

Effects of oxidative stress, exercise training, and aging in an animal model of autism spectrum disorder.

by

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Abstract

Background: Autism spectrum disorder (ASD) prevalence has increased 175% since 2000 with 1% of the world's population identified with ASD. Today, ASD affects 1 in 36 children and is characterized by delayed language, motor, and cognitive skills. ASD is characterized by two pillars of pathology: oxidative stress and inflammation. These have been identified as closely related to ASD and many intervention strategies in the literature target these parameters. **Purpose:** this project will evaluate brain and skeletal muscle oxidative stress via antioxidant expression in a rat model of ASD and the impact of exercise and aging. **Methods:** Sixteen Long-Evans pregnant rat dams were injected with a single dose of either saline (n=5) or VPA (Sodium Valproate (sigma), 250mg/ml, mixed in saline, 600 mg/kg, n=11). This prenatal exposure to VPA is known to increase the risk of ASD development in offspring. Rats were provided with free access to food and pair-housed with a light cycle of 7 am-7 pm. Rats were enrolled in experiment 1 (exercise study) or experiment 2 (aged ASD study). Experiment 1 animals began a 4-week exercise protocol on the treadmill (Harvard Apparatus, Holliston, MA) on post-natal day 40. Rats ran 5 days a week for 10 minutes at 4.98 m/min, 10 minutes at 7.98 m/min, and 10 minutes at 15.96 m/min for a total of 30 minutes at 0° inclination. Motor coordination was measured with a rotarod both pre and post-intervention. The rod maintained a constant 5rpm speed and the trial ended after 180 seconds. Latency time was recorded for each trial. The littermates of the animals enrolled in the exercise protocol were placed in experiment 2, and aged between 6-18 months to understand the effect of aging on autism. Before the end of life, aged animals performed the Novel Object Recognition task (NOR). Following behavioral testing for both experimental groups, brain and skeletal muscle tissues were collected. Skeletal muscle (soleus, red gastrocnemius) and brain tissue (hippocampi) were homogenized, and

western blots were conducted to quantify antioxidant expression. **Results:** Reduced motor coordination was identified in the experiment 1 VPA groups which was improved with exercise. To confirm an exercise effect, citrate synthase expression was quantified in the soleus muscles. Treadmill-trained animals resulted in higher citrate synthase (CS) activity compared to sedentary animals ($p=0.04$). Antioxidants superoxide dismutase 1 (SOD1) and catalase were measured in skeletal muscles (experiments 1 & 2), and hippocampi (experiment 2), resulting in declines to antioxidant expression in experiment 1 animals ($p<0.05$), and declines in aged male antioxidant expression ($p<0.05$). **Conclusion:** The protective effect of exercise against ASD pathology is important to understand both in its use as a therapy but also as a tool to highlight the protective pathways.

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Chapter 1 - Introduction

Autism is a neurodevelopmental disorder characterized by delayed language, repetitive behaviors, and impaired motor and cognitive skills. Autism prevalence has increased 175% since 2000 with 1 in 36 children affected, 1 in 45 adults affected, and 7.9 million individuals identified with Autism Spectrum Disorder (ASD) (CDC, 2024, p. 47)(CDC, 2022, 2024). Mortality is twice as high in individuals with ASD, compared to the general population (Hirvikoski et al., 2016; Mouridsen et al., 2008; Schendel et al., 2016). The age of death is estimated at 36 years earlier for those diagnosed with ASD when compared to the general population (Guan & Li, 2017).

Reduced lifespan is due to individuals with ASD being more susceptible to a host of comorbidities. These range from chronic illness to genetic and neurological disorders. An individual affected by ASD is 2.6 times more likely to develop early-onset Alzheimer's. Epilepsy, depression, and anxiety are also prevalent comorbidities, as well as, disruption in sleep and GI function (Al-Beltagi, 2021; Frye, 2015; Vivanti et al., 2021).

The cause of ASD is unclear and likely to fall under the three umbrellas of genetics, epigenetics, and environmental factors. Most notably, the use of valproate (VPA) during pregnancy, Fragile X Syndrome, and maternal immune activation creates a risk of ASD (*Autism Spectrum Disorder - Diagnosis and Treatment - Mayo Clinic*, n.d.; Basil et al., 2014; Chaste & Leboyer, 2012; Toscano et al., 2021).

Autism pathology is complex and presents hallmark features such as, behavioral deficits, neuronal count mismatch, oxidative stress, and inflammation. ASD also affects the individual physically, with impaired motor coordination and strength. Muscle strength as measured by hand grip force production and case severity have been correlated by Kern et. al. The more severe cases

produced lower levels of hand grip strength, whereas less severe cases produced higher levels (Kern et al., 2011).

Exercise has proved beneficial in alleviating both behavioral and physiological deficits found with ASD. Studies utilizing a rodent model of ASD have found that spatial awareness and memory improved as measured by the Morris Water Maze. Beyond this, Purkinje neuron counts were increased with exercise (Cho et al., 2016; Ghahremani et al., 2022). In humans, a jogging exercise intervention improved emotional regulation as well as behavior problems, as measured through better scoring on the Emotion Regulation Checklist (ERC) and the Child Behavior Checklist (CBCL) (Tse, 2020). The mechanisms of exercise-induced behavioral improvements in ASD are unclear, however, there is an inverse correlation between muscular strength and ASD case severity suggesting that skeletal muscle plays a role.

Finally, much remains to be understood regarding autism in aging and adulthood. To address the paucity in these areas of ASD research, the proposed study involves 2 experimental groups. The first will examine exercising and sedentary groups of ASD rats under the VPA model, with assessment of behavior through rotarod, and skeletal muscle through western blot analysis. The second will examine the VPA model of ASD, between 6 and 18 months of age. This period represents an older, or aged, model of ASD. These animals will perform the behavioral test, Novel Object Recognition task (NOR), with brain and muscle tissue analysis conducted through western blot analysis. This project will provide a better understanding of the pathology and the potential impact of exercise, the role of oxidative stress in autism, and the potential impacts of aging in autism pathology.

Chapter 2 - Literature Review

1). Background

Individuals with ASD are at higher risk of death due to unintentional injuries, the most common being drowning (Guan & Li, 2017). The National Autism Association approximates 91% of childhood autistic deaths occur under the age of 14 and are due to accidental drowning. It has been postulated that ASD children are at increased risk of drowning due to their instinct to run from a safe environment due to either fright or curiosity, this instinct is 4 times higher in ASD than their healthy peers (*Autism & Safety Facts - National Autism Association*, 2012). Aside from this, mortality rates are much higher in the ASD population. Individuals with an autism diagnosis are found to have twice as high risk of premature mortality when compared to the general population (Hirvikoski et al., 2016; Mouridsen et al., 2008; Schendel et al., 2016). Finally, individuals with ASD in the United States have been found to die approximately 36 years earlier than the general population (Guan & Li, 2017).

ASD also carries a high economic impact with the current lifetime cost per diagnosed individual estimated at \$3.6 million (Vivanti et al., 2021). Due to the nature of the disorder, there is a large societal impact on ASD as well. The family, community, and those involved in their education are also affected and responsible for aiding in the health and progress of the child, as well as caring for them throughout their lifespan. Which takes time, emotional strain, and the previously stated economic resources.

Autism is a neurodevelopmental disability that affects the brain in many ways. Particularly, the brain regions amygdala, prefrontal cortex, nucleus accumbens, cerebellum, cerebro-cerebellar circuits, involved with emotional regulation, social interaction, and motor coordination, are compromised in the case of autism (Psychiatry Simplified - Dr Sanil Rege, 2022; Sparks et al.,

2002) In neurotypical conditions, the Central Nervous System (CNS) development involves an overproduction of synapses which are then pruned by response to the neuronal activity occurring in early development. In the case of ASD, there is a normal overproduction of synaptic proteins, however, the pruning process causes abnormality in the brain (Cohen & Greenberg, 2008; Gkogkas et al., 2013; Kelleher & Bear, 2008). Thus, hyperconnectivity and CNS dysfunction are born (Courchesne et al., 2011)

One hallmark feature of autism is an enlarged brain with brain overgrowth reported at 9-18 months of age in ASD subjects (Courchesne et al., 2011). An early preliminary study by Courchesne et. al in 2011 evaluated brain overgrowth via mean neuron number and size in the prefrontal cortex (PFC) of male autistic postmortem brain tissues aged 2-16 years against age-matched controls. The PFC is related to the social aspects, communicative abilities, cognitive functions, and developments, as well as high-order social development, all of which are crucial to ASD behavior. The investigators specifically isolated the dorsolateral prefrontal cortex (DL-PFC) and medial prefrontal cortex (M-PFC). The mean mass of the ASD brain deviated from the control brain mass by 17.6%, and the control brain deviated by 0.2%. Statistically significant differences were found in PFC neuron counts, with 79% more neurons in the DL-PFC of ASD compared to control and 29% more in M-PFC. Together, these provided a 67% increase in PFC neurons, concluding that an increased number of neurons could be the result of impaired processes to remove unwanted, non-functioning, or old neurons (Courchesne et al., 2011).

In contrast, another study examining the autistic brain postmortem found fewer neurons in the amygdala (Schumann & Amaral, 2006). The amygdala holds special significance in the case of ASD. The amygdala is formed and practically matured at the time of birth. Since this is the key time frame for the initiation of ASD symptoms, abnormalities in this region would be logical

(Bachevalier & Loveland, 2006). Rodent studies in which the amygdala experienced damage resulted in behavioral deficits similar to those in autism, thus further bolstering the impact of the amygdala (Diergaarde et al., 2004). This damage could spread to nearby structures including the hippocampus, which has been observed to present as both enlarged and reduced in size (Fatemi et al., 2002; Mei et al., 2020). Beyond neuronal counts, abnormal increases in microglial activation, as well as proinflammatory cytokines have also been identified in ASD (Hutsler & Zhang, 2010; Varghese et al., 2017). In the cerebellum, there is glial activation and a reduced number of cerebellar Purkinje neurons (Fatemi et al., 2002; Mei et al., 2020).

While the areas of the brain appear abnormal in autism, there is still no single reason behind why autism occurs, or a clear model of the neurodevelopmental disorder in the brain regions. Beyond this, with autism presenting on a spectrum, abnormalities may differ from case to case.

Autistic individuals are more susceptible to a host of comorbidities. These range from chronic illness to genetic and neurological disorders. An individual affected by ASD is 2.6 times more likely to develop early-onset Alzheimer's (Vivanti et al., 2021). The reason behind this is not entirely understood. The hypothesis has been suggested that includes symptom overlap between the two disease states, such as depression and intellectual disability (Vivanti et al., 2021). Other studies suggest a mutual dysregulation in Amyloid- β Precursor Protein (APP) (Vivanti et al., 2021). APP has been long associated with Alzheimer's disease (AD), as it relates to amyloid- β , a hallmark feature of AD. In regards to ASD, APP is suggested to contribute to brain overgrowth in early life (Lahiri et al., 2021).

Individuals with ASD may experience seizures and seizure disorders (Besag, 2017). Due to the prevalence of seizures in many ASD individuals, the prevalence of epilepsy, a seizure disorder, in autistic individuals ranges from 5% to 38% (Danielsson et al., 2005; "The Incidence

of Seizures among Children with Autistic Symptoms,” 1979). For those not identified with ASD, this percentage is only 1-2% (Danielsson et al., 2005; “The Incidence of Seizures among Children with Autistic Symptoms,” 1979). Even day-to-day activities involving sleep (most likely attributed to poor quality sleep due to symptoms associated with ASD such as anxiety, seizures, and more. They are closely related and associated with the gastrointestinal dysfunction found in ASD as well. Mechanisms behind GI dysfunction in ASD include gut inflammation and immune dysfunction. GI symptoms have been associated with autism severity (Al-Beltagi, 2021).

2). Diagnosis

ASD diagnosis typically occurs between 18-24 months of age and involves primarily behavior observation. Early infancy symptoms include reduced eye contact, not being responsive to name calling and interactions, withdrawal, aggression, and in early years, no language or limited language (*Autism Spectrum Disorder - Diagnosis and Treatment - Mayo Clinic*, n.d.). Diagnosis relies heavily on parental observation and concern. Also existing in the medical field is a hesitation on the provider’s end to validate the concerns of parents and diagnose a child with ASD or refer to a specialist for the condition. There are delays between parental mention and provider action. This could be due to a lack of treatment options, and understanding of the disorder, however, ultimately may delay early interventions (Zuckerman et al., 2015). The treatment of ASD is still being developed and involves therapies, and drugs targeting secondary symptoms such as hyperactivity, aggression, and mood changes.

3). Causes

Although one cause of ASD has not yet been identified, individuals are put at risk for autism spectrum disorder through several avenues, all of which are encompassed within three

umbrellas 1) environment, 2) genetics, and 3) epigenetics. Environmental risks include neonatal hypoxia, maternal obesity, valproate (VPA), an antiepileptic drug, use during pregnancy, maternal diabetes, and advanced parental age. Sodium Valproate has been determined as an environmental risk in both humans and animals, producing autism-like behavior in rodent models (Toscano et al., 2021).

Genetic risks, ASD is related to Rett Syndrome, Fragile X Syndrome, the shank-3 gene, and tuberous sclerosis. All of which increase the chance of ASD. Rett syndrome is a disorder affecting motor and language skills. Rett syndrome is primarily related to a mutation in the Methyl-CpG-binding Protein 2 (MECP2) and is classified as an Autism Spectrum Disorder (Neul, 2012). Fragile X Syndrome is a genetic disorder, primarily related to intellectual disability. The syndrome involves developmental delays, learning disabilities, and social deficits, all of which are also found in ASD (CDC, 2022). SHANK3 is a synaptic protein involved with the function of synapses. Haplosufficiency of the SHANK3 gene results in developmental disorders presenting with autistic behaviors, hypotonia, and speech delays (Uchino & Waga, 2013). Tuberous sclerosis is found to be a risk factor for the development of both ASD and epilepsy, with the prevalence of ASD in patients with tuberous sclerosis found to be between 26-45% (Specchio et al., 2020).

Extremely premature babies of 26 weeks or earlier, as well as advanced parental age also contribute to ASD risk (*Autism Spectrum Disorder - Diagnosis and Treatment - Mayo Clinic*, n.d.).

Epigenetic risks include altering gene activity without changing DNA sequence. Epigenetics involves behavior and environment and their impact on genes and gene expression. Maternal stress, psychiatric disorders, chemical exposures and use in pregnancy, and maternal immune activation during prenatal development can induce epigenetic alterations in the brain correlated with ASD (Basil et al., 2014; Chaste & Leboyer, 2012). Pathological features of autism

include oxidative stress, inflammation, and Purkinje cell loss in the cerebellum (Pragnya et al., 2014).

Oxidative stress is of particular interest regarding ASD, due to its possible relationship with ASD severity. It was found that the increased presence of oxidative stress markers correlates to ASD severity (Ghezzi et al., 2013). Oxidative stress has been identified in both human and rodent models, and thus is recognized as a key feature of ASD pathology (Banji et al., 2011; Liu et al., 2022; Mirzavandi et al., 2023; Pangrazzi et al., 2020; Sandhya et al., 2012).

Activation of Endoplasmic Reticulum (ER) stress signals exists within the postmortem autistic brain. This activation could be the result of oxidative stress and relate to autophagy and apoptotic events. Different markers of ER stress were measured in postmortem autistic brain including PERK, phosphorylated PERK, ATF6, CHOP, IRE1 α , phosphorylated IRE1 α , caspase 8, PARP, 4-HNE, and nitrotyrosine. These were found to be either increased or upregulated at significant levels in the cerebellum and hippocampus (Dong et al., 2018).

Another key hallmark feature of ASD is inflammation. Elevated plasma levels of pro-inflammatory cytokines, specifically IL-6 and TNF-alpha were found in human children with ASD, and these cytokines were also correlated with the severity level of ASD (Inga Jácome et al., 2016). Inflammation and inflammatory cytokines are important in the case of ASD as the correct balancing of pro and anti-inflammatory markers is key to brain development (Li et al., 2009; Ormstad et al., 2018). Autistic postmortem cerebrospinal fluid was found to contain pro-inflammatory cytokines, microglia activation, and immune cell imbalances (Xiong et al., 2023). Microglia cells (immune cells) are chronically activated in the case of autism. Microglia are neuroglial cells that modulate neuronal function and homeostasis. During microglial activation, which is the result of injury or response to neuronal dysfunction, inflammation occurs. This can

contribute to central nervous system dysfunction. Abnormalities within microglia occur during developmental stages, and this relates to autism through synaptic dysfunctions (Vargas et al., 2005).

4). VPA Induced ASD

Autism has been demonstrated in rodents through several different models. These include genetic mutation models such as the SHANK3 mutated rodent model, as well as environmentally related models such as the VPA model. VPA, or Sodium Valproate exposure in pregnancy is a risk factor for autism in both humans and rodents. The result of the VPA model shows an increase in glutamatergic excitatory signaling, decreased PTEN gene expression, and increased p-AKT protein levels in the hippocampus and cortex (Huang et al., 2019; Mahmood et al., 2018).

Prenatal exposure to VPA in rodents is linked to behavioral and neuroanatomical deficits. Social interactions are reduced, and observed are repetitive behaviors, anxiety, reduced Purkinje cells, nucleus damage, and cortical synaptic changes. These are all related to the human condition of autism (Jiang et al., 2022).

The VPA rodent model was first validated in a 1997 study with Rodier (Rodier et al., 1997). Valproate was suggested to first injure the brain stem in utero, which has been shown in humans as well as related to ASD as a whole. VPA acts when the neural tube is closing, typically 11-12 days after conception. To validate the VPA model they first examined anatomical and physiological markers - specifically, Purkinje cell numbers as well as granule cells, neuron number in nuclei of cerebellar vermis, and measures of lobules in the brain. Cerebellar neurons and Purkinje cells were reduced in the VPA model (Rodier et al., 1997). The rodents under the VPA model had many features in common with autistic autopsy cases and thalidomide-induced autism

cases. Meaning, there was validity and pragmatic practicality behind the VPA model (Rodier et al., 1997). The greatest strength of the VPA model is that rodents experience similar impairments as humans (Favre et al., 2013; Jiang et al., 2022).

5). Skeletal Muscle and Brain Health

There exists a connection between the skeletal muscle and the brain that is still being researched, with current knowledge suggesting a strong connection between the activity at the level of the muscle and cognition, brain health, and brain disease.

Low muscle tone has been linked to cognitive impairments (Pasco et al., 2015; Sui et al., 2020). We see this in our aged populations through Sarcopenia, as well as in the disease state of Alzheimer's (AD). With AD, there is both muscle loss and cognitive decline. Due to the possible overlap of pathology between AD and ASD as discussed previously, there is a possibility of overlap concerning the muscle and its connection to cognitive function (Pasco et al., 2015).

While autism is primarily known for its cognitive and social deficits, there are many physical impairments associated with the disorder. Abnormalities with gait, balance, fine motor skills, and posture stability are just a few examples. Hypotonia, or decreased muscle tone, is reported and said to be an early indicator of autism (Ming et al., 2007; Serdarevic et al., 2017). Those individuals experience a delay in the onset of independent walking, unlike their autistic peers without generalized hypotonia (Gabis et al., 2021). Another physical characteristic of ASD is known as Tip Toe Behavior (TTB). It is hypothesized that a lack of range of motion in the Achilles as well as shortened soleus and gastrocnemius muscles produce walking and running with minimal use of the heel. TTB is present in 20% of ASD individuals (Valagussa et al., 2020). Muscle strength (as measured by hand grip) and case severity have been correlated by Kern et. al.

The more severe cases produced lower levels of hand grip strength, whereas the less severe cases produced higher levels (Kern et al., 2011).

Skeletal muscle, through exercise, can impact brain function and health. This brain-to-muscle connection influenced investigators to examine the effects exercise could have on brain health. It was found that cognitive benefits occur as early as 20 minutes after a bout of exercise (Chang et al., 2012). Beyond this, investigators have discovered an exercise-induced increase in hippocampal volume (Firth et al., 2018). Additional benefits induced by exercise include increased BDNF and APP in an Alzheimer's disease rodent model brain (Alkadhi, 2018). Angiogenesis, and memory improvements in mice (Guerrieri et al., n.d.). One hypothesis to explain the skeletal muscle: the brain axis can be demonstrated through the release of exercise-induced myokines.

Myokines are signaling proteins released by the skeletal muscle into the bloodstream and can cross the blood-brain barrier. Cathepsin-b and interleukin-6 (IL-6) are just two examples of myokines which are also involved in autism pathology. Both cathepsin B and IL-6 are found to be increased in ASD (Ni et al., 2022; Wei et al., 2012). Myokines are involved in brain function as it relates to memory, mood behaviors, and learning (Ranieri et al., 2023; Wang et al., 2018). Myokines also have a role in the upregulation of local neurotrophic expression such as BDNF anti-inflammation (Morley, 2018).

The benefits of physical exercise as they relate to brain health through attention, learning, mood, sleep, and memory - are due to the release of these myokines (Morley, 2018).

6). ASD and Exercise

The effect of exercise in combination with autism has been examined although the research evaluating its impact is limited. A study analyzing the effect of exercise on motor dysfunction utilized a rodent model of ASD to examine the linkage between exercise and behavioral as well as neurological outcomes. Purkinje neurons were examined due to their association with cognitive function, coordination, and movement. Purkinje neurons are found within the cerebellar cortex and are involved with the integration of information, as well as the coordination of motor movements. They are also identified as the sole output from the cerebellar cortex. These neurons are found to be reduced in autistic individuals. Through a 4-week treadmill exercise protocol, it was found that Purkinje neuron count was improved in the case of the ASD model (Cho et al., 2016).

In another study, both continuous and interval training were examined with the use of a treadmill and an 8-week exercise protocol (Ghahremani et al., 2022). The findings from this study are expressed through the Morris Water Maze, an analysis of spatial memory and learning. A pool filled with water holds a clear platform at the center. The animals are to navigate to the platform by swimming, and then crawl atop the platform to escape the water. During the formal testing periods, animals are timed for how long it takes them to find the platform based on their memory and spatial awareness. Video footage is utilized to analyze movement patterns. The ASD rats took an increased amount of time to find the platform during the formal trials. After the exercise protocol, the ASD groups performed better and reduced the time it took to escape the water (Ghahremani et al., 2022). The exercise was also examined in a thimerosal (THIM) rodent model of autism and was found to improve behavior as expressed through animal grooming in a four-week treadmill exercise protocol beginning on PND 51 through 80 (Alipour et al., 2022).

Activity levels expressed by autistic individuals were analyzed over COVID-19 by Demirci and Phytanza. During this time, a pool of subjects was gathered, and data collected revealed that

those who were autistic were less active and experienced reduced activity levels (*Investigation of Obesity, Physical Activity and Sedentary Behaviors of Individuals with and Without Autism Spectrum Disorder during the Covid-19 Pandemic Process | JUMORA: Jurnal Moderasi Olahraga*, n.d.).

A study by Ludyga et. al examined muscular strength, body mass index, and autism as well as their possible interlinkages. In healthy human subjects, higher scores in executive functioning were linked to high cardiorespiratory fitness levels and muscular strength. Human participants who were already diagnosed with ASD completed cognitive assessments, as well as physical fitness tests including measurements for body composition through body mass index, and testing muscle strength through exercises such as push-ups and grip strength (Ludyga et al., 2021). It was found that reduced strength in ASD was linked to poor cognitive performance and function, and the ASD patients reported a higher body mass index than their non-diagnosed counterparts (Ludyga et al., 2021).

Finally, in a human study, the effects of a jogging intervention were examined. Autistic individuals who participated in the jogging protocol saw improvements in emotional regulation as well as behavior problems (Tse, 2020).

7). Aging in ASD

The impact of ASD in adulthood is a new and emerging area of research. In 2023, the death of the first person to be diagnosed with ASD was recorded (Spectrum, n.d.). The ASD population has just begun to breach adulthood and geriatric years. This emphasizes understanding the changes to pathology and lifestyle that might occur in individuals diagnosed

with ASD. This necessitates additional research on aging and ASD. This was due to the great lack of studies and resources on aged autism pathology (Jasien et al., 2014).

There exists a lack of research on aged ASD individuals. This was stressed in 2010 by a multidisciplinary conference that was held to determine gaps in knowledge regarding adult autism (Piven et al., 2011). They concluded within the conference that additional human and rodent model studies were necessary to understand aging in ASD. With the rising prevalence of autism, at 1 in 36 children and 1 in 45 adults, this means that individuals over the age of 65 will drastically increase as well (Dietz et al., 2020; Jasien et al., 2014; Maenner, 2023). Aging in ASD has been explored by Jasien et. al and a BTBR +tf/j rodent model. Animals were 15 months old when they underwent behavioral testing followed by cortex and hippocampi tissue extraction. Behavioral deficits were identified through the open field and three-chamber tests, but not the novel object recognition task or the rotarod (Jasien et al., 2014).

To our knowledge, no studies exist that have explored aging in the VPA rodent model of ASD.

Statement of Hypothesis

There exists a physical element to ASD that has not been explained at the level of the skeletal muscle. Additionally, exercise has been shown to improve behavior and neurological deficits. The question remains, how does this occur?

The studies throughout this review suggest a knowledge gap in explaining the persistent inverse correlation between muscular strength and case severity ASD. These deficits relate to the skeletal muscle, and the possible phenotypic differences existing at baseline for ASD versus healthy controls.

To gain insight into ASD pathology, we ask what is the role of oxidative stress in autism. How does exercise modulate improvements through antioxidant expression in both skeletal muscle and the brain? And, is exercise a tool that should be implemented in therapy?

We hypothesize that the VPA model of ASD will present with behavioral deficits in young and old animals (as measured by the rotarod, and NOR). It is suspected that antioxidant expression will be reduced in the VPA animals and upregulated with exercise in both the skeletal muscle and the brain.

Chapter 3 - Methods

Experimental Design

Subjects:

Sixteen Long-Evans pregnant rat dams were delivered from Charles River on day 6 of gestation. On day 12 of gestation, injection of a single dose of saline was given to 5 dams, and VPA (Sodium Valproate (sigma), 250mg/ml, mixed in saline, 600 mg/kg) was given to 11 dams. There was a higher number of VPA included because previous work has shown that litter sizes for VPA may be smaller than the litter sizes for control. Rats were anesthetized with isoflurane gas (5% isoflurane, 95% oxygen) to give an I.P. injection.

All procedures were completed according to the Kansas State University IACUC as well as the NIH guide for the care and use of laboratory animals. Rats were provided with free access to food except for the preparation for the set-shifting task. The light cycle was 7 am-7 pm. Before weaning, rats were reared along with litter mates. After weaning, rats were pair-housed with a littermate of the same sex.

Timeline (Experiment 1, Young VPA):

Weaning took place on postnatal day (PND) 21, where 1-2 male and female rats from each litter were assigned at random to the different experiment groups: VPA exercise, VPA sedentary, exercise control, and sedentary control. PND (postnatal day) 28 involved rats performing the rotarod behavioral test, as well as beginning the 4-week exercise protocol on the treadmill (Harvard Apparatus, Holliston, MA). Treadmill training included an air puff production to promote running. The treadmill protocol involved rats running 5 days a week for 10 minutes at 4.98 m/min speed, 10 minutes at 7.98 m/min speed, and 10 minutes at 15.96 m/min speed. The Novel Object

Recognition task (NOR) was completed on PND 48-50. The rotarod was performed in the final week of treadmill training. Twenty-four hours following their final treadmill bout, rats were euthanized and tissues were collected. The Soleus and red portion of the gastrocnemius muscles were isolated and snap-frozen in liquid nitrogen. All tissues were first frozen in liquid nitrogen and then transferred to a -80°C freezer for later processing. Researchers who collected behavioral data were blinded to conditions.

Timeline (Experiment 2, Aged VPA):

The littermates of the animals assigned to experiment 1 were placed in experiment 2, examining the effect of aging on the VPA rodent model of autism. Animals were aged between 6 and 18 months and performed behavioral testing by the Novel Object Recognition task (NOR) after their lifespan (6-18 months). Animals were euthanized, and tissues were collected. The brain was excised and weighed before isolation of the hippocampi. The hippocampi were lightly blotted and weighed after isolation. Soleus skeletal muscle was also excised, weighed, and snap-frozen in liquid nitrogen.

Behavioral Tests

Rotarod Procedure

The use of the rotarod is a behavioral test to measure coordination. Rats were properly acclimated to the rod. During testing, rats were placed upon the rotarod (Harvard Apparatus, Holliston, MA) in the first and last week of the exercise protocol. The rod maintained a constant 5 revolutions per minute (rpm) speed and time as animals were placed upon it. Falling was logged. If the rats ran for 180 seconds they were removed to reduce stress and fatigue.

Novel Object Recognition

The Novel Object Recognition task (NOR) is a measure of sociability and anxiety. The test takes place over two days (PND 48-50). Animals are habituated in a double-wide cage (40 x 43 x 20 cm) for 5 minutes, during which the cage is empty. On day two, animals undergo a sample and test phase. The sample phase consists of the cage arena containing two identical objects placed in opposite corners. Animals explore for a total of 10 minutes. The test phase consists of one familiar object and one novel object. Animals are given 10 minutes to explore. Blind-to-condition researchers hand-coded the time of interactions with the objects. Interactions were defined as touching, smelling, and chewing. The total time spent exploring objects (percent exploration) was measured. Additionally, the difference in exploring time between the novel object and familiar object as a fraction of the total time exploring (discrimination ratio) was calculated.

Tissue Analysis

The soleus and gastrocnemius muscles (experiment 1), soleus, gastrocnemius, and extensor digitorum longus (EDL) muscles (experiment 2) were extracted when animals were euthanized. Biomolecular analysis followed to examine biomarkers related to ASD pathology.

Markers included SOD1 (antioxidant), catalase (antioxidant), and citrate synthase (exercise adaptation). **Protein quantification** Hippocampi, gastrocnemius, and soleus tissue samples were lysed in radio-immune precipitation assay (RIPA) buffer (Sigma-Aldrich) with protease inhibitor (Pierce Protease Inhibitor Mini Tablet, ThermoFisher Scientific) prepared according to manufacturer guideline. Lysate protein concentrations were determined using the Qubit 4 Fluorometer with Protein Broad Range Assay (ThermoFisher Scientific). Protein quantification

was completed with Jess Simple Western (ProteinSimple) automated western blot analysis to quantify SOD1 (Novus, NBP1-90186) and catalase (Abcam, ab209211) per the manufacturer's protocol with 2 mg/ml of sample protein per well and antibody concentration 1/100. Protein expression was quantified with Compass for Simple Western software.

Statistical Analysis

For statistical analysis, significance was indicated with a p-value less than 0.05. GraphPad by Prism was utilized for statistical analysis. An unpaired t-test was utilized for citrate synthase analysis. The rotarod data was analyzed using a fixed effects test, and mixed model, with condition, treatment, and trial as factors. Novel Object Recognition (NOR) in experiment 2, aged VPA group, was analyzed using a one-sample t-test and Wilcoxon test. All other analyses were completed with an ordinary one-way ANOVA with a Tukey post-hoc.

Chapter 4 - Results

Experiment 1

To test the hypothesis that exercise in a VPA rodent model of autism improves antioxidant status in the skeletal muscle, experiment 1 utilized a young VPA rodent model of Autism Spectrum Disorder (ASD). The animals were divided into 4 groups, control sedentary, control exercise, VPA sedentary, and VPA exercise. Animals in the exercise groups underwent 4 weeks of treadmill exercise training, with all groups completing behavioral testing. Following the intervention, skeletal muscle tissues were collected and analyzed for oxidative stress markers.

Citrate Synthase Activity

First, we determined that the exercise training protocol was effective through an increase in citrate synthase activity, measured in the red portion of the gastrocnemius muscle (Figure 1).

Behavioral Testing

Rotarod: Exercise improved motor coordination in the males.

The rotarod test was used to measure motor coordination in all animals before and after the exercise intervention (post-natal day 28 (P28), fourth week of treadmill training). Both trial 1 and 2 data are represented in Figure 2. Significant improvements were noted in all groups between trials 1 and 2, this notes a learning effect and was expected. There were no significant differences in trial 2 between control females and VPA females, however, the exercising females did display a trend towards significant motor coordination improvements over the sedentary groups. In the males, the exercised control and VPA groups increased their latency time

significantly within trial 2. This indicated an exercise-induced improvement in motor coordination.

Novel Object: VPA animals spent less time in exploration indicating reduced recognition memory. Exercise attenuated this effect in VPA females.

The Novel Object Recognition task (NOR) was performed to measure recognition memory and learning. Animals completed the behavioral task between P48-50. While no significant differences were present between males and females, there existed a trend in the male VPA animals. Such that, the VPA males' exploration time decreased in comparison to control males (not significant). Further, VPA males showed a trend to spend less time exploring the novel object compared to control males (not significant). In the females, total object exploration time, as well as novel object recognition time, was found to be significantly reduced in the VPA groups, with exercise improving exploration time significantly. These results indicate an effect of the VPA condition.

Biochemical Analysis

Antioxidants: VPA condition caused decreased antioxidant expression, exercise rescued antioxidant levels.

Oxidative stress has been identified as a part of ASD pathology. Because of this, we examined antioxidant biomarkers to understand the effect of ASD on antioxidant expression, as well as the influence of exercise to counter the effects seen with ASD.

There were no significant differences found between males and females regarding antioxidants. Because of this, data was pooled, and sexes were combined with even distribution to identify differences.

Red Portion of the Gastrocnemius Muscle

Significant declines in superoxide dismutase 1 (SOD1) and catalase were found in the gastrocnemius muscle of VPA animals (control sedentary vs. VPA sedentary, $p < 0.0001$, figure 3a-b). The exercise provided a significant increase in SOD1 expression in the gastrocnemius of VPA animals (VPA sedentary vs. VPA exercise, $p < 0.007$, figure 3a). Decreases in SOD1 expression were identified in the control exercisers. Trends towards increased catalase expression in VPA exercise groups were noted, yet not significant.

Soleus

The soleus muscle presented similar results. Significant decreases in SOD1 expression were identified within the VPA sedentary groups (control sedentary vs VPA sedentary $p < 0.004$, figure 4a). A significant increase in SOD1 expression was identified in the VPA-exercised animals (VPA sedentary vs. VPA exercise $p < 0.04$, figure 4a) There were no differences in catalase across groups.

Experiment 2 (Aged VPA)

Experiment 2 examined the effect of aging on ASD through a VPA rodent model. These animals underwent behavioral testing, followed by tissue extraction of the brain, and skeletal muscles.

Behavioral Testing

Novel Object Recognition: Reduced exploration time found in VPA females.

VPA resulted in a trend toward a decrease in total exploration time (NOR % exploration) for female animals when compared to control (not significant, figure 5). No change was found between control and VPA males. There were similar trends noted for females in the discrimination ratio, although not significant. No significant differences were identified between the discrimination ratio for each group.

Body, Brain, Muscle Masses: VPA and control animals presented comparable brain and muscle masses.

Brain and muscle masses were measured as a proxy for atrophy in the aged VPA model of ASD.

Brain and hippocampus mass

In the condition of ASD, there is typically a hypergrowth in the brain in young animals, and with age there is a sharp decline. Thus, we sectioned and weighed the whole brain and the isolated hippocampi in our aged cohort. Brain mass, measured in grams, did not display changes across groups and sexes (figure 7a). There were no changes in hippocampus mass across the control and VPA groups (figure 7b).

Skeletal muscle mass

The process of aging involves the loss of skeletal muscle. The condition of ASD is expected to exacerbate this because of the prevalence of muscle-related side effects and comorbidities in ASD. These include hypotonia (low muscle tone), tip-toe behavior, and trouble with balance and coordination. The control and VPA soleus masses were taken as a percentage of body weight. No trends existed, nor were significant differences found (figure 8a). EDL mass remained the same across groups, with no significant differences (figure 8b).

Biochemical Analysis

To better understand the effect of aging on ASD, antioxidants, indicative of oxidative stress, were assessed in the skeletal muscle and brain tissues of aged VPA and control rats.

Antioxidants: VPA males presented with reduced antioxidant expression

Soleus

Superoxide Dismutase 1 (SOD1) was assessed in the soleus skeletal muscles of aged VPA and control rats. Expression of SOD1 was found to be reduced in the VPA group for males only (figure 9a, control male vs. VPA male $p < 0.01$). An additional comparison was found between VPA females and males, with decreases in SOD1 observed in males only (figure 9a, VPA females vs. VPA males $p < 0.004$). No differences in SOD1 expression were found between control and VPA females (figure 9a). Catalase produced similar trends in results. Significant decreases in catalase were identified in VPA animals (figure 9b, control male vs. VPA male $p < 0.04$). Additionally, significant decreases between VPA males and females were identified (figure 9b, VPA female vs. VPA male $p < 0.007$). No differences were found in control and VPA-aged females with regards to catalase.

Hippocampus

This sex-specific trend remained in the hippocampal tissue, with significant decreases in SOD1 expression identified in VPA males (figure 10a, VPA females vs. VPA males, $p < 0.02$, figure 10a). No differences were found between control and VPA groups of either sex. There were no differences in catalase expression found in the hippocampus (figure 10b). These results indicate a sex difference in the aged VPA animals.

Citrate Synthase Activity

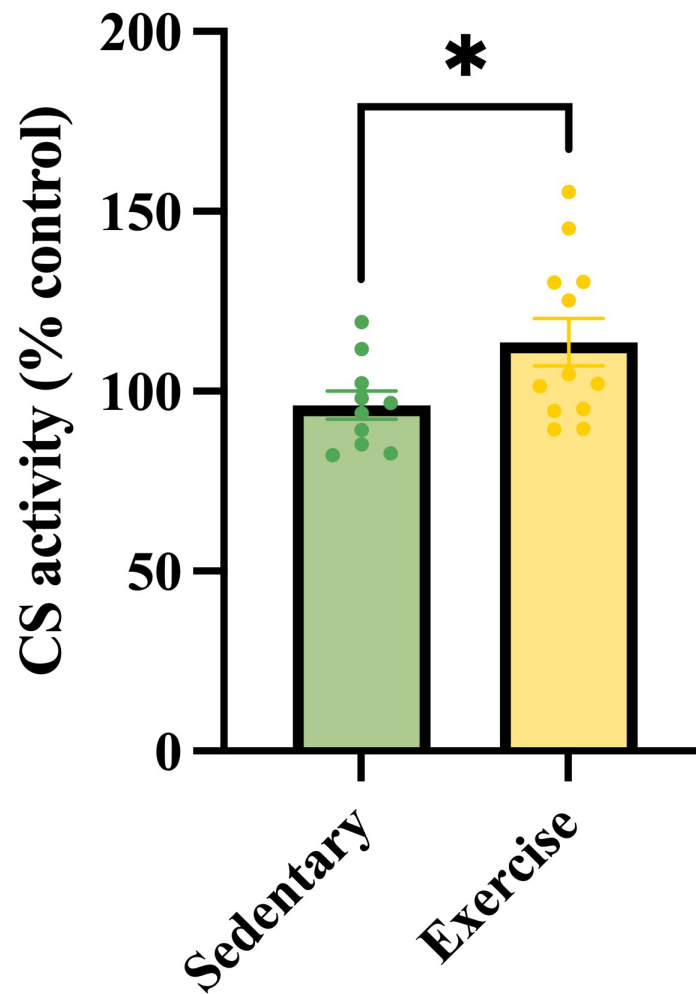


Figure 1. Citrate Synthase

Citrate synthase activity in the gastrocnemius muscle was significantly increased in the control exercised animals compared to the control sedentary animals (* $p < 0.05$)

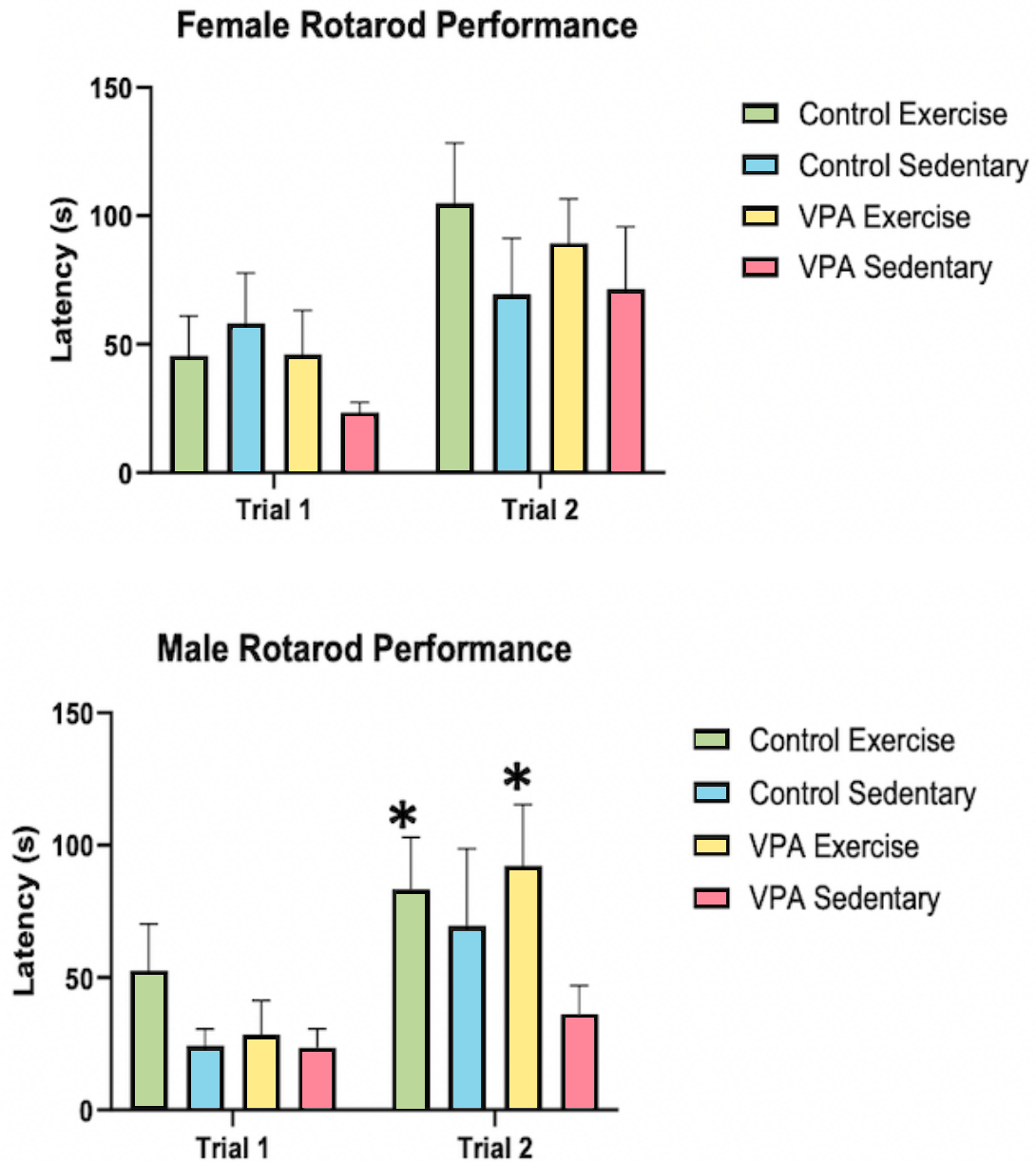


Figure 2. Rotarod

Exercise increased latency time in VPA and control exercise males. Trial 2 male exercised animals improved significantly compared to trial 1 (* $p < 0.05$). Further, exercised males performed better than sedentary animals within trial 2 (* $p = 0.03$).

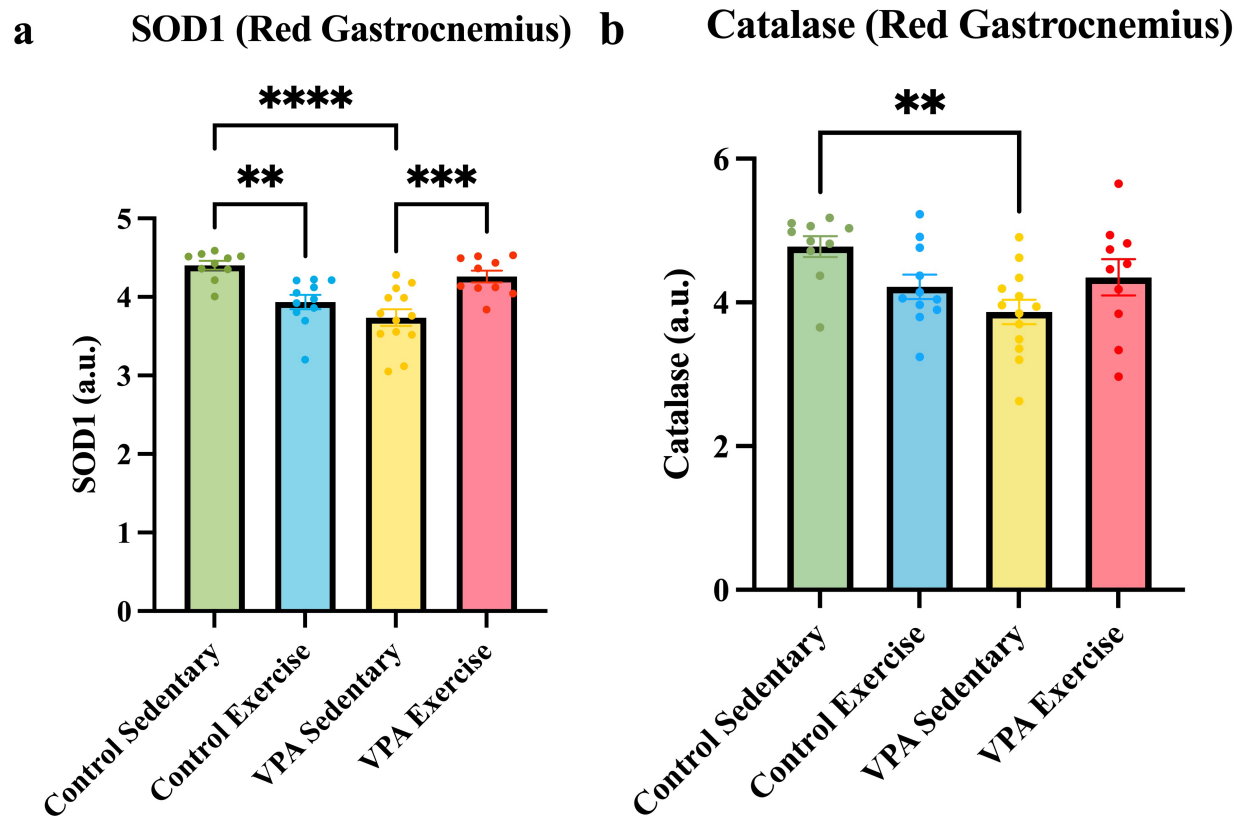


Figure 3. Red Gastrocnemius Antioxidant Expression

a) SOD1 expression in the gastrocnemius was significantly declined in VPA sedentary animals, compared to control sedentary animals (***p=0.0001). There was a significant increase in SOD1 expression in the VPA exercise group compared to VPA sedentary group (***p=0.0007). b) Catalase presented a significant decline in expression for VPA sedentary animals compared with control sedentary (**p=0.006)

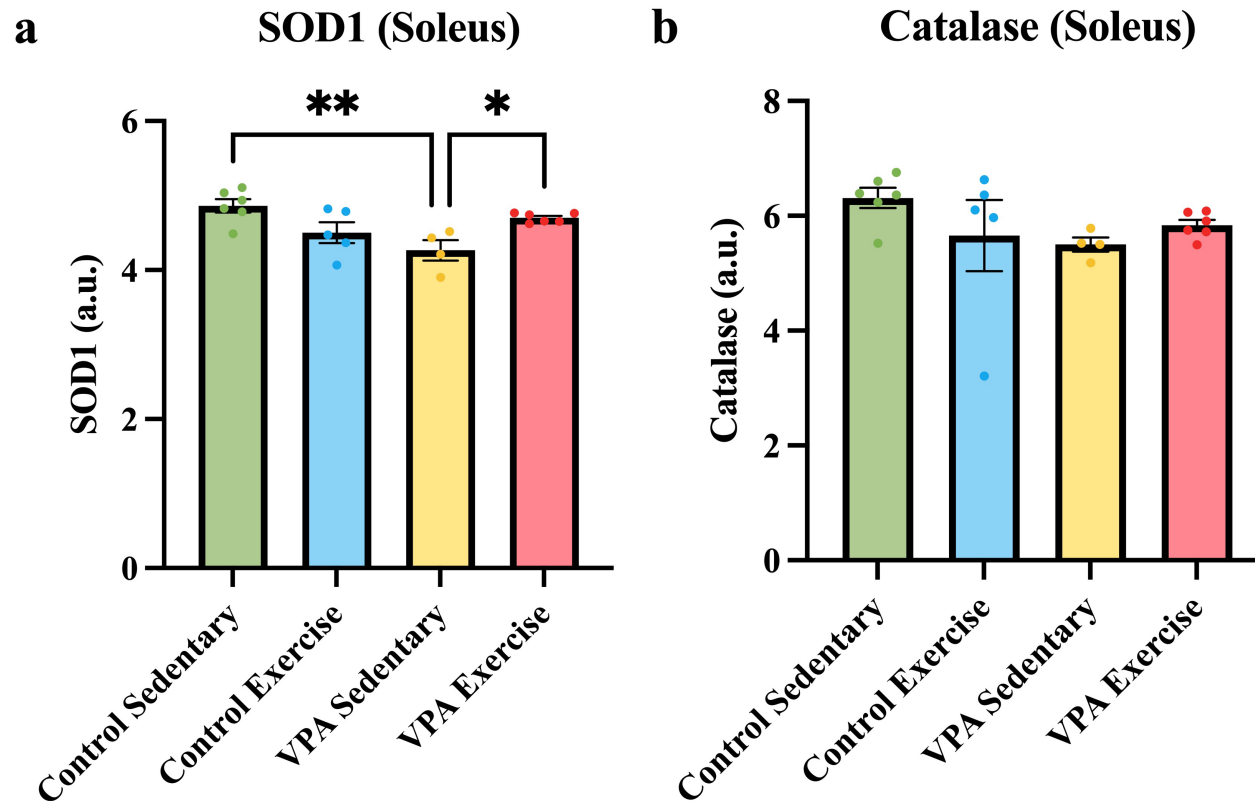


Figure 4. Soleus Antioxidant Expression

a) SOD1 expression was found to be significantly decreased in the VPA sedentary animals compared with control sedentary (** $p=0.004$), indicating an effect of ASD condition. VPA exercise groups had a significant increase in SOD1 expression compared with VPA sedentary (* $p=0.04$). b) Catalase expression presented no significant differences between groups (figure 5b).

NOR: % Exploration

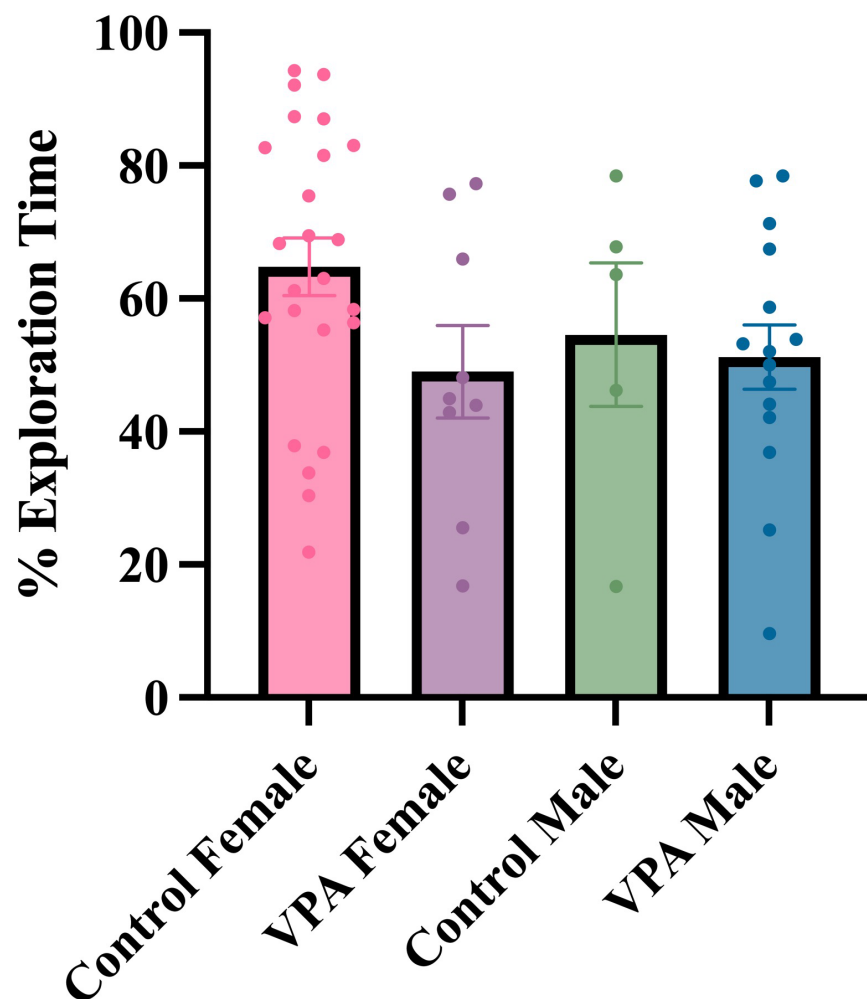


Figure 5. NOR % Exploration

NOR % exploration pertains to the exploration time of novel object as a percentage of the total object exploration time (Novel Exploration Time (s) / Total Time (s)). Trends towards decreased exploration time were identified in females (not significant). There were no differences found between VPA and control males.

NOR Discrimination Ratio

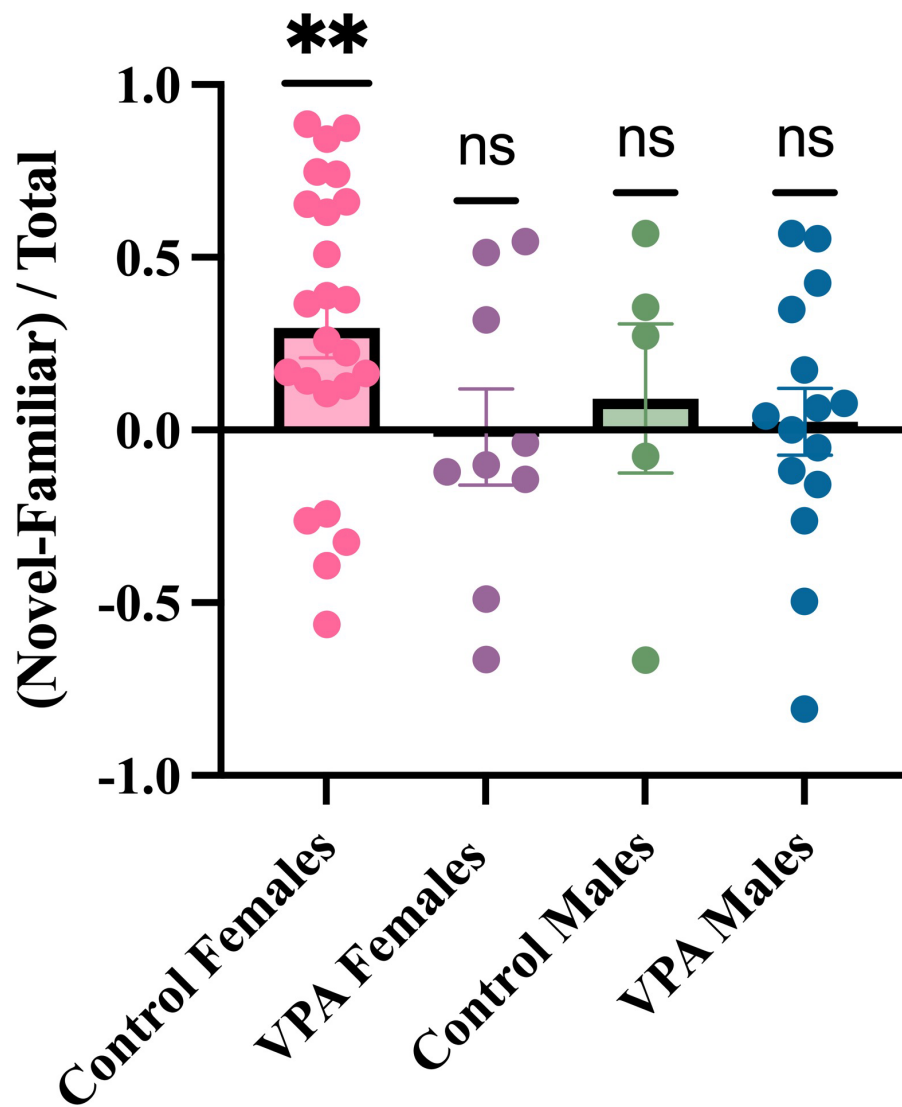


Figure 6. NOR Discrimination Ratio

The discrimination ratio is defined as the difference in exploration time between novel and familiar objects as a fraction of the total object's exploration time ((novel exploration time (s) – familiar exploration time (s) / (novel exploration time (s) + familiar exploration time (s))). No differences were identified for the discrimination ratio between groups. When compared to a hypothetical constant of 0, control females appeared significantly different in their discrimination ratio (** $p=0.002$), whereas VPA were not significantly different.

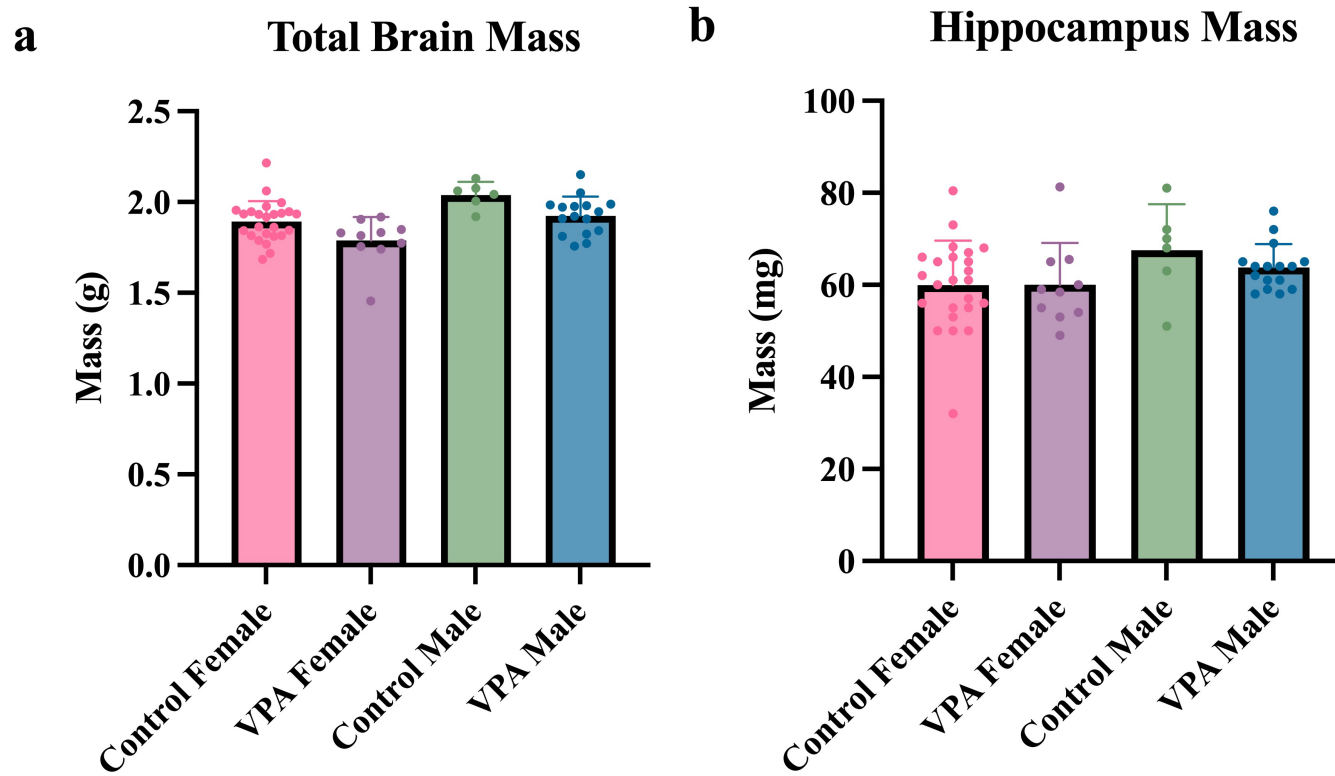


Figure 7. Brain and Hippocampus Mass

a) Total brain mass was measured in grams, resulting in no significant differences across groups. b) Hippocampus mass was measured in milligrams, with no significant differences identified.

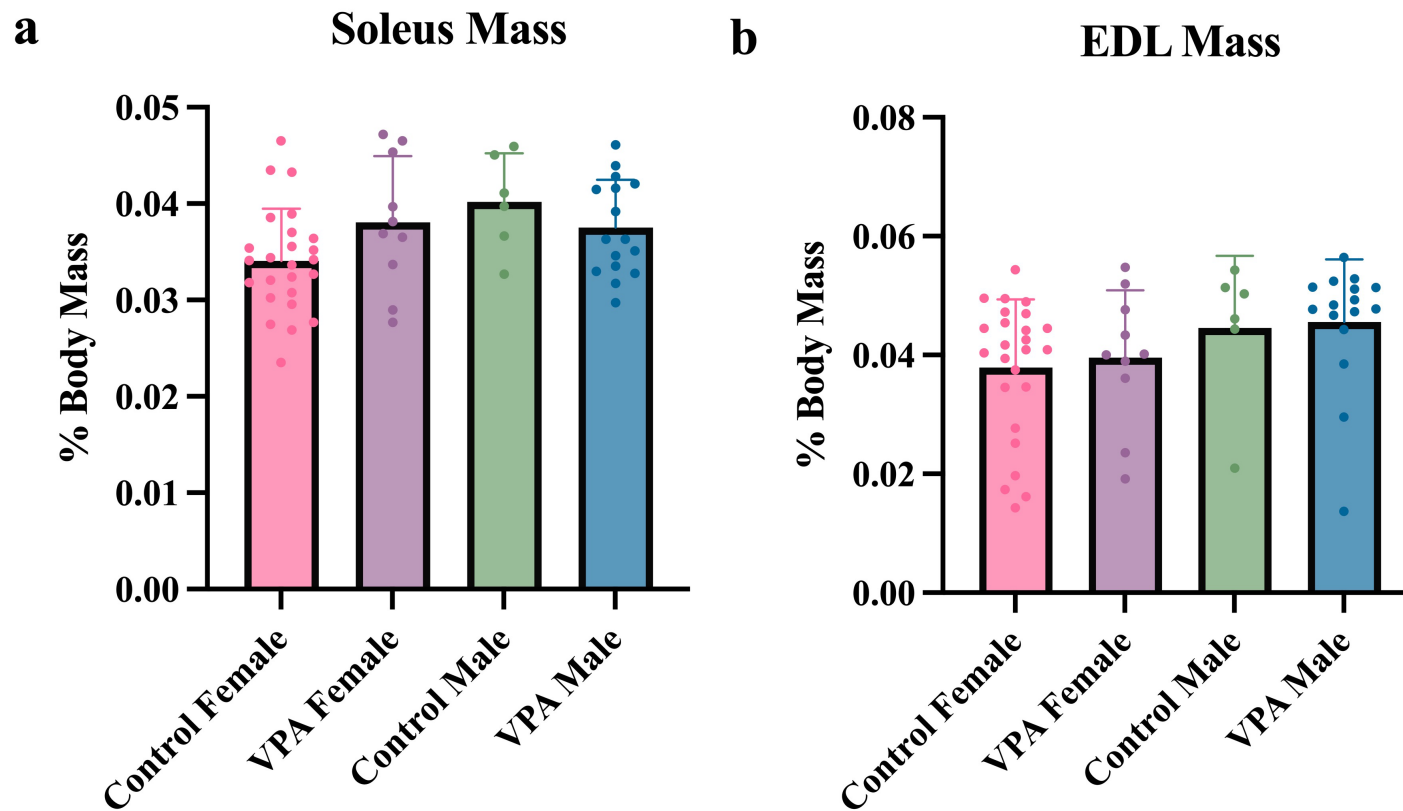


Figure 8. Skeletal Muscle Mass

a) Soleus mass, measured as a percentage of body mass, did not show significant differences between groups. b) EDL mass did not present any differences across groups.

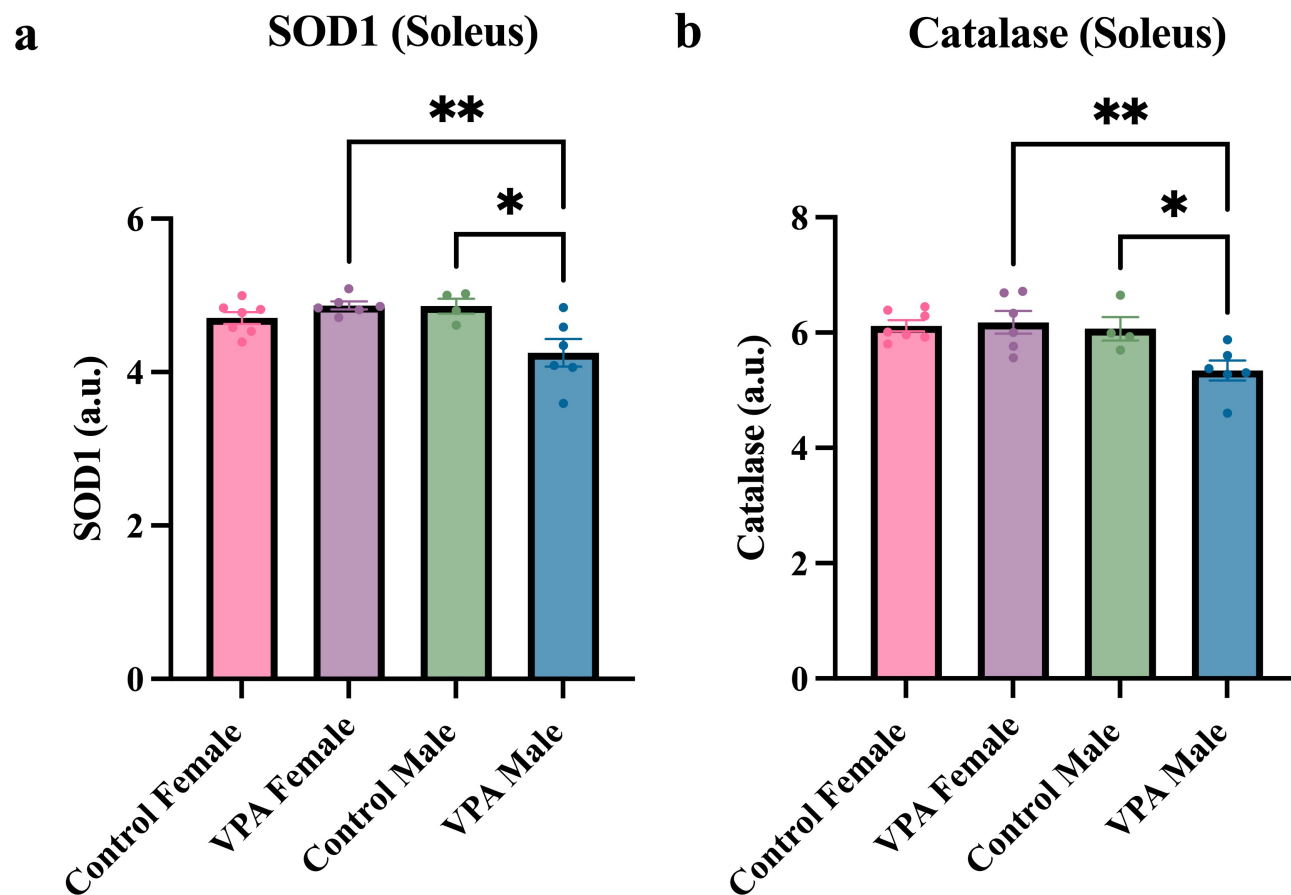


Figure 9. Soleus Antioxidant Expression

a) SOD1 expression, measured in the soleus, was found to be decreased in the VPA males, when compared to VPA females (** $p=0.004$) and control males (* $p=0.01$). b) Catalase expression, measured in the soleus presented decreased expression in VPA males compared to control males (* $p=0.04$), and when compared to VPA females (** $p=0.007$).

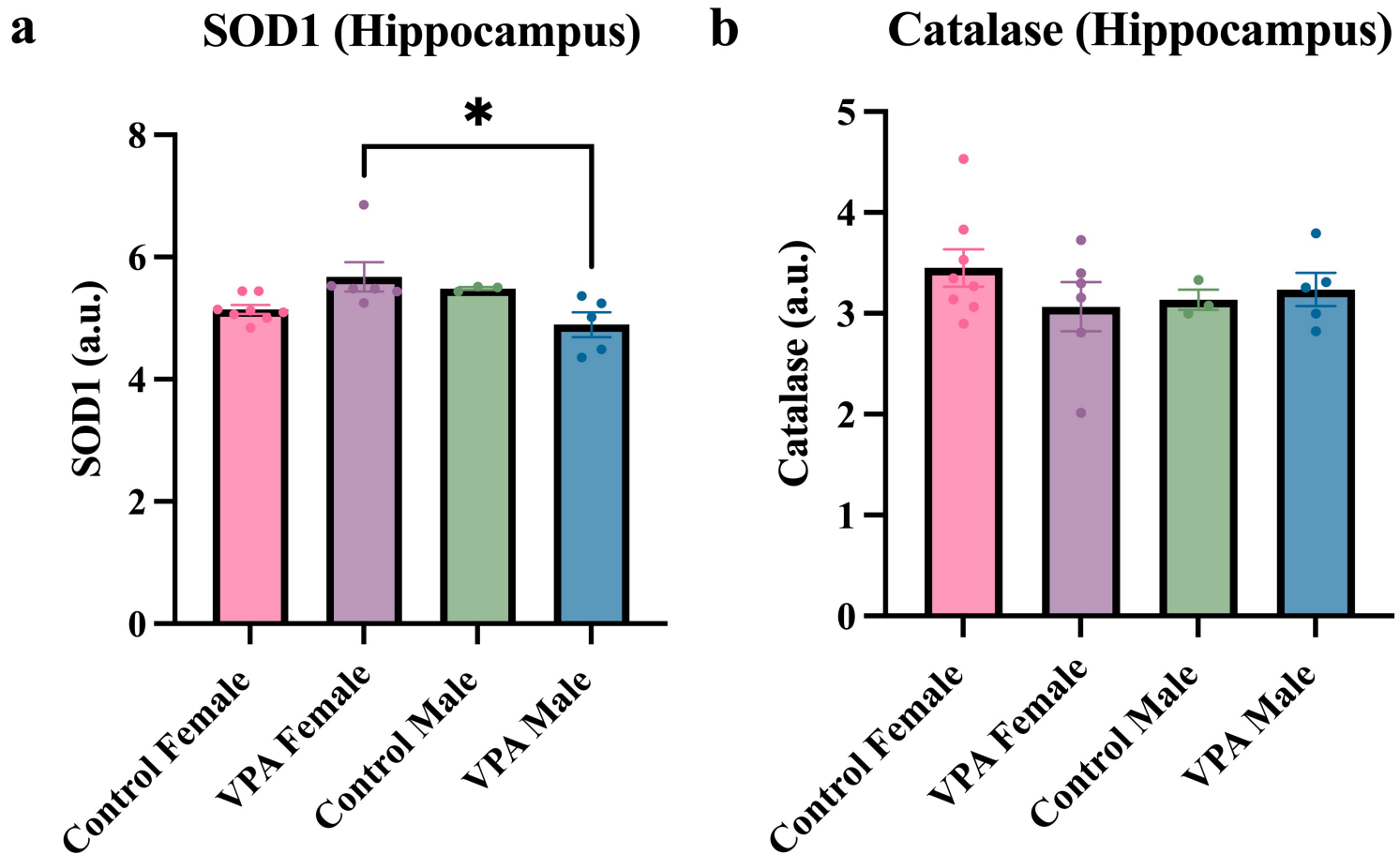


Figure 10. Hippocampus Antioxidant Expression

a) SOD1 hippocampal expression was decreased in the VPA males when compared to VPA females (* $p=0.02$). b) No differences were found between groups for catalase expression.

Chapter 5 - Discussion

To our knowledge, this was the first study to examine brain and skeletal muscle antioxidant status and the effects of exercise training and aging in the VPA rodent model of Autism Spectrum Disorder (ASD).

ASD in Human Behavior

ASD presents with impaired social interaction and cues, cognitive functioning, repetitive behaviors, language deficits, and stimuli sensitivities (Pragnya et al., 2014). These behaviors are hypothesized to occur through abnormalities within the brain, specifically the amygdala, prefrontal cortex, nucleus accumbens, and cerebro-cerebellar circuits. Environmental exposures in early childhood continue to exacerbate these abnormalities (Bachevalier & Loveland, 2006; Courchesne et al., 2011; Jasien et al., 2014; Schumann & Amaral, 2006).

ASD diagnosis follows the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). Here, symptoms of ASD are divided into two categories: communication and social interaction deficits, and restricted or repetitive patterns of activities and/or behaviors. Patients must present with symptoms in both categories to be diagnosed (McCarty & Frye, 2020; Rosen et al., 2021).

Current treatments to rectify these behavior deficits include behavioral, language, and social therapies. Prescription drugs and supplements will be used to treat the associated symptoms found with ASD such as anxiety, aggression, and insomnia (CDC, 2024; DeFilippis & Wagner, 2016; LeClerc & Easley, 2015; Sharma et al., 2018).

ASD in Rodent Behavior

In the present study, we found that ASD modulated behavior in the young ASD rats, as measured with rotarod and novel object recognition tasks. Due to the heterogeneity found in the human presentation of ASD, rodent models are often utilized to eliminate variability. The behaviors observed in humans with ASD translate to rodent models. This is expressed through performance in behavioral tasks. Three-chamber, elevated plus maze, rotarod, Morris water maze, open field activity, and novel object recognition, are among the most popular behavioral tasks utilized in the research (Banji et al., 2011; Sandhya et al., 2012).

As supported by the present research, ASD animal models perform poorly in these behavioral tasks when compared to control counterparts (Banji et al., 2011; McKinnell et al., 2021; Sandhya et al., 2012). Specifically, this study confirmed motor coordination declines in the VPA model of ASD animals as well as novel object recognition task data that demonstrated reduced social interaction behavior in the ASD animals compared to controls. These behaviors were normalized with the addition of exercise training. Indeed, previous literature has demonstrated this exercise effect; ASD behaviors as measured by novel object recognition task, rotarod, three-chamber task, and Morris water maze, are reversed, and become comparable with control groups (Cho et al., 2016; Xu et al., 2023). The behavioral improvements mediated through exercise give credence to exercise as a lifestyle intervention for ASD treatment.

ASD and Exercise Training

Exercise improves behavior impairments as assessed with novel object recognition tasks for both sexes and increased rotarod latency time for the male ASD animals. Exercise training also increased antioxidant expression in young VPA animals of both sexes and aged VPA males.

Exercise training has gained momentum as a more common form of therapy for ASD. It has been found that exercise improves the condition of ASD through behavioral outcomes, as well as neurological deficits (Cho et al., 2016; Xu et al., 2023). In addition, exercise significantly increases cerebellum Purkinje cell counts. Purkinje cells are involved with coordination, movement, and cognitive function. Significant decreases in Purkinje cell counts have been identified in the ASD post-mortem cerebellum (Bailey et al., 1998; Cho et al., 2016). The treadmill exercise protocol lasted 4 weeks. Animals ran 30 minutes a day, 5 days a week, with a maximum speed of 8 m/min. Following exercise the VPA-induced motor coordination impairments were attenuated. The Purkinje cells were measured in the cerebellar vermis, where they found a neuronal loss in the VPA-induced autism. Treadmill exercise rescued Purkinje neuronal count through inhibition of neuronal loss (Cho et al., 2016).

Various forms of exercise were explored by Xu et. al (2023) by implementing wheel running and swimming exercises. Utilizing a VPA model of ASD, animals underwent a 4-week wheel running training and swimming training. The novel object recognition task was performed for behavior analysis. ASD animals showed reduced time spent exploring novel object compared to control animals. The exercise protocol enhanced short-term recognition memory through increased time spent with the novel object. It was noted that the male VPA animals presented more obvious ASD-like behavioral phenotypes (Xu et al., 2023).

These findings are consistent with the behavioral findings in the present study. Additionally, these findings support our VPA model, as well as the exercise protocol of 4 weeks as sufficient to reduce ASD-like behavioral phenotypes.

Human studies have also identified the benefits with exercise. In a study implementing exercise through a trampoline, 16 children were enrolled in a 32-week exercise study utilizing a

trampoline. Strength, as measured by a long jump with both feet landing together, improved after the study (Lourenço et al., 2015). Jogging has also been explored as a therapy intervention for autistic individuals in a 12-week moderate to vigorous jogging intervention. The result of this improved emotional regulation in ASD subjects (Tse, 2020).

There remains much to be understood regarding exercise and the mechanism by which exercise improves ASD pathology. Thus, in the present study, exercise was utilized to understand its impact on skeletal muscle antioxidant status, as well as behavioral outcomes in young ASD rodents. Exercise was able to counter the deficit in antioxidants in the disease state of ASD. These results are indicative of a change modulated through the muscle which ultimately affects brain function and the condition of ASD.

It is important to connect exercise and antioxidants, as oxidative stress is a key pathological feature of ASD. This furthers our knowledge and understanding of the power of exercise in the context of ASD pathology.

Antioxidants in ASD

In the present study, reduced expression of the antioxidants superoxide dismutase 1 (SOD1) and catalase were found in young VPA skeletal muscles, and in aged VPA male skeletal muscle and hippocampi. Exercise training reversed this decrease in the young VPA muscles.

Examining antioxidants allowed us to understand the skeletal muscle as it relates to oxidative stress – a hallmark of many disease states, including autism (Ghezzi et al., 2013; Liu et al., 2022). One proposed mechanism for VPA-induced autism is the occurrence of oxidative stress, ultimately leading to CNS damage (Radavi-Asgar et al., 2022). VPA is a pro-oxidant factor, among others such as mercury, air pollutants, toxins, thalidomide, lead, and viruses (Chauhan & Chauhan,

2006; Kern & Jones, 2006; Sweeten et al., 2004; Zoroglu et al., 2004). Oxidative stress has been identified in humans with ASD as well (Al-Gadani et al., 2009; Assessment, 2009; Rose et al., 2012) Importantly, increased oxidative stress markers have been linked to ASD severity, meaning the presence of oxidative stress indicates a more severe case of ASD (Ghezzi et al., 2013).

Based on similar studies examining antioxidant supplementation, we suspect that improvements in antioxidants contributed to the behavioral improvements found in the present study (Banji et al., 2011; Pragnya et al., 2014; Sandhya et al., 2012). Pragnya et. al examined antioxidant power through the supplementation of piperine in a VPA mouse model of ASD (Pragnya et al., 2014). Piperine, present in black pepper, is known to possess strong antioxidant qualities. Administering 20mg of piperine to VPA animals caused significant change and improvement to behavior, as tested through negative geotaxis, thermal nociception, rotarod, elevated plus maze, and Morris water maze.

Antioxidant supplementation, via green tea extract (GTE), has also been shown to attenuate VPA-induced behavioral deficits (Banji et al., 2011). The VPA-treated mice performed poorly on the rotarod while the VPA mice supplemented with 300mg/kg GTE performed with increased latency times. Interestingly, they also examined the cerebellum in sagittal sections, finding damage to the Purkinje cell layer in the VPA-exposed animals. This was reversed with GTE supplementation, as indicated by an intact Purkinje layer examined on the GTE VPA animals (Banji et al., 2011). These results support the findings in the present study, regarding motor coordination results measured by the rotarod.

Exercise Training and Antioxidants

Exercise training has been shown to increase skeletal muscle expression of antioxidants, in both health and disease. In healthy rodent models, Hyatt et. al (2016) compared exercised and sedentary skeletal muscle in rats, finding increased antioxidant expression in the exercised soleus and plantaris muscles. Criswell et. al (1993) examined the difference between moderate (8-10 m/min) and high-intensity (20 m/min) exercise training and antioxidant production. Their finding was that both exercise intensities provided increased SOD antioxidant production. Lastly, exercise can protect against ROS-generating toxins. In a model of oxidative damage and muscular atrophy, aerobic treadmill exercise was able to counter this damage through the production of antioxidants. Exercise was found to be a protective mechanism through neutralizing ROS through exercise-induced antioxidant production (Smuder et al., 2011).

A separate study showed that VPA exposure in rats decreased the antioxidant levels of catalase and SOD in the liver ($p<0.001$) (Mirzavandi et al., 2023). Liver disorders are found in the case of ASD and thus explored through the study. They implemented an exercise training protocol consisting of mild and moderate intensity exercise. Mild-intensity exercise training consisted of four weeks of 30-minute runs a day at 8-10 m/min speed. Moderate intensity exercise training group ran five times a week for four weeks at 20m/min for 30 minutes. The exercised animals presented increased levels of antioxidants ($p<0.001$). These results are consistent with the present study (Mirzavandi et al., 2023).

With this knowledge, we hypothesized that exercise training would generate antioxidant production in the skeletal muscle in the disease state of ASD. Our results supported this hypothesis with increases in the antioxidant, SOD1, in the gastrocnemius and soleus muscles of exercised ASD rats.

Antioxidants and skeletal muscle

The antioxidants SOD1 and catalase, assessed in the present study, are naturally occurring in the body. The ability to enhance antioxidant expression naturally through lifestyle intervention is valuable as a drug-free and cost-free method of treatment.

SOD1 is expressed within the mitochondria, while catalase is expressed outside the cytosol. Significance for SOD1 appeared across the board between muscle type, as well as in young and aged animals on conclusion. Because of the consistency in our findings with SOD1, we can assess that ASD pathology is related to mitochondrial dysfunction. This theory is supported in the literature (Castora, 2019; Genovese & Butler, 2020; Legido et al., 2013). Frye and Rossignol, (Frye & Rossignol, 2011) proposed mitochondrial dysfunction as a candidate for ASD pathology. Their reasoning behind this is connected to the whole-body pathology of ASD. The brain, musculoskeletal system, and gastrointestinal system, among other systems are affected (Balachandar et al., 2020; Frye & Rossignol, 2011)

ASD and Muscle

Beyond antioxidant expression, we expected a potential difference in the physical phenotype of ASD skeletal muscle. This suspicion was based on the high prevalence of hypotonia and associated muscular impairments found in ASD. Low muscle tone, or hypotonia, has been identified in ASD and presents alongside autistic traits (Serdarevic et al., 2017). Hypotonia has even been identified as an early indicator of ASD. Hypotonia and ASD occur together in up to 51% of cases examined (Ming et al., 2007; Serdarevic et al., 2017). Beyond this exists the inverse relationship discovered between ASD case severity and muscle strength (Kern et al., 2011). These comorbidities and connections suggest a physical aspect of ASD that could be manifested in the muscles. Furthermore, because of the success exercise has shown with ASD, this tells us that

muscle is a powerful tool to improve ASD behavior and pathology. This raises questions regarding the skeletal muscle phenotype in ASD.

Pathogenic variants to the gene dystrophin have been associated with ASD risk. Dystrophin is expressed in skeletal and cardiac muscle, as well as the CNS, and contributes to Duchenne Muscular Dystrophy (DMD). There is a clear connection between intellectual disability and abnormality with dystrophin. The recent cohorts with DMD have presented with a higher frequency of neurodevelopmental disorders, including ASD (Passos-Bueno et al., 2022).

Megaconial muscular dystrophy presents with early-onset hypotonia, muscle wasting, weakness, cardiac involvement, delay in gross-motor developmental milestones, autistic features, and behavioral problems, and is often involved with an ASD diagnosis (Kutluk et al., 2019).

To gauge the potential phenotypic differences in skeletal muscle, weights of the gastrocnemius, extensor digitorum longus (EDL), and soleus muscles were excised and analyzed in the aged VPA animals. While no significant differences were found between muscle masses, our results support a particular function of the muscle in ASD pathology as indicated in our skeletal muscle antioxidant findings.

ASD Impact in Aging

Through the present study, we demonstrated the effects of aging through reduced antioxidant expression in VPA males. Understanding aging in ASD is highly important, due to its rising prevalence, increased mortality, and lack of medical knowledge. Furthermore, the population of adults with autism has been found to have a higher mortality rate when compared with their control counterparts (Akobirshoev et al., 2020). More concerning, individuals identified

with ASD are 2.6 times more likely to develop early-onset dementia than the general population (Vivanti et al., 2021). Because of this, we chose to explore an aged VPA rodent model.

In the present study, we employed the behavioral task, Novel Object Recognition (NOR). This did not result in significant change across groups, indicating that behavioral impairments were not affected by aging for the VPA model of ASD.

In a separate study, an aged model of autism was analyzed. The BTBR + *tf/j* (black and tan, brachyuric) rodent model of autism was analyzed at 15 months to examine autism in old rats. The animals underwent a battery of behavioral tasks. They discovered that the social behaviors in ASD persisted into adulthood, as gauged by the social preference in the three-chamber test. In the open field test, ASD animals preferred to remain in the periphery during exploration. Interestingly, there was no change identified in the NOR or rotarod data between ASD and control animals. Beyond behavioral data, they also examined BDNF in the cortex and hippocampus, finding significantly decreased levels of mature BDNF in the aged ASD animals.

The molecular data taken from our aged animals in the current study presented with decreases in antioxidant expression in the muscle and brain. However, this result was only identified in the males. This indicated a sex difference in the VPA rodent model that developed through the aging process.

To begin to explain this sexual dimorphism, we can turn to the statistic that males are more likely to develop ASD than females, with a 4:1 ratio (Loomes et al., 2017). Beyond this, there are theories regarding female protection against ASD, indicating that additional genetic abnormalities must be present for ASD behaviors to present themselves in females (Werling & Geschwind, 2013; Zhang et al., 2020). What we know about sex differences in ASD has been primarily explored in

young human and rodent studies rather than old. Because of this, the reason behind the sex difference discovered through the present study requires further research.

Study Limitations

One limitation that could have impacted results in experiment 1 animals is the exercise training treadmill performance. Animals were treadmill trained on a motorized treadmill with air puff sensor. Animals were not always motivated by the air puff on the treadmill leading to breaks in their session. This low-stress environment was intentional, albeit still a limitation. While the citrate synthase activity indicated that an exercise training effect did occur, it was a modest increase. Exploring alternative exercise training methods and treadmill speeds in the future could provide further insight into the impact of exercise on ASD.

In experiment 2, the number of animals enrolled in the experimental protocol were littermates from experiment 1 and thus, limited in number. The groups in experiment 2 were not evenly distributed and could have impacted results.

As with all translational research, the goal is to design a rodent experiment that translates to the human condition. Age is a practically hard aspect to translate and thus observations made in these old rats may not represent the human condition of aging.

ASD displays behaviors that range from social deficits, to repetitive actions, to cognitive impairments. The behavioral tasks implemented in the present study measured sociability and motor coordination, leaving many other behavioral parameters to account for. It is possible that exercise and age would have a greater impact on behavior outcomes other than sociability and coordination.

Future Directions

Exploring alternative exercise methods could provide value in the field of exercise and ASD. Future studies could look into the impact of resistance training, as well as varying treadmill intensities in ASD (Dudley et al., 1982).

Exploring potential sex differences in the 6-18 month VPA model of ASD could provide further insight into the male-specific changes that were identified in the present study.

Finally, measuring reactive oxygen species (ROS) in the skeletal muscle would allow for a full understanding of oxidative stress in ASD skeletal muscle.

Conclusion

In the present study, the expression of antioxidants in skeletal muscle and brain tissue was assessed across young and old VPA animals. We demonstrated that VPA-induced autism decreased antioxidant expression in the skeletal muscle of young rats and in the muscle and brain of aged males. We also demonstrated the protective effect of exercise via increased antioxidant expression in young VPA animals. Additionally, VPA-induced ASD caused decreased motor coordination in the males (as measured by the rotarod). This impairment was rescued with exercise.

Decreased antioxidant expression was indicative of oxidative stress. This is important to identify in our VPA animals as oxidative stress is a core pathological feature of ASD. This provides further validity to the study as well as the animal model. ASD-like behaviors were identified in the young VPA sedentary animals, which were brought closer to control baselines with the addition of exercise.

Employing exercise training as the intervention in young VPA animals increased antioxidant expression significantly. This is impactful to the field of ASD research by shedding light on the benefits of exercise as a lifestyle intervention and treatment form.

Finally, while much is yet to be understood, the present study began to provide insight into an animal model of ASD and the potential impacts of aging. While the ASD behavior did not persist into adulthood, a decreased antioxidant expression in the brain and skeletal muscle did. This change was identified in the male VPA animals only and warrants further investigation into the sex differences that occur with aging.

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