



Assessment of the protective effects of high doses of prenatal L-carnitine on the development of valproic acid induced autism

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Abstract

Autism Spectrum Disorder (ASD) is a developmental disorder in which an individual has difficulty with social interaction, impaired communication skills, and stereotyped repetitive behaviors. The current etiology is not known but the administration of teratogens, such as valproic acid (VPA), during a critical time during development has been shown to produce characteristic symptoms of autism in rats. It has been hypothesized that the dysfunction of the biochemical carnitine, due to VPA, plays a role in the development of autism. Sprague-Dawley rats were tested in adolescence and adulthood to determine if pre-natal carnitine supplementation prevented the development of autism due to VPA administration during a critical time of development. The results were inconclusive, as three out of the four behavioral tests did not show the presence of autism in subjects treated with VPA. It was determined, based on only one behavioral test, that carnitine did not have a protective effect against the development of autism, though further testing is needed to confirm these results before carnitine supplements can be ruled out as a possible protective treatment against the development of autism.

Introduction

Autism is a developmental disorder in which an individual has difficulty with social interaction, impaired communication skills, and stereotyped repetitive behaviors. The large range of severities of the disorder has resulted in the label, Autism Spectrum Disorder (ASD). The autism spectrum is currently divided into three categories: Autistic disorder, Asperger syndrome, and Pervasive Developmental Disorder Not Otherwise Specified (or atypical autism). According to National Institute of Child Health and Human Development, symptoms of autism can be identified in children as early as 18 months of age (Autism 2011).

According to the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM IV), there are three core symptoms of Autism Spectrum Disorder, each of which manifest in a number of different ways. The three core systems are: impairments in social interaction, impairments in communication (mainly spoken language), and restricted repetitive and stereotyped patterns of behavior (American 2000). People affected by autism also exhibit behaviors including lower sensitivity to pain and lower exploratory activity (Schneider and Przewłocki, 2005).

Currently the etiology of autism is not known, but research has shown that there are genetic and developmental components. Males, siblings of those with autism, and people with certain other genetic disorders, such as Fragile X syndrome, are at a higher risk of developing autism. Exposure to one of at least three different teratogens (valproic acid, thalidomide, and ethanol) during early development can result in the development of autism (Christianson et al., 1994; Nanson 1992; Stromland et al., 1994). It may be that a genetic susceptibility, combined with local factors such as teratogens, is what results in the increased frequency of autism.

Treatments for autism range from behavioral therapy (such as behavior management and speech language therapy) to medications. Medications, such as selective serotonin reuptake inhibitors (SSRIs), antipsychotics, and antianxiety drugs, can reduce some of the symptoms in many of those affected by autism. However, to date, according to National Institute of Child Health and Human Development, there is no specific medication that treats or prevents the dysfunction underlying autism (Autism 2011).

Animal models of autism are important in furthering the understanding of the disorder and in the assessment of experimental treatments to minimize risk to humans. A study of patients in the Swedish thalidomide registry found that of about 15 patients exposed to the teratogen, specifically during the 20th to 24th day of gestation, five had autism. This finding suggests that there is a specific window during gestation in which the fetus is especially vulnerable to the development of autism. Before the 20th and 24th day of development the neural tube closes and neurons begin forming the motor nuclei of the cranial nerves. The autistic patients in this study had evidence of injury to the cranial nerve motor nuclei and their projections.

The relationship between the development of autism and teratogen administration during a critical window of development, led to the development and increasingly common use of the valproic acid (VPA) model of autism in laboratory animals (Rodier et al., 1997).

Administration of VPA to pregnant dams on day 12.5 of gestation causes characteristic pathological features of autism in the offspring, such as abnormalities in the cerebellum and cranial nerve nuclei (Rodier et al., 1997). The animals have also been reported to exhibit behavior that is similar to that seen in humans affected by autism, including lower sensitivity to pain and higher sensitivity to non-painful stimuli, locomotor and repetitive behavior hyperactivity with lower exploratory activity and decreased social behaviors (Schneider and

Przewłocki, 2005). Together, the pathological and behavioral abnormalities support that administration of VPA at a critical window in development results in a valid animal model of autism.

Valproic acid is a widely used and highly effective medication for the treatment of epileptic seizures and bipolar disorder. It can reduce seizure frequency by 25 percent or more, even producing complete seizure freedom, in people with previously uncontrolled epilepsy (Mattson et al., 1978). Although VPA is extensively used, it has a broad range of adverse effects. VPA acts as a teratogen when women take it during the first few weeks of pregnancy. It is associated with a one to two percent increase in neural tube closure defects, including spina bifida and more severe forms of abnormal neural tube formation, which can result in paralysis or brain malformations, in newly born infants (Lammer et al., 1987). The means by which VPA interferes with the neural tube or creates autistic symptoms are not fully understood.

VPA has a variety of metabolic effects, including interference with carnitine metabolism. Carnitine has a crucial role in energy production in mitochondria. It helps transport long-chain fatty acids into the mitochondria, allowing the chains to be oxidized to produce ATP. Carnitine also helps with the transport of cellular waste out of the mitochondria. One effect of VPA on carnitine metabolism was demonstrated in 25 patients, which happened to be severely handicapped, ranging in age from 3 to 21 years, treatment with VPA was shown to lower plasma carnitine concentrations. A significant inverse relationship was found between the dosage of VPA and the plasma carnitine concentration. In these patients, it took four weeks of oral administration of D-, L-carnitine (50mg/kg/day) to correct the carnitine deficiency (Ohtani et al., 1982).

Carnitine occurs in two isomer forms, D- and L-, with only L-carnitine being active in the body. This is the isomer present in food, with the highest concentrations found in red meat, fish, and poultry. According to the Office of Dietary Supplements, most carnitine that is ingested is absorbed in the small intestine, stored in the kidneys, and released into the blood stream to maintain stable blood concentrations. Excess carnitine is not stored but excreted in the urine (Dietary 2006).

Two categories of carnitine deficiency exist, labeled primary and secondary carnitine deficiency. Primary carnitine deficiency is due to a genetic disorder of the cellular carnitine-transporter system, which results in an intracellular carnitine deficit. Symptoms include skeletal-muscle weakness, hypoglycemia, and cardiomyopathy. The secondary deficiency arises from external factors, including the use of certain drugs or the existence of certain disorders, such as chronic renal failure, that reduce carnitine absorption or increase carnitine excretion.

Carnitine supplements at low dosages are a safe treatment for both types of carnitine deficiencies. According to the Office of Dietary Supplements, at higher dosages of carnitine (about 3 grams/day), side effects such as nausea, vomiting, abdominal cramps, and diarrhea can occur (Dietary 2006). L-carnitine, acetyl-L-carnitine, and propionyl-L-carnitine are all approved by the Food and Drug Administration to treat primary and certain types of secondary carnitine deficiency syndromes. Clinicians generally prefer to use acetyl-L-carnitine, which the body converts to the active L-carnitine form within the cytosol. Acetyl-L-carnitine is more readily absorbed and more efficiently crosses the blood-brain barrier.

Carnitine deficiency itself may play a role in the development of autism. Autistic children ranging in age from 2-13 years of age were found to have significantly lower free and total blood carnitine levels than normal children. In addition these children had slight elevations in lactate

and significant elevations in alanine and ammonia levels, suggestive of mild mitochondrial dysfunction, possibly related to the carnitine deficiency (Filipek et al., 2004).

Thus, there are several lines of evidence suggesting that carnitine plays a role in the development of autism. It is conceivable that there is a critical prenatal window of time, suggested by the effects of teratogens affecting carnitine, where suboptimal carnitine function could result in the development of autism.

I hypothesize that carnitine plays a vital role in the development of the brain and that disruption of this development, at a critical time, by reduced carnitine levels or function, could result in autism and is the mechanism underlying the VPA model of autism.

Materials and Methods

Subjects

Healthy male and female offspring of Sprague-Dawley rats were studied in adolescence (day 45 – 55 of life) and adulthood (day 75 – 85 of life). The offspring were divided into two treatment conditions and a control condition. The conditions each consisted of four male and four female offspring randomly selected from the pool of offspring produced by healthy dams in each of their respective conditions.

Condition 1 consisted of the offspring of dams that had received an intraperitoneal injection of VPA (470 mg/kg) during day 12.5 after conception, to produce an animal model of autism. Condition 2 consisted of the offspring dams that had received oral treatments of L-carnitine (250 mg/kg/day) on days 10 through 13 after conception and an intraperitoneal injection of VPA (470 mg/kg) during day 12.5 after conception. Condition 3, the control group, consisted of offspring of dams who did not receive any treatment.

Materials

To create the treatments for the different conditions, valproic acid, L-carnitine powder, and peanut butter (to help the rats with eating the L-carnitine powder) were used. The rats' behavior was characterized by a tail-flick nociception test, a novel environment repetitive behavior test, a social behavior test, and a Y-maze exploratory test.

The tail-flick nociception test consisted of a platform, a hotplate, and a 500 mL beaker of water (maintained at 52 degrees Celsius). For the novel environment repetitive behavior test, an Opto-Varimex test cage (Columbus Instruments, Columbus, OH) was used (35x29x16cm). A computer running the associated Opto-Varimex software automatically recorded the movement of the subject and reported the total beam breaks every 2.5 minutes. Each trial was 15 minutes long. For the social behavior test, a clear test cage (50x26x32cm) was used. Four separate observers individually assessed and recorded the social behavior of the subjects. For the Y-maze test, a Y-maze with three equal (27x10x29cm) arms was used along with a novel isolation cage, to hold the subjects between trials.

Procedure

Prenatal and Postnatal Treatment

All dams in same condition were placed into mating cages. On the 10th through 13th day after conception, the dams in Condition 2 were weighed and given a daily oral dose of L-carnitine (250mg/kg/day).

The carnitine pills (Spring Valley L-Carnitine 500mg) were not pure; each pill contained inactive ingredients and weighed approximately 1.3 grams. To make it easier for the subjects to ingest the carnitine, the pills were crushed and mixed with peanut butter. A stock mixture was made with a ratio of 8 grams of peanut butter for every 1.3 grams of carnitine powder. For every

100 grams of body weight, the dams received 0.4 grams of the carnitine/peanut butter mixture to attain the desired amount of 250mg/kg of L-carnitine.

On day 12.5 after conception, dams in treatment Conditions 1 and 2 received a single intraperitoneal injection of 470mg/kg of VPA solution. The solution had a concentration of 370mg/ml of VPA. Dams were housed individually and allowed to raise their own litters. All offspring were weaned on postnatal day 23.

Behavioral Tests

Behavioral testing was performed in two rounds, once during adolescence (day 45-55 of life) and once during adulthood (day 75- 85 of life). All of the offspring went through each of the tests once during adolescence and once during adulthood, with the exception of the control condition, which was only tested during adolescence due to time constraints. All data sheets used can be found in Appendix A.

Nociception – Tail-Flick Test

The Tail-Flick Nociception Test consisted of a platform, a hotplate, and a 500 mL beaker of water maintained at a temperature of 52 degrees Celsius. Each rat was placed on the Tail-Flick platform, in a rodent holder tube with its tail given a free range of motion. The rat was allowed to stay in the rodent holder tube for one minute to relax. After the minute its tail was placed in the water. The time it took for the rat to flick its tail away from the water (due to the pain threshold being reached) was recorded. Tail-flick measurements were taken for two consecutive trials separated by a 60 second interval. The average time of the two trials was analyzed. If the rat did not flick its tail after ten seconds, the trial was ended to prevent tissue damage.

Novel-Environment Repetitive Behavior Test

The Novel-Environment Repetitive-Behavior Test was conducted in an Opto-Varimex test cage. Infrared beams along the x-, y-, and z-axis were used to assess the subject's motion. A corner of the box was labeled as the starting point. Subjects were individually placed in the box at the starting point with their heads facing the corner and allowed to explore for 15 minutes. Motion was recorded by the Opto-Varimex software with totals being reported every 2.5 minutes. The cage was cleaned after every trial. This test was used to assess the subject's repetitive behavior in a novel environment. The amount of repetitive behavior was determined by subtracting the total ambulatory movement from the total movement. In an attempt to reduce error due to low sample sizes, the proportion of repetitive behavior was reported and analyzed to counter large differences in total movement among individuals. The proportion of repetitive behavior was calculated by taking the total repetitive behavior and dividing it by the total movement (Figures 4, 5 and 6).

Social Behavior Test (Adolescence)

The test was performed in a novel test cage. Prior to the test, the subjects were socially isolated, by placing them in separate cages, for 3.5 hours. The isolation period has been shown to produce an increase in the amount of social interaction (Niesink and Van Ree, 1989). The test consisted of placing two animals, from the same condition and of the same sex, into the test cage for 15 minutes. Four separate observers individually assessed and recorded the social behavior of the pair of subjects. Behavior of individual subjects was not analyzed. Frequency of pinning (one of the subjects lying with its dorsal surface on the floor of the test cage with the other subject standing over him/her), total duration of pinning, frequency of social non-play behavior (following or approaching the test partner, mounting or crawling over the test partner, sniffing or

grooming any part of the body of the test partner), and total duration of social non-play behavior were measured.

Social Behavior Test (Adulthood)

The test was performed in a novel test cage. Prior to the test, the subjects were socially isolated, by placing them in separate cages, for 3.5 hours. The isolation period has been shown to produce an increase in the amount of social interaction (Niesink and Van Ree, 1989). The test consisted of placing two animals, from the same condition and of the same sex, into the test cage for 10 minutes. Four separate observers individually assessed and recorded the social behavior of one of the two subjects. The target subject of the pair was chosen randomly immediately prior to the test. Behavior of the second subject was not analyzed. Frequency of social exploration behavior (sniffing or licking any part of the body of the test partner except the anogenital area, crawling or mounting by placing the forepaws on the dorsal or ventral surface of the test partner, approaching or following the test partner), total duration of social exploration behavior, frequency of anogenital investigation (sniffing or licking the anogenital area of the test partner), and total duration of anogenital investigation were measured.

Y-Maze Exploratory Test

Prior to the test, the subject was isolated for 10 minutes in a novel cage. For the test, one of the arms was marked as the starting arm and one of the other arms was blocked off and marked as the novel arm. The subject was allowed to explore the initial two arms for 10 minutes. The subject was then isolated for 10 minutes and the third arm was unblocked and the subject was again allowed to explore for 10 minutes. The time spent within the boundaries of the novel arm and the boundaries of the familiar two arms was recorded as exploratory behavior.

Data Analysis

Data from all of the behavioral tests were analyzed using uni-variant and multi-variant two-way ANOVAs in the SPSS statistical software. If a significant difference was found, a Tukey's HSD post-hoc test was performed by hand.

Results

Data for adult control rats were not obtained due to time constraints. All condition vs. age and adult condition vs. adult gender statistical tests were only performed between the VPA conditions and Carnitine/VPA conditions for all adult behavioral tests.

Tail Flick Test

Adolescents

A two-way ANOVA was conducted to examine the effects of condition and gender on latency to tail flick in a hot water bath in adolescent rats. There was a significant interaction between the effects of condition and gender, $F(2, 18) = 3.544, p = 0.050$. A Tukey's HSD post-hoc test did show a statistically significant difference between VPA males and VPA females ($p < 0.05$). No significant difference was found between conditions ($F(2, 18) = 0.015, p = 0.985$) but there was a significant difference between genders ($F(1, 18) = 3.486, p = 0.078$), with males (4.74 ± 1.47 sec) displaying longer latency than females (3.50 ± 1.98 sec). However, a Tukey's HSD post-hoc test did not show a statistically significant difference ($p > 0.05$) (Figure 1).

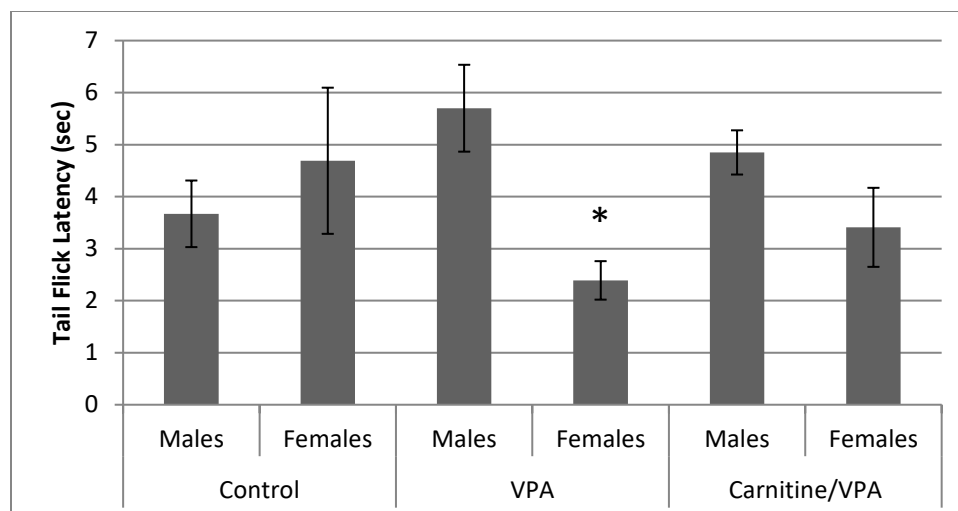


Figure 1: Nociception – Tail Flick Test (adolescent). The data presented are means of the two trials of the hot water tail flick latency in adolescent rats. Data are expressed as mean $\pm$ SEM, $n=4$ for each group. * $p < 0.05$ male rats prenatally exposed to VPA compared to female rats prenatally exposed to VPA (Tukey's HSD Test).

Adults

A two-way ANOVA was conducted that examined the effect of condition and gender on latency to tail flick in a hot water bath in the adult rats. There was no significant interaction between the effects of condition and gender on pain threshold, $F(1, 12) = 0.059$, $p = 0.812$. No significant difference was found between conditions ($F(1, 12) = 1.560$, $p = 0.235$) or between genders ($F(1, 12) = 0.260$, $p = 0.619$) (Figure 2).

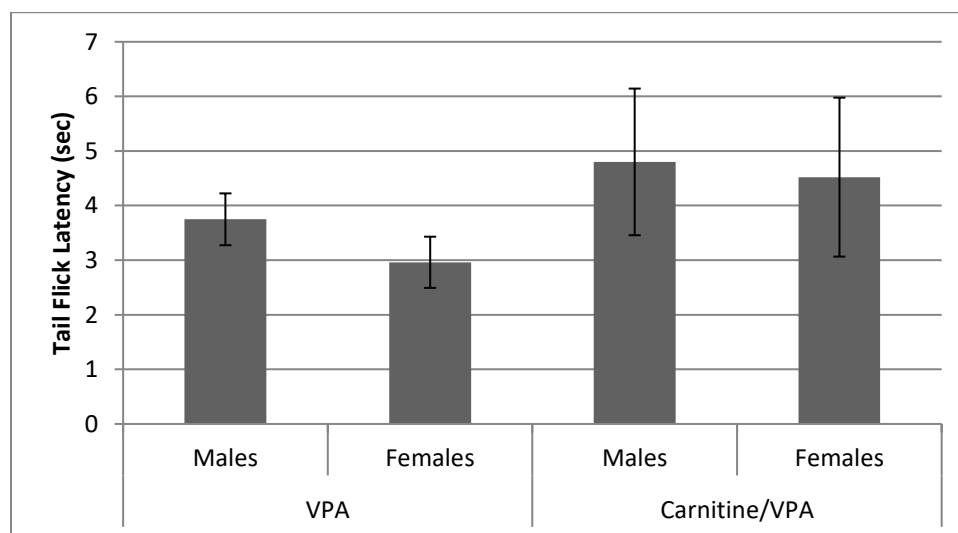


Figure 2: Nociception – Tail Flick Test (adult). The data presented are the means of the two trials of the hot water tail flick latency in adult rats. Data are expressed as mean $\pm$ SEM, $n=4$ for each group.

Adolescents vs. Adults

A two-way ANOVA was conducted that examined the effect of condition and age on latency to tail flick in a hot water bath. There was no significant interaction between the effects of condition and age on pain threshold, $F(1, 28) = 0.838$, $p = 0.368$. No significant difference was found between conditions ($F(1, 28) = 1.096$, $p = 0.304$) or between ages ($F(1, 28) = 0.014$, $p = 0.906$) (Figure 3).

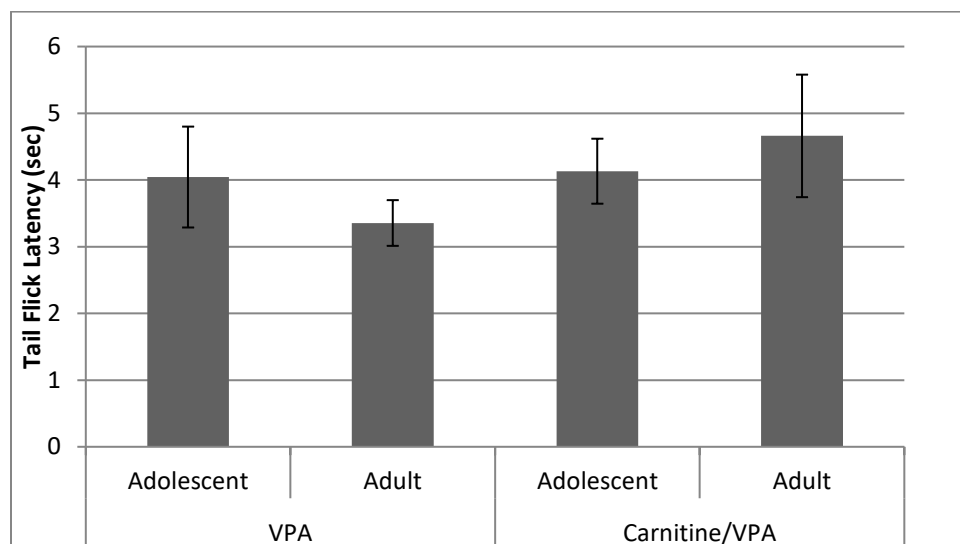


Figure 3: Nociception – Tail Flick Test (adolescent vs. adult). The data presented are the means of the two trials of the hot water tail flick latency. Data are expressed as mean $\pm$ SEM, $n=8$ for each group.

Novel Environment Locomotion Test

Adolescents

A two-way ANOVA was conducted that examined the effect of condition and gender on the proportion of repetitive behavior performed in a novel environment. There was no significant interaction between the effects of condition and gender on the proportion of repetitive behavior performed in a novel environment, $F(2, 18) = 0.410$, $p = 0.670$. No significant difference was found between conditions ($F(2, 18) = 1.523$, $p = 0.245$) but a statistically significant difference between genders ($F(1, 18) = 5.463$, $p = 0.031$) was found, with males ($30.65 \pm 2.84\%$) performing proportionally more repetitive behavior than females ($27.90 \pm 2.90\%$). However, a

Tukey's HSD post-hoc test did not show a statistically significant difference ($p > 0.05$) (Figure 4a).

A two-way ANOVA was conducted that examined the effect of condition and gender on the amount of ambulatory movement performed in a novel environment. There was no significant interaction between the effects of condition and gender on the amount of ambulatory movement performed in a novel environment, $F(2, 18) = 0.018$, $p = 0.982$. A significant difference was found between conditions ($F(2, 18) = 11.786$, $p = 0.001$). A Tukey's HSD post-hoc test did show a statistically significant difference ($p < 0.05$) between the control condition (1309.13 ± 319.88) and the VPA condition (631.63 ± 282.91) and the control condition (1309.13 ± 319.88) and the Carnitine/VPA condition (798.50 ± 261.16). No statistically significant difference was found between genders ($F(1, 18) = 2.702$, $p = 0.118$) (Figure 4b).

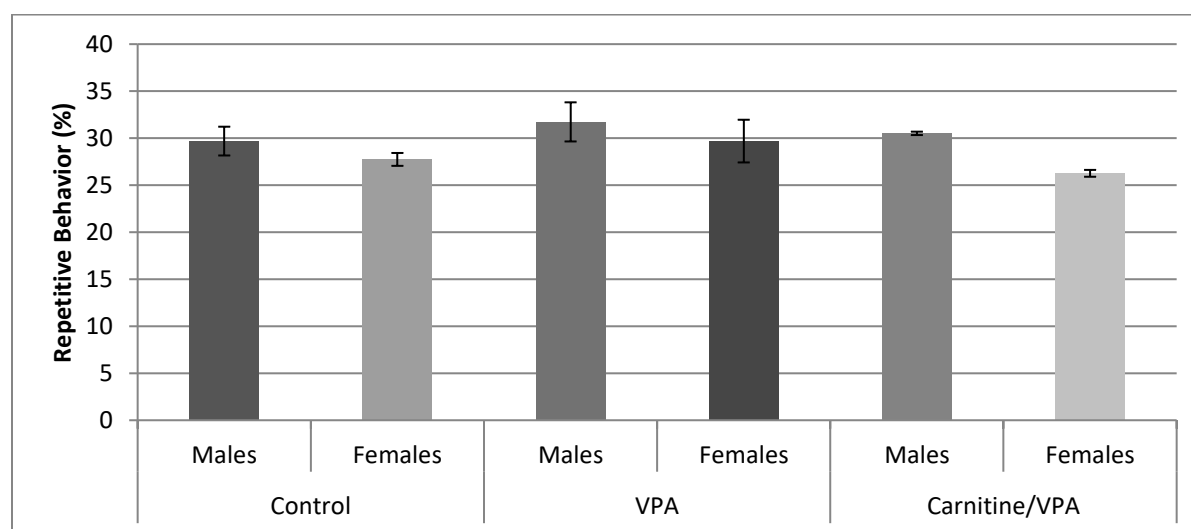


Figure 4a: Novel Environment Locomotion Test (adolescent). Proportion of repetitive behavior performed in a novel environment by adolescents. The data presented was calculated for each group by taking the total amount of repetitive behavior movement divided by the total movement in the Opto-Varimex and multiplying by 100. Data are expressed as mean $\pm$ SEM, $n=4$ for each group.

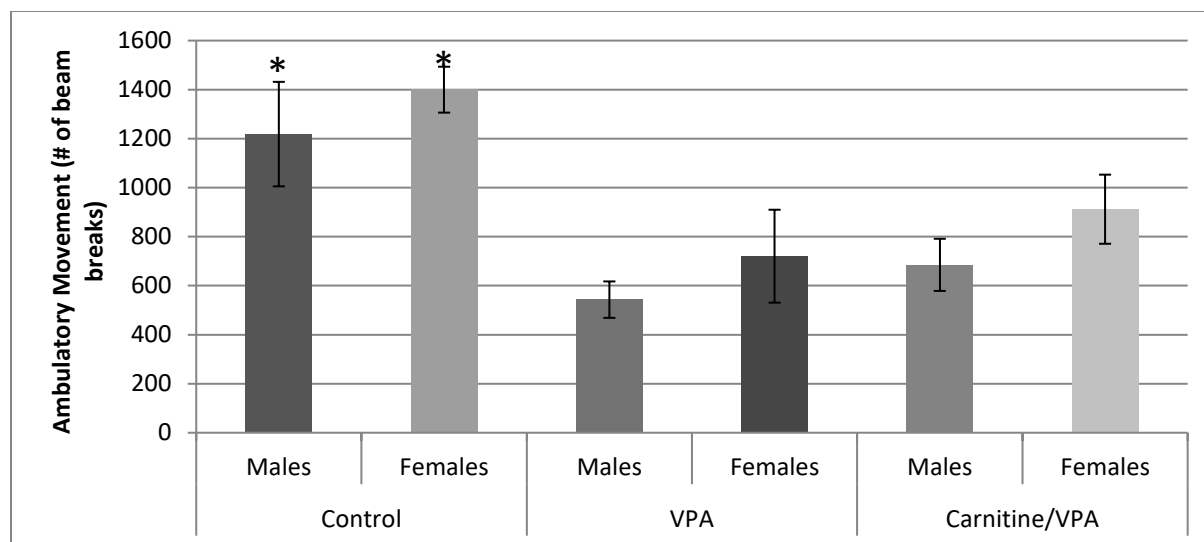


Figure 4b: Novel Environment Locomotion Test (adolescent). Ambulatory movement performed in a novel environment by adolescents. The data presented was determined by the number of infrared beam breaks in the Opto-Varimex test cage during a 15 minute test. Data are expressed as mean $\pm$ SEM, $n=4$ for each group. * $p < 0.05$ control rats had more beam breaks compared to VPA rats and Carnitine/VPA rats (Tukey's HSD Test).

Adults

A two-way ANOVA was conducted that examined the effect of condition and gender on the proportion of repetitive behavior performed in a novel environment. There was a significant interaction between the effects of condition and gender on the proportion of repetitive behavior performed in a novel environment, $F(1, 12) = 4.768$, $p = 0.050$, with VPA males ($30.22 \pm 1.75\%$) performing proportionally more repetitive behavior than VPA females ($22.51 \pm 1.43\%$) and carnitine/VPA males ($28.04 \pm 2.55\%$) performing proportionally more repetitive behavior than carnitine/VPA females ($27.60 \pm 5.72\%$). A Tukey's HSD post-hoc test did show a statistically significant difference ($p < 0.05$) between VPA males and VPA females. No significant difference was found between conditions ($F(1, 12) = 0.765$, $p = 0.399$) but a statistically significant difference between genders ($F(1, 12) = 6.009$, $p = 0.031$) was found, with males ($29.13 \pm 2.33\%$) performing proportionally more repetitive behavior than females ($25.05 \pm 4.72\%$). A Tukey's HSD post-hoc test did show a statistically significant difference ($p < 0.05$) between males and females (Figure 5a).

A two-way ANOVA was conducted that examined the effect of condition and gender on the amount of ambulatory movement performed in a novel environment. There was no significant interaction between the effects of condition and gender on the amount of ambulatory movement performed in a novel environment, $F(2, 18) = 0.861$, $p = 0.372$. No significant difference was found between conditions ($F(2, 18) = 1.115$, $p = 0.312$). No statistically significant difference was found between genders ($F(1, 18) = 3.170$, $p = 0.100$) (Figure 5b).

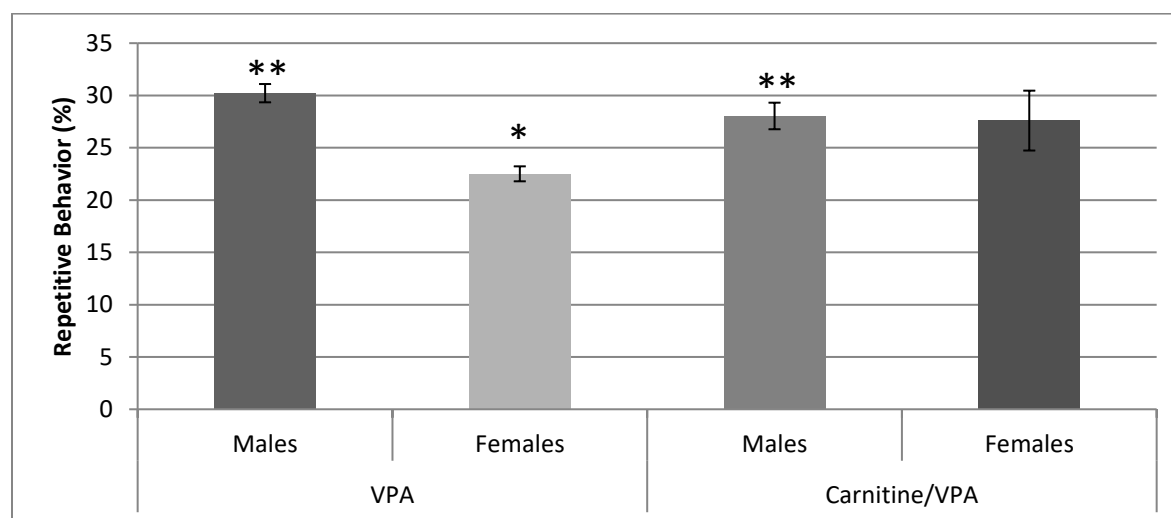


Figure 5a: Novel Environment Locomotion Test (adult). Proportion of repetitive behavior performed in a novel environment by adults. The data presented was calculated for each group by taking the total amount of repetitive behavior movement divided by the total movement in the Opto-Varimex and multiplying by 100. Data are expressed as mean $\pm$ SEM, $n=4$ for each group. * $p < 0.05$ for male rats prenatally exposed to VPA produced more repetitive behavior compared to female rats prenatally exposed to VPA (Tukey's HSD Test). ** $p < 0.05$ for male rats produced more repetitive behavior compared to female rats (Tukey's HSD Test).

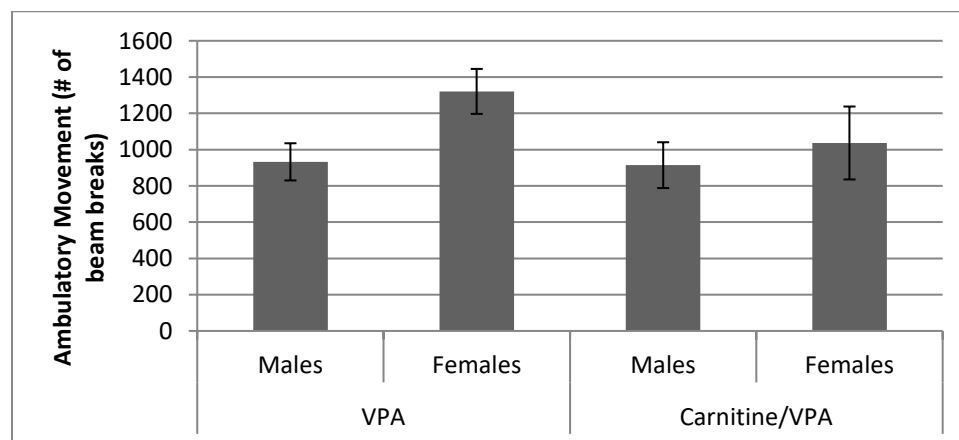


Figure 5b: Novel Environment Locomotion Test (adult). Ambulatory movement performed in a novel environment by adults. The data presented was determined by the number of infrared beam breaks in the Opto-Varimex test cage during a 15 minute test. Data are expressed as mean $\pm$ SEM, $n=4$ for each group.

Adolescents vs. Adults

A two-way ANOVA was conducted that examined the effect of condition and age on the proportion of repetitive behavior performed in a novel environment. No significant interaction between the effects of condition and age on the proportion of repetitive behavior performed in a novel environment was found, $F(1, 28) = 1.937, p = 0.175$. No significant difference was found between conditions ($F(1, 28) = 0.102, p = 0.752$) but a statistically significant difference between ages ($F(1, 28) = 3.269, p = 0.081$) was found, with adolescents ($29.55 \pm 3.48\%$) performing proportionally more repetitive behavior than adults ($27.09 \pm 4.17\%$). However, a Tukey's HSD post-hoc test did not show a statistically significant difference ($p > 0.05$) between adolescents and adults (Figure 6a).

A two-way ANOVA was conducted that examined the effect of condition and gender on the amount of ambulatory movement performed in a novel environment. There was no significant interaction between the effects of condition and gender on the amount of ambulatory movement performed in a novel environment, $F(1, 28) = 2.406, p = 0.132$. No significant difference was found between conditions ($F(1, 28) = 0.006, p = 0.940$). A statistically significant difference was found between ages ($F(1, 28) = 10.730, p = 0.003$), with adolescent rats (715.06 ± 276.78) performing less ambulatory movement than adult rats (1051.06 ± 306.69). A Tukey's HSD post-hoc test did show a statistically significant difference ($p < 0.05$) between adolescents and adults (Figure 6b).

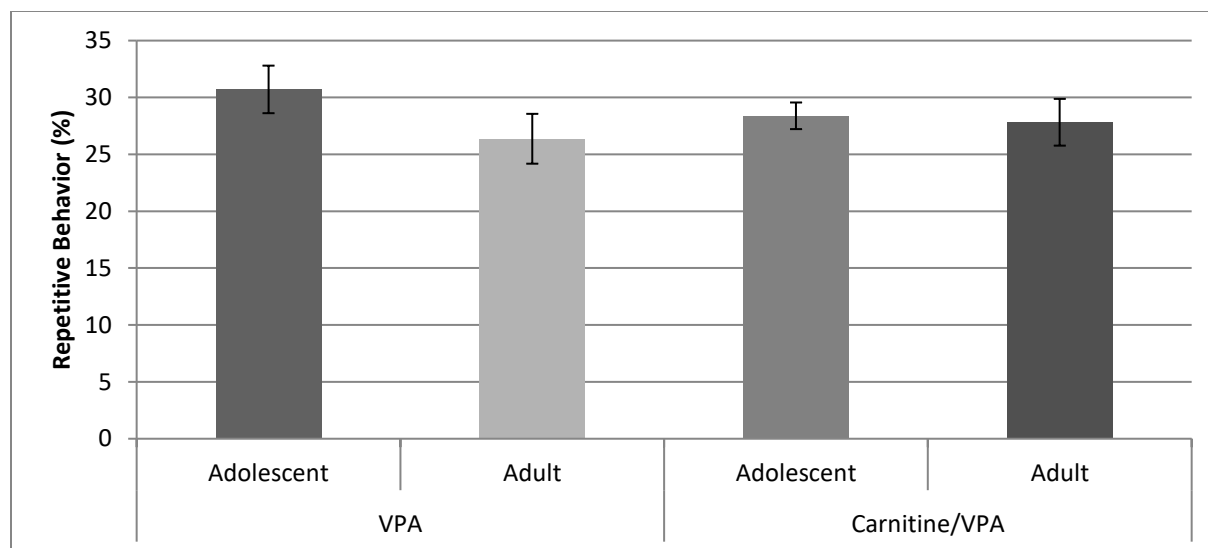


Figure 6a: Novel Environment Locomotion Test (adolescent vs. adult). Proportion of repetitive behavior performed in a novel environment. The data presented was calculated for each group by taking the total amount of repetitive behavior movement divided by the total movement in the Opto-Varimex and multiplying by 100. Data are expressed as mean $\pm$ SEM, $n=8$ for each group.

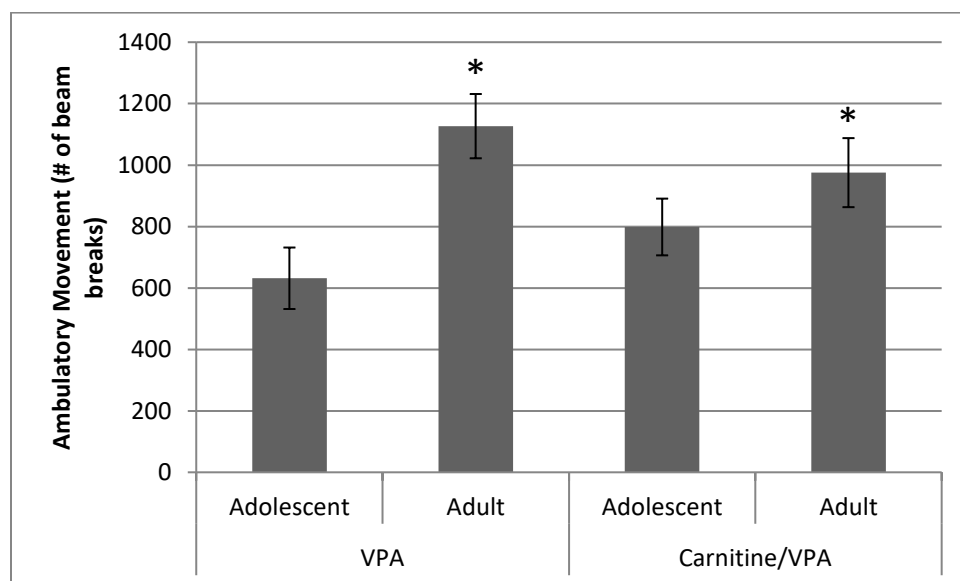


Figure 6b: Novel Environment Locomotion Test (adolescent vs. adult). Ambulatory movement performed in a novel environment by adults. The data presented was determined by the number of infrared beam breaks in the Opto-Varimex test cage during a 15 minute test. Data are expressed as mean $\pm$ SEM, $n=8$ for each group. * $p < 0.05$ for adult rats making more beam breaks compared to adolescent rats (Tukey's HSD Test).

Social Behavior Test

Adolescents

A two-way ANOVA was conducted that examined the effect of condition and gender on social behavior of the adolescent rats. There was no significant interaction between the effects of condition and gender on frequency of social non-play behavior ($F(2, 6) = 0.356, p = 0.714$), total duration of social non-play behavior ($F(2, 6) = 0.136, p = 0.876$), frequency of pinning ($F(2, 6) = 0.019, p = 0.982$), or total duration of pinning ($F(2, 9) = 0.298, p = 0.752$). No significant difference was found between conditions for the frequency of social non-play behavior ($F(2, 6) = 1.437, p = 0.309$), total duration of social non-play behavior ($F(2, 6) = 0.435, p = 0.666$), frequency of pinning ($F(2, 6) = 1.736, p = 0.254$), or total duration of pinning ($F(2, 6) = 0.439, p = 0.664$) or between genders for the frequency of social non-play behavior ($F(1, 6) = 3.118, p = 0.128$), total duration of social non-play behavior ($F(1, 6) = 0.207, p = 0.665$), frequency of pinning ($F(1, 6) = 0.523, p = 0.497$), or total duration of pinning ($F(1, 6) = 0.765, p = 0.415$) (Figures 7 and 8).

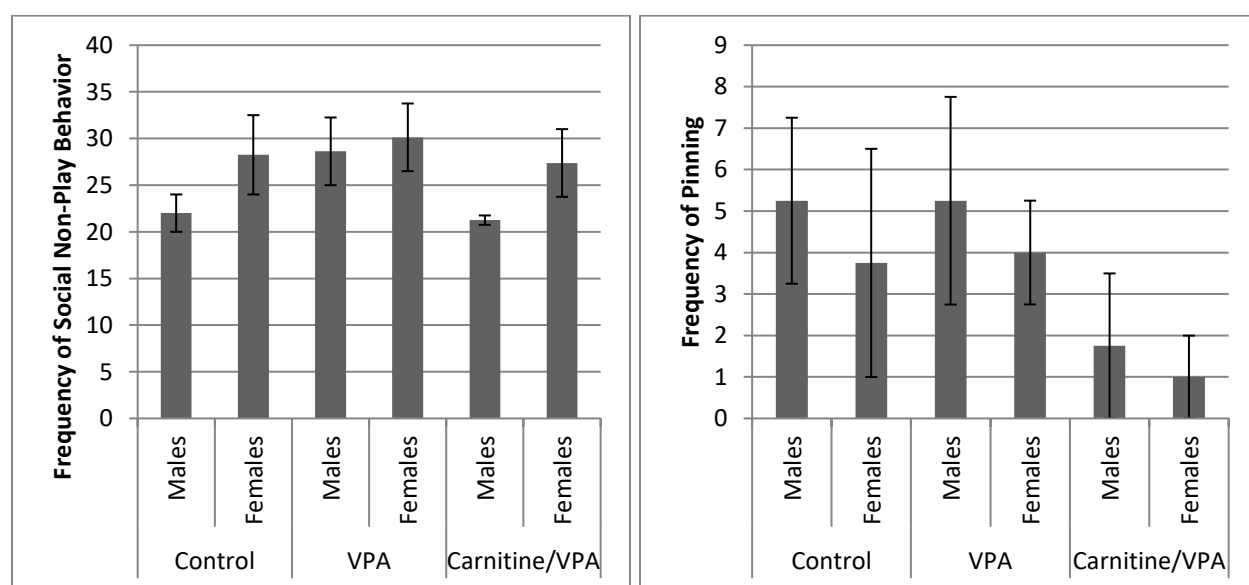


Figure 7: Social Behavior Test (adolescent). Frequency of social non-play behavior (left) and frequency of pinning (right) measured for a pair of subjects of the same condition during a 15 minute test. Data are expressed as mean $\pm$ SEM, $n=2$ for each group.

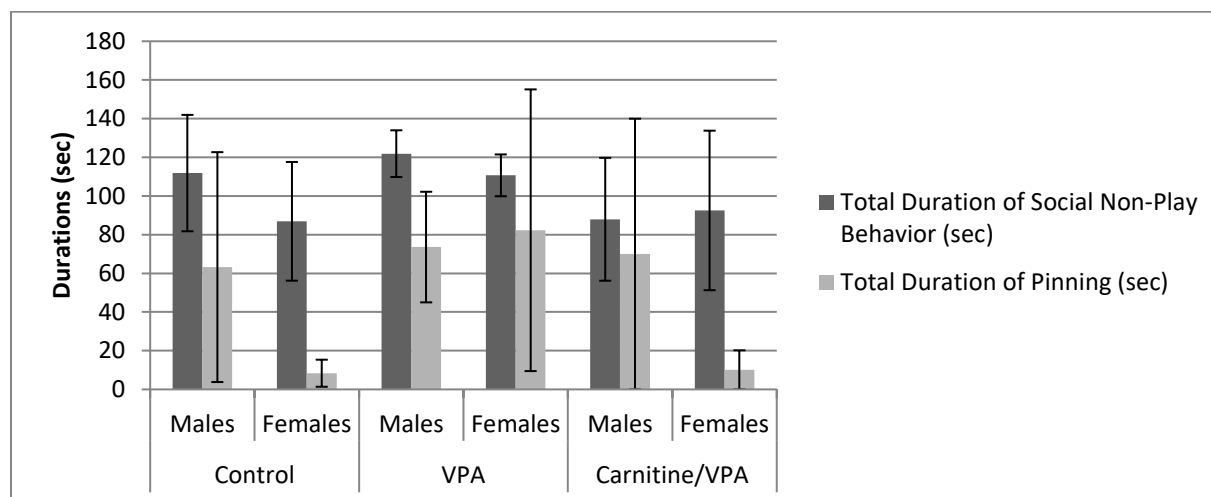


Figure 8: Social Behavior Test (adolescent). Duration of social non-play behavior and duration of pinning measured for a pair of subjects of the same condition during a 15 minute test. Data are expressed as mean $\pm$ SEM, $n=2$ for each group.

Adults

A two-way ANOVA was conducted that examined the effect of condition and gender on social behavior of the adult rats. There was no significant interaction between the effects of condition and gender on frequency of social exploration ($F(1, 4) = 3.829, p = 0.122$), total duration of social exploration ($F(1, 4) = 0.093, p = 0.775$), frequency of anogenital investigation ($F(1, 4) = 1.063, p = 0.361$), or total duration of anogenital investigation ($F(1, 4) = 0.019, p = 0.896$). No significant difference was found between conditions for frequency of social exploration ($F(1, 4) = 0.114, p = 0.753$), total duration of social exploration ($F(1, 4) = 0.107, p = 0.760$), frequency of anogenital investigation ($F(1, 4) = 0.632, p = 0.471$), or total duration of anogenital investigation ($F(1, 4) = 0.003, p = 0.961$) or between genders for frequency of social exploration ($F(1, 4) = 0.146, p = 0.722$), total duration of social exploration ($F(1, 4) = 0.702, p = 0.449$), frequency of anogenital investigation ($F(1, 4) = 0.306, p = 0.610$), or total duration of anogenital investigation ($F(1, 4) = 0.242, p = 0.648$) (Figures 9 and 10).

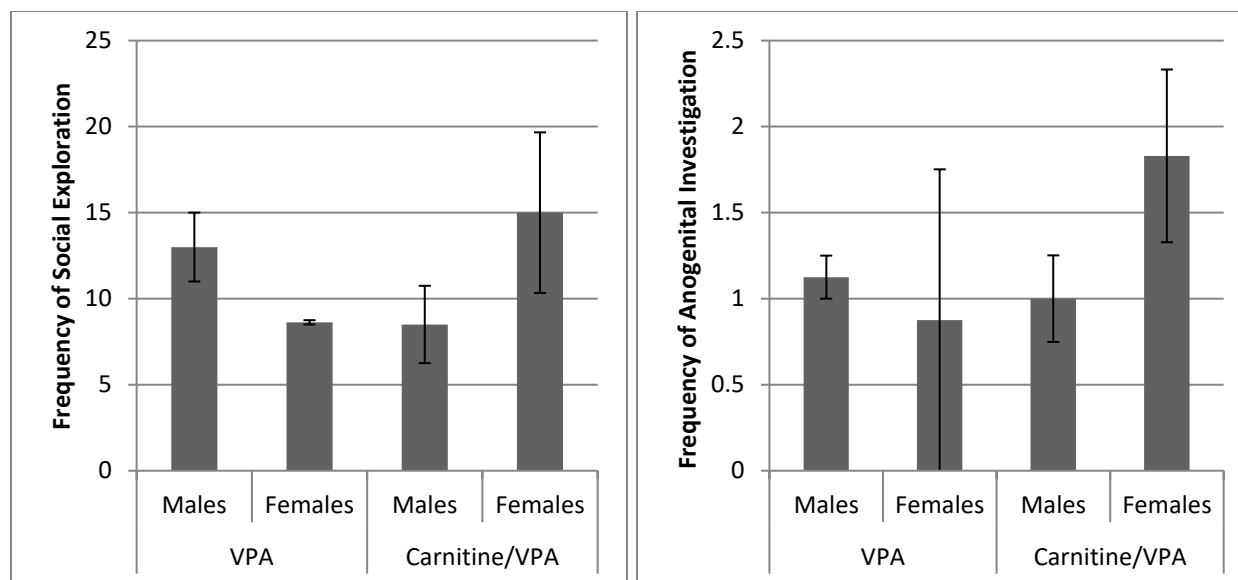


Figure 9: Social Behavior Test (adult). Frequency of social exploration (left) and frequency of anogenital investigation (right) measured for a target subject (paired with a subject of the same condition) during a 10 minute test. Data are expressed as mean $\pm$ SEM, $n=2$ for each group.

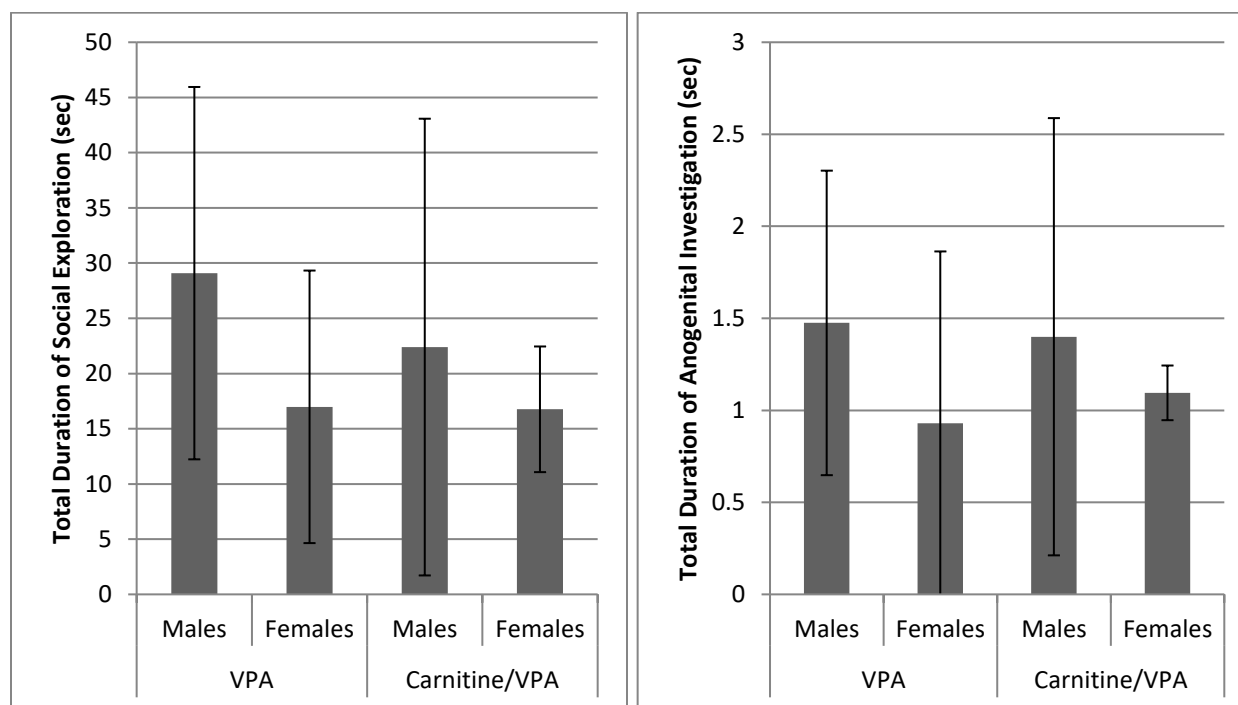


Figure 10: Social Behavior Test (adult). Total duration of social exploration (left) and total duration of anogenital investigation (right) measured for a target subject (paired with a subject of the same condition) during a 10 minute test. Data are expressed as mean $\pm$ SEM, $n=2$ for each group.

Y-Maze Test

Adolescents

A two-way ANOVA was conducted that examined the effect of condition and gender on the amount of time spent in the novel arm in adolescents. There was no significant interaction between the effects of condition and gender on the amount of time spent in the novel arm, $F(2, 18) = 0.258$, $p = 0.776$. A significant difference between conditions ($F(2, 18) = 4.452$, $p = 0.027$) was found. A Tukey post-hoc test revealed that the amount of time spent in the novel arm was significantly higher ($p < 0.05$) for the control (230.26 ± 169.19 sec) condition compared to the VPA (67.63 ± 81.20 sec) condition and the Carnitine/VPA (58.49 ± 95.75 sec) condition. There was no statistically significant difference between the VPA and Carnitine/VPA conditions ($p > 0.05$). No significant difference was found between genders ($F(1, 18) = 0.000$, $p = 0.995$) was found (Figure 11).

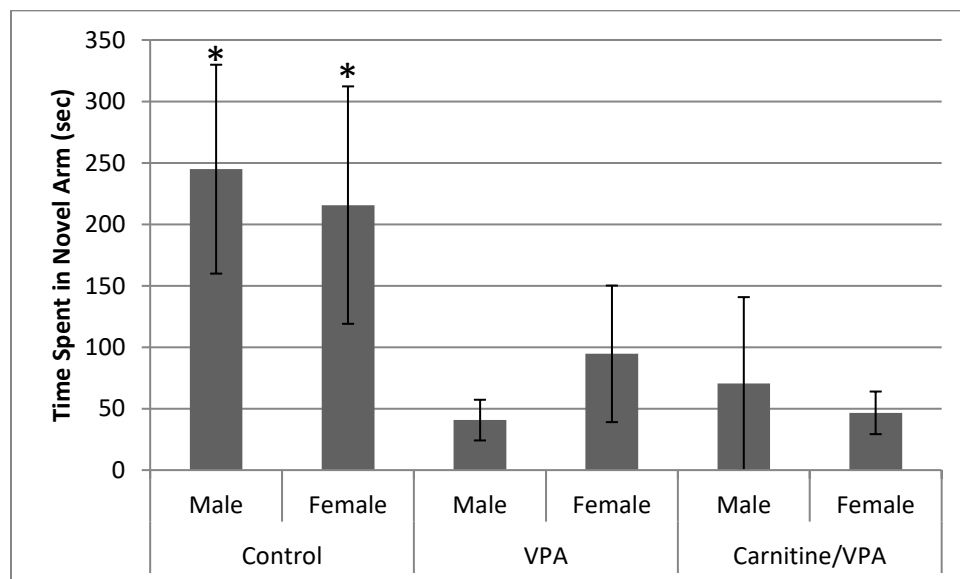


Figure 11: Y-Maze Exploratory Test (adolescent). The data presented is the amount of time spent in novel arm by the adolescent subjects during a 10 minute test. Data are expressed as mean $\pm$ SEM, $n=4$ for each group. * $p < 0.05$ for control rats spending more time in novel arm compared to the VPA and Carnitine/VPA rats (Tukey's HSD Test).

Adults

A two-way ANOVA was conducted that examined the effect of the condition and gender on the amount of time spent in the novel arm by adults. There was no significant interaction between the effects of condition and gender on the amount of time spent in the novel arm, $F(1, 12) = 1.060$, $p = 0.323$. No significant difference was found between conditions ($F(1, 12) = 2.728$, $p = 0.125$) or between genders ($F(1, 12) = 2.424$, $p = 0.145$) (Figure 12).

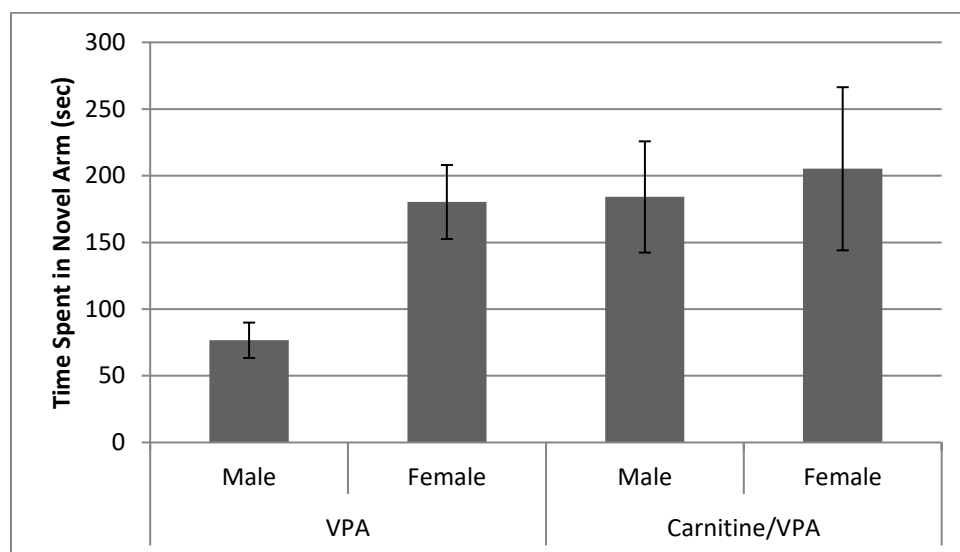


Figure 12: Y-Maze Exploratory Test (adult). The data presented is the amount of time spent in novel arm by the adult subjects during a 10 minute test. Data are expressed as mean $\pm$ SEM, $n=4$ for each group.

Adolescents vs. Adults

A two-way ANOVA was conducted that examined the effect of the condition and age on the amount of time spent in the novel arm. There was no significant interaction between the effects of condition and age on the amount of time spent in the novel arm, $F(1, 28) = 1.516$, $p = 0.228$. No significant difference was found between conditions ($F(1, 28) = 0.868$, $p = 0.360$) but, a significant difference was found between ages $F(1, 28) = 10.355$, $p = 0.003$, with adults (161.56 ± 88.33 sec) spending more time in the novel arm than adolescents (63.075 ± 85.89 sec). A Tukey HSD post-hoc test did show a statistically significant difference ($p < 0.05$) between ages (Figure 13).

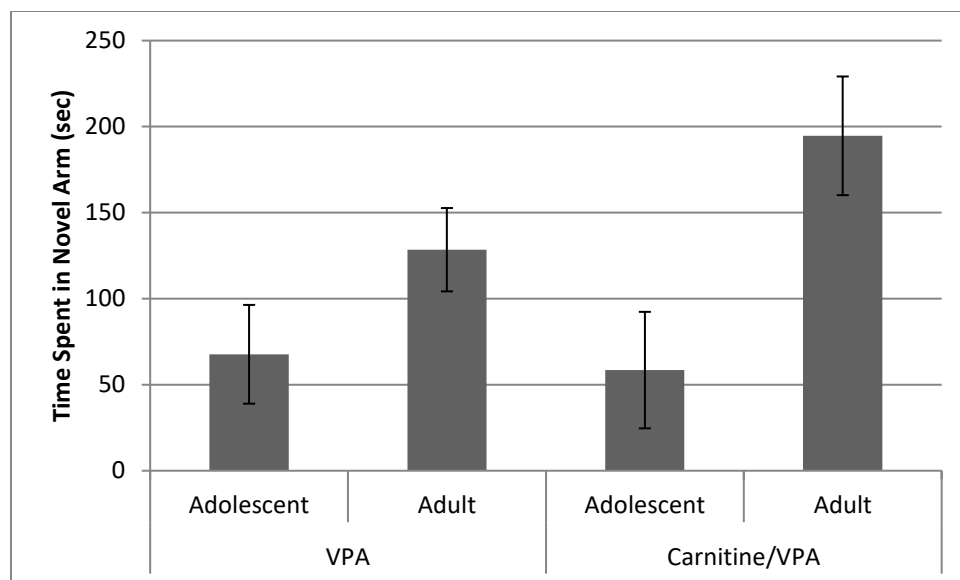


Figure 13: Y-Maze Exploratory Test (adolescent vs. adult). The data presented is the amount of time spent in novel arm by subjects during a 10 minute test. Data are expressed as mean $\pm$ SEM, $n=8$ for each group.

Discussion

Performance of VPA Model of Autism

The results of this study are somewhat equivocal because three of the tests used, the tail-flick, repetitive-behavior, and social behavior tests, did not produce results consistent with previous findings of the basic characteristics of the VPA animal model of autism. Nevertheless, the results obtained in the other test, the Y-maze, do not support the hypothesis that carnitine plays a critical role in the mechanism underlying the VPA model of autism.

The tail-flick test was used to measure the previous findings that autistic subjects displayed higher tolerances to pain compared to control subjects. However, the results of the tail-flick test did not agree with previous studies in the VPA model of autism, which found statistically significant differences between the control and the VPA condition's pain thresholds (Figure 1 and 2). It is possible that the lack of an observed statistical difference was due to differences in procedures. While this study used a hot water bath to induce the painful stimulus,

previous studies used radiant heat as a pain stimulus (Schneider and Przewłocki, 2005). This difference in pain stimulus may account for the differences in results.

Similarly, the repetitive movement test in the Opto-Varimex was used to measure one of the core symptoms of autism, repetitive behavior. It was expected that the proportion of repetitive performed during the test would be significantly higher in the VPA condition compared to the control condition. However, this core characteristic of autism was not observed to be statistically different between the control and the VPA conditions (Figure 4a). It was also observed that rats in the control condition displayed a higher amount of ambulatory movement than either the VPA or the Carnitine/VPA conditions, which is suggestive of lower anxiety (Figure 4b). This observation does agree with other findings that individuals affected by autism display a higher amount of anxiety in novel environments.

The social behavior test was chosen to test one of the most recognizable features of autism, a lack of or an inhibition of social interaction. The results from the social behavior test also did not agree with the findings of previous VPA autism model studies, which found a significantly lower number of pinnings for the VPA condition compared to the control conditions (Schneider and Przewłocki, 2005).

Based on the lack of observed accepted characteristics of autism it was concluded that the tail-flick, repetitive-movement, and social behavior tests cannot be used in the determination of the protective effects of carnitine against the development of autism in this study.

The results from the Y-Maze test did agree with previous findings with the VPA condition preferring the familiar area over the novel area (Markram et al., 2008). Thus, this was the only test that could appropriately be used to determine the effects of carnitine.

Carnitine Effects on VPA Model of Autism

The Y-maze produced the only results that were consistent with previous findings in the VPA model of autism and suggested that there was partial success in producing the animal model of autism induced by VPA. The results of this single test suggest that carnitine supplements during days 10 through 13 after conception did not prevent the development of autism, as both the VPA and Carnitine/VPA conditions were found to be statistically the same (Figure 11).

No other conclusions about the mitigating the effects of VPA with maternal carnitine supplementation in the VPA autism model can be drawn from the other three behavioral tests performed in this study due to the lack of evidence of the development of the VPA model of autism in the subjects.

Limitations

There were a number of limitations in this study that could account for the absence of accepted autism characteristics in three of the four behavioral tests. The low sample size in each of the conditions could have been a factor in the lack of statistically significant differences between the control conditions and the VPA conditions. The animals used in this study, although of a type used by other researches in the VPA induced animal model of autism, may have been resistant in some way to the effects of VPA. It is also possible that there were unforeseen environmental or other factors different in the particular animals studied compared to other researches; such as the particular feed supplied or the light/dark cycle of the housing room, which may have had an unrecognized protective effect against VPA inducing autism in the animals.

In the Y-Maze test, the one test that was consistent with the VPA model of autism in the study, it is possible that an improper operational definition in the Y-Maze led to a large amount of error in the results. The operational definition used in the Y-Maze test was that when the rats simply entered the novel arm they were marked down as exploring the novel area. However, at the conclusion of the test, it was apparent that this operational definition may have been flawed. A number of the rats entered the novel arm and then remained still in a fear-reaction state. The amount of time spent in this fear-reaction was not separated but included in exploratory behavior time in the novel area. This fear-reaction also occurred in some individuals in the familiar arms as well. This potentially flawed operational definition combined with the low sample sizes ($n=8$ per condition), could have produced false results that led to the conclusion that the subjects were autistic in the Y-maze test. Thus, it is possible, that none of the tests used in this study revealed an effect from the VPA and that the animals studied did not have any degree of autistic behaviors caused by VPA.

A major aspect of the VPA model and the attempted carnitine intervention is that there is a critical developmental time point where the fetus is vulnerable to VPA administration. It is possible that this time point was missed, which could account for the lack of data supporting the development of the VPA model of autism in the tail-flick, repetitive-movement, and social behavior tests in the subjects. Similarly, the supplemental carnitine dose may have been insufficient, either in quantity or timing to prevent the development of autism in the one test that was consistent with autism (Y-Maze test) in this study.

Future Research

Further research that should be considered is the examination of the effects of varying dosages and time points of administration of L-carnitine supplements during gestation on the

development of autism. In addition, larger population sizes might substantially improve the possibility of finding a significant result. Along the same lines, experimenting with other potential treatment agents, such as folic acid, should be considered, as recent research has provided promising results on maternal folic acid supplements resulting in lower rates of autism in humans (Suren et al., 2013).

Additional exploration of the relationship of carnitine levels to autism could be obtained by correlating maternal levels of carnitine during early pregnancy with the likelihood that their children develop autism. If such an analysis was positive, then the need to further explore the role of carnitine in animal models of autism would be much greater.

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Appendix A

Y-Maze Test Data Sheet

Round 1: 10 minute explore time (right arm blocked off) **[Do not Record Data]**

10 minutes isolated in covered cage

Round 2: 10 minute explore time (all arms available) **[Record Data]**

Condition: Control

[illegible]

Condition: VPA

[illegible]

Condition: Carnitine + VPA

[illegible]

Tail-Flick Test (Nociception) Data Sheet

Round 1: Drop tail in water

1 minute break

Round 2: Drop tail in water

Condition: Control

[illegible]

Condition: VPA

[illegible]**Condition:** Carnitine + VPA[illegible]

Social Play Behavior Data Sheet

Prep: Isolate Subjects for 3.5 hours

Place the two subjects from same condition into test cage

Measure: Total Duration and Frequency

- Latency to **pinning** -- one of the animals is lying with its dorsal surface on the floor of the test cage with the other animal standing over him
- Latency to **social non-play behavior**
 - following or approaching the test partner
 - mounting or crawling over the test partner
 - sniffing or grooming any part of the body of the test partner

Condition: Control

Date	IDs #1 and #2	Pinning			Social Non-Play Behavior		
		Latency	Total Duration (sec)	Frequency	Latency	Total Duration (sec)	Frequency

Condition: VPA

Date	IDs #1 and #2	Pinning			Social Non-Play Behavior		
		Latency	Total Duration (sec)	Frequency	Latency	Total Duration (sec)	Frequency

Condition: Carnitine + VPA

Date	IDs #1 and #2	Pinning			Social Non-Play Behavior		
		Latency	Total Duration (sec)	Frequency	Latency	Total Duration (sec)	Frequency

Social Play Behavior -Adult- Data Sheet

Prep: Isolate Subjects for 3.5 hours

Place the two subjects from same condition into test cage

Measure: Target Animal (chosen randomly) –10min–

- **Latency** to social behavior- Any social interaction
- Total Duration and Frequency of **Social Exploration Behavior**
 - Sniffing or licking any part of the body of the partner **except the anogenital area**
 - Crawling or mounting
 - Putting forepaws on the back of the partner or climbing over the partner
 - Approaching or following the partner
- Total Duration and Frequency of **Anogenital Investigation**
 - Sniffing or licking the anogenital area of the partner

Condition: VPA

Date	IDs	Latency to Social Behavior	Social Exploration		Anogenital Investigation	
			Total Duration (sec)	Frequency	Total Duration (sec)	Frequency

Condition: Carnitine + VPA

Date	IDs	Latency to Social Behavior	Social Exploration		Anogenital Investigation	
			Total Duration (sec)	Frequency	Total Duration (sec)	Frequency