans. The study also tested the effectiveness of environmental enrichment (EE) as a post-weaning rearing treatment. EE is a procedure which involves housing groups of rodents in cages containing toys, tunnels, and running wheels for various amounts of time to produce neurophysiological and behavioral changes, which are generally interpreted as beneficial (Brenes et al., 2006). Francis et al. (2002) found that environmental enrichment is able to prevent these deficits and anxious behaviors.

Following suit of this research, our lab has conducted research that involves MS and EE. The following study was conducted as a continuation of past work that indicated MS induced autistic-like behaviors in animal models. We focused on assessing anxious behavior as an indirect symptom of autism based on the comorbidity between the two disorders. We maternally separated CD-1 mice in order to assess whether MS would induce anxious behavior in the mice as measured by the elevated plus task. We also environmentally enriched some of these mice in order to assess if the enrichment could reduce the extent of anxious behaviors in CD-1 mice on the elevated plus task. In addition, we investigated whether environmental enrichment of BTBR mice would reduce anxious behavior in these mice on the elevated plus

task. Both the MS CD-1 mice and the BTBR mice were compared to controls of non-maternally separated CD-1 mice as they serve as a better model of the genetic diversity in humans (Hsieh, Wen, Miyares, Lombroso, and Bordey, 2016). We had three hypotheses for these experiments. Hypothesis 1 and 2 pertained to the MS experiments and hypothesis 3 pertained to the BTBR experiments. Hypothesis 1 was that maternal separation would increase anxious behavior on the elevated plus task when compared to controls. Hypothesis 2 was that environmental enrichment would reduce anxious behavior on the elevated plus task in MS CD-1 mice. Hypothesis 3 was that environmental enrichment would reduce anxious behavior on the elevated plus task in the BTBR mice.

Methods

This study used 41 CD-1 mice that were born in the lab to breeder mice received from Charles River Laboratories. The CD-1 mice were split into four groups—maternally separated then enriched (MSE) mice ($n = 14$), maternally separated and then standard reared (MSS) mice ($n = 16$), enriched controls ($n = 9$), and standard reared controls ($n = 10$). The mice were born on postnatal day 0. During postnatal days 1-14, the maternal separation procedure was implemented for the MS mice and control mice were left undisturbed with their mothers. Postnatal days 15-20 all mice were left with their mothers. On postnatal day 21 all mice were sexed and weaned, and they were placed into same-sex cohorts of 3-5 mice. Their housing was determined to be either enriched or standard. During days 22 through 35 or 36, the mice were

left in their respective environments and the enriched environments had their dirty toys replaced with clean ones every other day. The animals were tested on postnatal day 36 or 37.

This study used 13 BTBR mice that were born in the lab from breeder mice procured from Jackson Laboratories. The BTBR mice were split into two groups—enriched ($n = 6$) and standard ($n = 7$) housed. They were compared to 8 non maternally separated CD-1 mice that were used as controls. Half of the controls were enriched ($n = 4$), and half were reared in standard environments ($n = 4$). All mice were left undisturbed with their mothers during postnatal days 1-20. On postnatal day 21 they were sexed, weaned, and placed into same-sex cohorts of 3-5 mice that were determined to be either enriched or standard housed. They were tested on postnatal days 37- 40.

Maternal Separation

The maternal separation process that we used mimicked that of the Francis et al study. The rodent pups were separated from their dams for three hours a day from postnatal day 1 to postnatal day 14. After the dam was placed into a holding cage away from the pups, the pups were weighed, and their general health was assessed. During the three hours, the pups were placed in a heat-controlled room.

Environmental Enrichment

The environmental enrichment procedure that was followed also mimicked the Francis et al. study. It entailed altering the housing of the mice after weaning at postnatal day 21. The enriched cages were larger than standard cages at 18.5" x 10" x 8.25" and 17.5" x 9" x 6.25", respectively. The enriched cages also contained toys. Each enriched cage had a running wheel,

a tube, a small ball, a star-shaped container, and a ring or cookie cutter. The used toys were replaced with clean toys every other day (see Figures 1 and 2).



Figure 1. Enriched Housing. This figure displays CD-1 mice in an enriched housing environment.



Figure 2. Standard housing. This figure displays CD-1 mice in a standard housing environment.

Elevated Plus Task (EP)

The elevated plus task is an assessment of anxious behavior in rodents. It has four arms that are connected by a center square to form a cross-like shape (see Figure 3). Two of the arms are walled on three sides. These are considered the closed arms. Two of the arms are not walled and are considered the open arms. The two open arms are across from each other and

the two closed arms are across from each other. For the purpose of testing, the EP apparatus was elevated 25 inches off the ground. The mouse was left to explore the enclosure without interference for five minutes. The amount of time spent in the open arms and the number of entries into the open arms was recorded. The percentage of time spent in the open arms is then calculated. The premise of the task is that fear of falling will encourage the mouse to spend most of its time in the closed arms. Generally, about 25% of the time is spent in the open arms. The more time spent in the open arms, the less anxious the mouse is perceived to be (Cornwell, 2017).



Figure 3. Elevated plus task. This the set-up of the of the elevated plus apparatus used for testing in our lab.

Results

Independent samples analysis of variance (ANOVA) tests were conducted to compare the effect of environmental enrichment on time in the open arms, entries into the open arms, and percent of time spent in the open arms based on strain and treatment for both the MS

mice and the BTBR mice. In the MS study, we found that there was no significant effect of pre-weaning on the amount of time spent in the open arms, the number of entries into the open arms, or the percent of time spent in the open arms, $F(1, 45) = 0.17$ $p = .68$; $F(1, 45) = 0.006$ $p = .94$; $F(1, 45) = 0.19$ $p = .67$, at the $p < .05$ level (see Table 1). For each measure, time in open arms, entries into the open arms, and percent of time spent in the open arms, a significant effect treatment was found at the $p < .05$ level, $F(1, 45) = 9.8$, $p = .003$; $F(1, 45) = 16.8$, $p < .001$; $F(1, 45) = 9.8$, $p = .003$, (see Table 1). These treatment effects were due to the enriched MS mice spending more time in the open arms than standard MS mice ($p = .025$), entering into the open arms more than standard MS mice ($p = .003$), and spending a larger percent of time in the open arms than standard MS mice ($p = .025$). These treatment effects were also due to the enriched control mice spending more time in the open arms than standard control mice ($p = .046$), entering into the open arms more than standard control mice ($p = .016$), and spending a larger percent of time in the open arms than standard control mice ($p = .046$). Data for the various comparisons are presented in Figures 4-6.

		df	F	p
Open Arm	Pre-weaning	(1,45)	.171	.681
	Enrichment	(1,45)	9.818	.003
	Pre-wean*Enrichment	(1,45)	.014	.906
Open Entries	Pre-weaning	(1,45)	.006	.941
	Enrichment	(1,45)	16.823	< .001
	Pre-wean*Enrichment	(1,45)	.006	.941
% Open Arm Time	Pre-weaning	(1,45)	.188	.666
	Enrichment	(1,45)	9.843	.003
	Pre-wean*Enrichment	(1,45)	.019	.892

Table 1- Maternally Separated vs. Control CD-1 Mice F Table

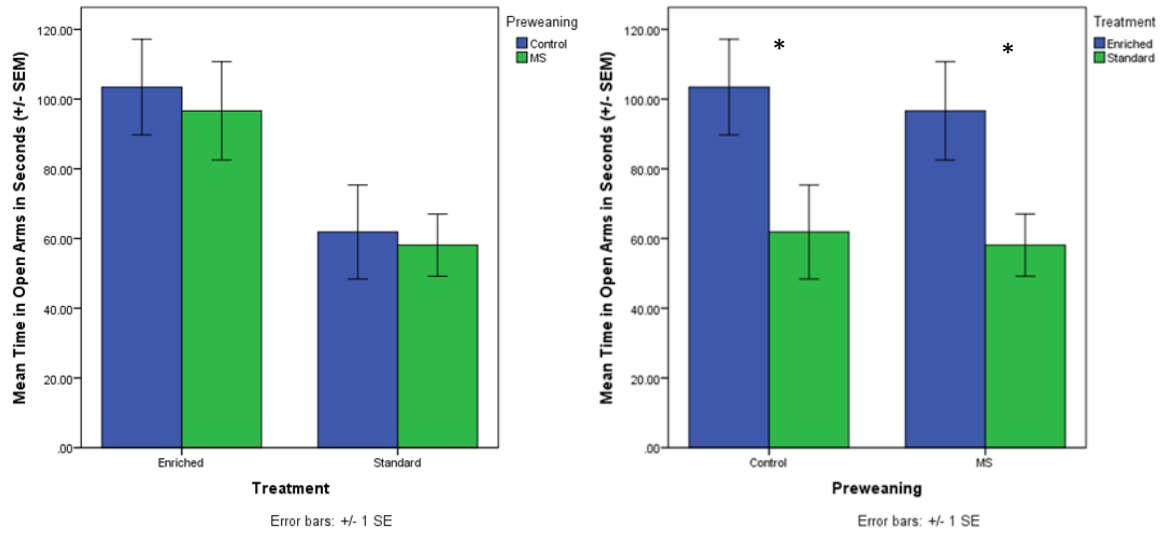


Figure 4. MS vs Controls: Time in Open Arms. This figure displays the mean amount of time spent in the open arms of the elevated plus apparatus for enriched and standard reared maternally separated and non-maternally separated CD-1 mice. Note: * indicates significance.

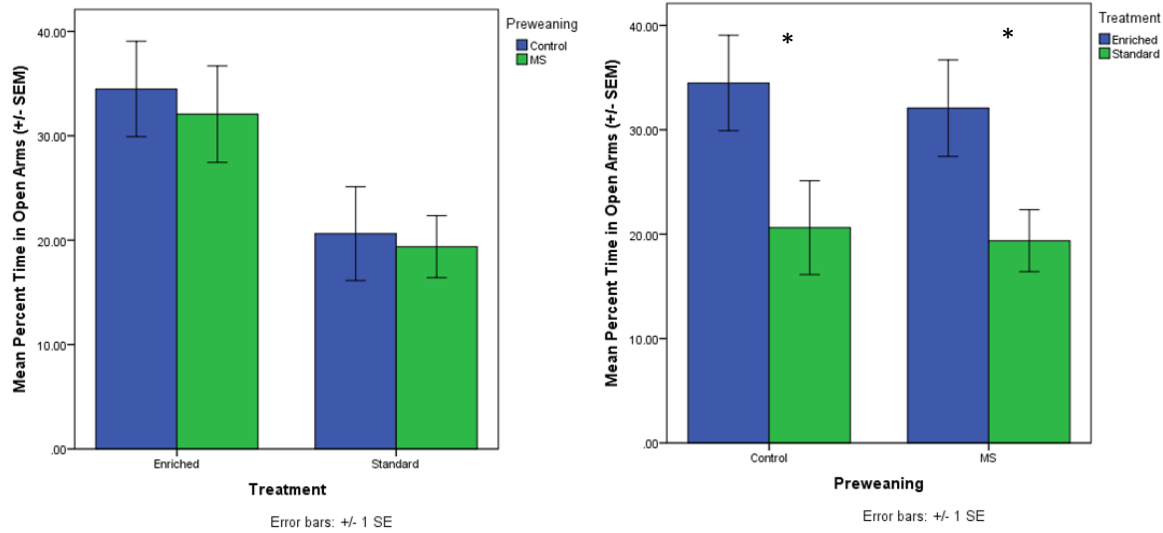


Figure 5. MS vs Controls: Percent Time in Open Arms. This figure displays the mean percent of time spent in the open arms of the elevated plus apparatus for enriched and standard reared maternally separated and non-maternally separated CD-1 mice. Note: * indicates significance

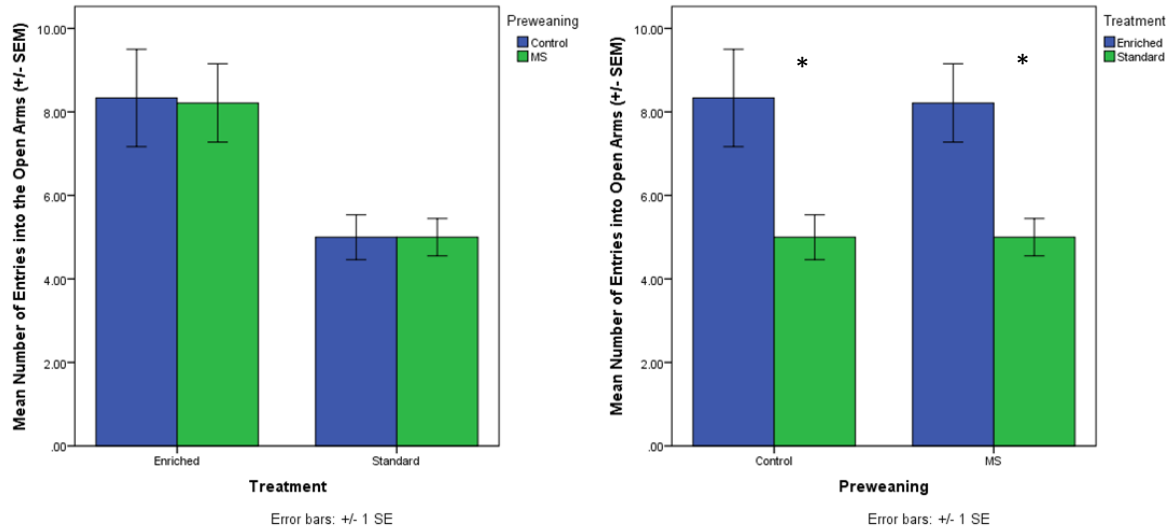


Figure 6. MS vs Controls: Open Arm Entries. This figure displays the mean number of entries into the open arms of the elevated plus apparatus for enriched and standard reared maternally separated and non-maternally separated CD-1 mice.

Note: * indicates significance

In the BTBR study, there was a significant effect of strain on the time spent in the open arms at the $p < .05$ level, but there was no significant effect of treatment on time in the open arms, $F(1, 17) = 4.56, p = .048$; $F(1, 17) = 0.55, p = .33$. The strain effect was due to the enriched BTBR mice spending less time in the open arms compared to enriched controls. This difference was not significant at the $p < .05$ level but was numerically close to significance ($p = .066$). We found no significant effects of strain or treatment on number of entries into the open arms, $F(1, 17) = 1.66, p = .22$; $F(1, 17) = 1.01, p = .48$. A significant strain effect was found at the $p < .05$ level for percent of time spent in the open arms, $F(1, 17) = 5.16, p = .036$. This effect was also due to the enriched BTBR mice spending smaller percentage of the overall time exploring the open arms. This difference was not statistically significant but was numerically close to significance ($p = .065$). No significant effect of treatment was found for the percent of time in the open arms, $F(1, 17) = 2.41, p = .14$.

		df	F	p
Open Arm	Strain	(1,17)	4.558	.048
	Enrichment	(1,17)	1.008	.329
	Strain*Enrichment	(1,17)	0.463	.505
Open Entries	Strain	(1,17)	1.658	.215
	Enrichment	(1,17)	0.553	.467
	Strain*Enrichment	(1,17)	2.377	.142
% Open Arm Time	Strain	(1,17)	5.164	.036
	Enrichment	(1,17)	2.413	.139
	Strain*Enrichment	(1,17)	.793	.386

Table 2- BTBR vs Control CD-1 Mice F Table

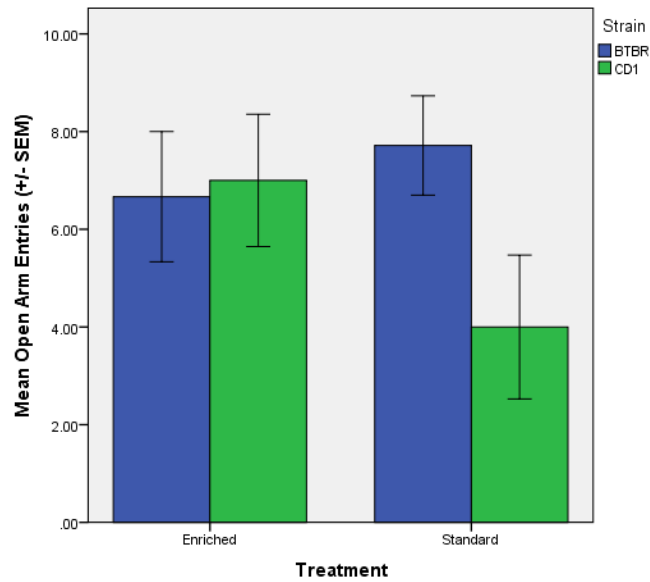


Figure 7. BTBR vs Control: Open Arm Entries. This figure displays the mean number of entries into the open arms or the elevated plus apparatus for both enrich and standard reared BTBR mice and control CD-1 mice.

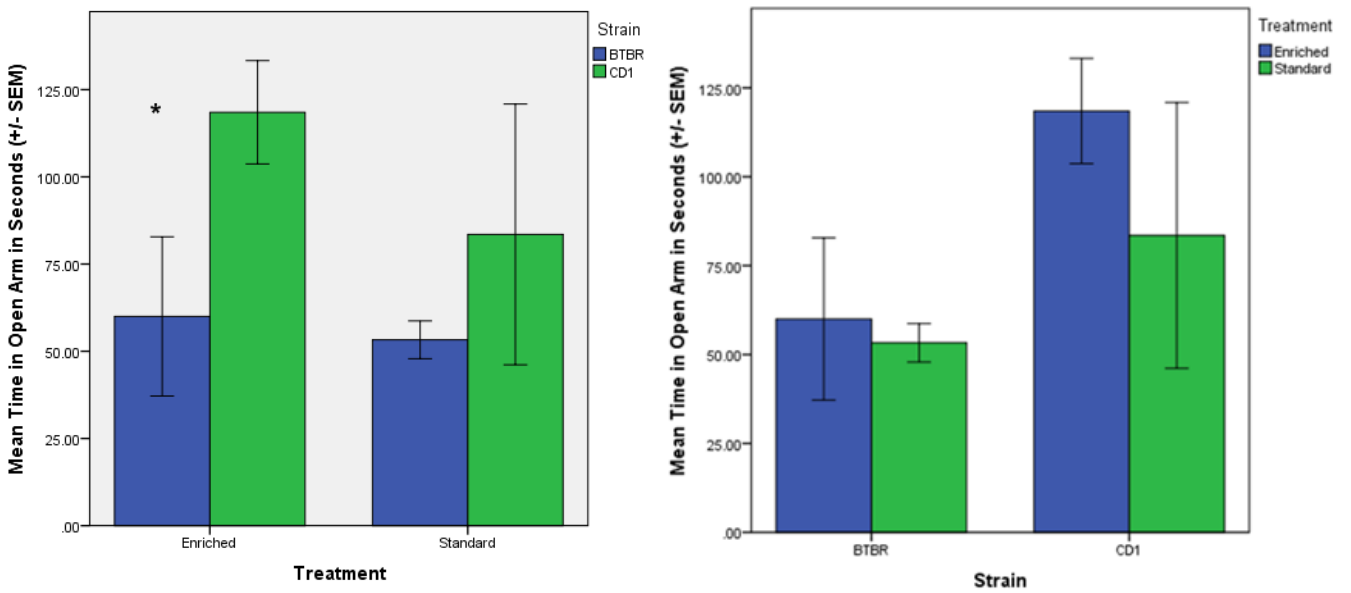


Figure 8. BTBR vs Control: Time in Open Arms. This figure shows the mean amount of time spent in the open arms of the elevated plus apparatus for both enriched and standard reared BTBR and control CD-1 mice.
 Note: * indicates close to significance

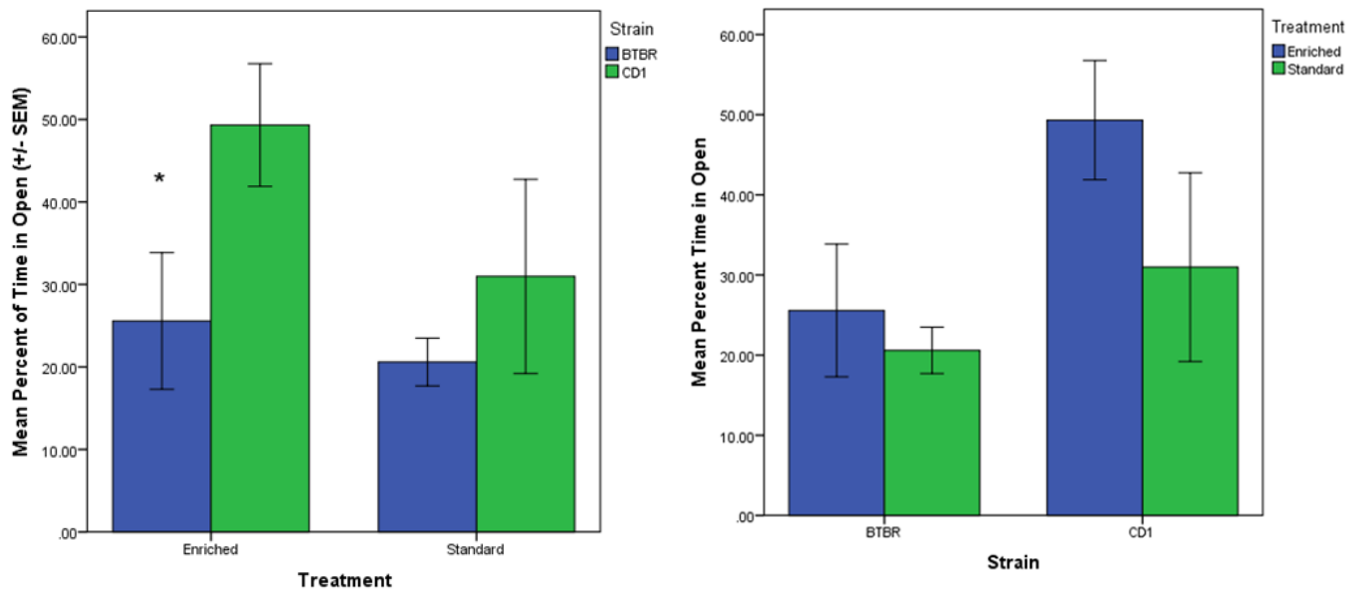


Figure 9. BTBR vs Control: Percent in Open Arms. This figure shows the mean percent of time spent in the open arms on the elevated plus apparatus for both enriched and standard reared BTBR and control CD-1 mice.
 Note: * indicates close to significance

Discussion

This research has some interesting real-world implications. The results of the completed experiments can be summarized in three main points: 1) Maternal separation does not increase anxiety, as measured by behavior on the elevated plus, in CD-1 mice; 2) Anxiety, as measured by the elevated plus is not increased in standard BTBR mice vs standard CD-1 mice; 3) Environmental enrichment reduces anxiety for CD-1 mice, but not BTBR mice.

The first point is indicated by that fact that all standard reared mice in this experiment, regardless of pre-weaning environment, spent approximately the same amount of time in the open arms, approximately the same percent of time in the open arms, and made approximately the same number of entries into the open arms. Standard reared MS mice and standard reared control mice performed similarly on all three measures of the elevated plus task. The same was found for all of the enriched mice, regardless of pre-weaning environment. These findings are directly in contrast with results of the previously discussed Francis et al. study, done on rats, in which the results indicated that maternal separation increased stress reactivity. This may indicate that rats and mice develop and react differently in terms of stress reactivity, and results between the two cannot be generalized. This may also indicate that MS is not as effective for generating anxiety in mice. This suggests that further testing with samples comparing mice and rats would need to be completed.

The second main point is indicated by the fact that the standard reared BTBR mice did not have very different scores on the elevated plus when compared to the standard reared control mice. Both standard BTBR mice and standard control mice spent a percent of time in

the open arms that was not significantly different from the normal 25 percent. Because BTBR mice are an already established model of autism, and anxiety is comorbid with autism, we expected to see increased anxious behavior in the BTBR mice. In this, we were attempting to understand anxiety as an indirect symptom of autism. However, the results garnered in these experiments are consistent with the fact that anxiety is not a core symptom of autism. Other BTBR mice have been tested in our lab on other apparatus that assess social behaviors. These mice have displayed poor results on these tasks. The lack of a significant finding in the elevated plus could indicate that the BTBR strain of mice do not manifest higher levels of anxiety.

The third main conclusion is consistent with the fact that social motivation – seeking and deriving pleasure from social interaction– is reduced in people with autism (Chevallier, Kohls, Troiani, Brodtkin, & Schultz, 2012). We found that environmental enrichment was effective in reducing anxiety on the EP for CD-1 mice, but not BTBR mice. A possible reason for the lack of treatment effects in the BTBR studies is due to the behavior of the BTBR mice in the enriched enclosures being less socially inclined than the controls. This was reflected in the observations made of the BTBR mice during rearing. Researchers in our lab noticed that the BTBR mice took less advantage of the social opportunities in the cage in comparison to the controls. The control mice were very excited to play with the toys, run in the running wheel, and play with each other; while the BTBR mice behaved differently. BTBR mice were not using the toys and running wheels as often and were less prone to playing with each other. If this behavior was consistent, it would mean that the environmental enrichment had less of a chance to influence the mice and would explain the smaller effect.

It is also possible that the lack of treatment effects in the BTBR vs. control studies were due to low numbers of subjects in these groups. The low numbers were due to the seasonal effects on breeding. Breeding is less successful and produces less pups during the winter, which is when our mice were born. The severity of the winter in Syracuse may have also influenced this further. In the case of the BTBR mice, there were also even more specific difficulties with breeding due to their genetics and behavior. Multiple breeding sessions failed with the female breeders not becoming pregnant, which may be due to the less social behavior of the breeder mice. We also had the death of a female breeder. These factors served as limitations in the study, but with the continuation of our work, more animals will be tested, and our power to detect significant effects will be increased.

Based on this research, I believe that looking towards measures of anxiety such as the EP may be inadequate as indications of autism. Further, MS may not be the best model of environmentally influenced autism in CD-1 mice. However, use of these methods may offer some ability to test anxious behaviors in model animals, as well as the effects of enrichment on various behaviors. The treatment effects we found in this study are promising for the development of environmental enrichment as a cheaper, non-pharmacological treatment for humans suffering from anxiety disorders. Our BTBR research has solidified our use of this type of mice as a genetic autistic model. The deficits that they have displayed in the study and others are akin to autistic-like symptoms in humans. If our further testing of BTBR mice garners results that display significant treatment effects, this will provide support for environmental enrichment as a possible treatment for autism.

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