PROTECTIVE EFFECTS OF ANTIOXIDANT PRETREATMENT IN VPA-TREATED NRF2 KNOCKOUT MICE

by

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ABSTRACT OF THE THESIS

Protective effects of antioxidant pretreatment in VPA-treated Nrf2 knockout mice

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Genetic and environmental factors associated with oxidative stress have been implicated in the etiology of autism. The present study attempted to mimic the factors in an animal model of autism. Specifically, mice with a deletion of the Nrf2 gene, a master regulator for downstream enzymes associated with management of toxicant-generated reactive oxygen species, were administered valproic acid (VPA), a toxicant known to engender oxidative stress and one that has been associated with autism in humans. Prior studies revealed that VPA treatment induces functional and pathological changes in mice akin to autism. Further, previous work has established that pretreatment with antioxidants has the ability to protect mice from these VPA-induced functional deficits. The present study extended these observations to mice with alterations in NRF2 expression. On postnatal day 14 knockout (KO) and wild type (WT) mice were exposed to VPA (400 mg/kg) or saline and pretreated with either Trolox, a water-soluble form of vitamin E, or saline 1 hour prior to VPA. The behavioral tasks employed assessed maturation of normal social, cognitive, and motor skills and classified toxicant-induced deficits along a developmental timeline. Treatment with VPA resulted in deficits in mid-air righting and Morris water maze learning. Further, Trolox pretreatment prior to VPA
provided partial protection from deficits associated with VPA treatment and this protective effect was more apparent in the Nrf2 KO mice. The results of the present study, at least in part, indicate the importance of Nrf2 during development as it might relate to autism and, more generally, the effects of oxidative stress during early development as well as the potential protective effects of antioxidant pretreatment.
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INTRODUCTION

Background

Autism spectrum disorder (ASD) is a neurodevelopmental disorder affecting about 1 in 68 children in the United States (CDC, 2014). Exposure to environmental toxicants, infection or genetic alterations are hypothesized to contribute to the development of ASD, although the underlying cause is still unclear. As a result, much current research is focused upon attaining a better understanding of potential risk factors and the mechanisms through which they might disrupt neurobehavioral development. One such hypothesized mechanism involves the role of oxidative stress.

Oxidative Stress and Development

The process of oxidation is a necessary chemical event in cells in which electrons are removed from target molecules to enable necessary biochemical reactions to generate energy for the cell and for metabolism of proteins and carbohydrates through various cellular functions (Muralidharan et al., 2013). The byproduct of these reactions results in free radicals, or reactive oxygen species (ROS). Under normal conditions, cellular stability is maintained by endogenous regulatory antioxidant responses which have the ability to neutralize ROS; these include glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase. When environmental demands such as toxicant exposure, infection, or genetic alterations cause increased oxidation, ROS levels may exceed the antioxidant capacity of a cell, a process referred to as oxidative stress. This excess of ROS can lead to cytotoxic damage to cell membranes, proteins, and even DNA. The brain is particularly vulnerable to excess ROS as it has a smaller antioxidant capacity compounded with high energy requirements for cell function compared to other organs.
Therefore, the brain seems to be quite susceptible to the deleterious effects of oxidative stress, particularly during development when there is a high rate of cell proliferation (Chauhan et al., 2006).

Antioxidants are necessary for survival of neurons during critical periods early in life (Perry et al., 2004). Children from conception throughout infancy may be more susceptible to the effects of oxidative stress as they have lower levels of antioxidants including glutathione (Erden-Inal et al., 2002). Additionally, evidence suggests antioxidant ability is acquire over time (Bernhardt et al., 2017). This lack of defense mechanisms in addition to the fact that environmental toxicants found in the mother are also detectable in the fetus, indicates the brains of developing children are at increased risk of the deleterious effects of oxidative stress.

**Oxidative Stress and Autism**

The link between oxidative stress and autism has been identified through examination of markers of oxidative stress in individuals with autism along with increased body burdens of environmental toxicants associated with induction of oxidative stress (Edelson & Cantor, 1998; Dietert & Dietert, 1997). ASD has been linked to low levels of glutathione peroxidase, superoxide oxidase and catalase activities and total glutathione and cysteine levels (James et al., 2004; Sogut et al., 2003; Yorbik et al., 2010; Zoroglu et al., 2004). Further, decreased antioxidant levels were present in plasma of individuals with ASD and there is evidence of impaired energy metabolism and mitochondrial dysfunction as well (Filipek et al., 2003; Rossignol and Frey, 2014). Finally, children with autism have increased levels of oxidative stress biomarkers in their urinary excretions indicating they are experiencing increased oxidative stress relative to
age-matched controls (Ming et al., 2005). Collectively, these data indicate that individuals with autism may have difficulty managing oxidative stress due to environmental demands such as toxicant exposure and/or genetic alterations.

**Oxidative Stress and NRF2**

Excessive ROS exert a cellular burden that can cause functional decline. Since ROS accumulation is inevitable given the energy demands of the developing organism, its removal or limits on oxidation are controlled by endogenous antioxidant mechanisms. A master regulator for the induction of antioxidant events is nuclear factor erythroid 2-related factor 2 (NRF2) (Wakabayashi et al., 2010; Sandberg et al., 2013). This is a transcription factor that is normally restrained in the cytoplasm by the protein KEAP1. However, in response to metabolic or toxic stimuli, NRF2 migrates to the nucleus and binds to an antioxidant response element (ARE) on DNA, initiating downstream induction of antioxidant genes (Wakabayashi et al., 2010; Sandberg et al., 2013). This effect is a critical response to environmental demands (including toxicants), which necessitate its activation and the downstream induction of antioxidant genes. Our preliminary data confirm this at the behavioral level using Nrf2-deficient mice, and suggest that NRF2 may serve to protect the developing animal from oxidative stress (Furnari et al., 2014).

**Valproic Acid, Oxidative Stress and Autism**

Valproic acid (VPA) is a GABAergic anticonvulsant that has been shown to increase the risk of autism-like behaviors in offspring if taken during pregnancy (Christensen et al., 2013; Singh et al., 2014). VPA acts as an indirect GABA agonist as it non-specifically blocks the enzymatic degradation of GABA, thereby resulting in GABA
buildup. Recent work by Ben-Ari (2014) suggests a shift in GABA action, which is regulated by development. This work suggests an excitatory action of GABA during fetal development and into early postnatal life followed by a shift to inhibitory action around the third week of life in rodents (Ben-Ari, 2014). This initial excitatory role of GABA may underlie excitotoxicity during this critical window of development.

We, and others, developed an animal model of autism involving exposure to VPA, which results in behavioral and neuroanatomical deficits similar to autism in humans, both pre- and postnatally, targeting the time window of GABA shift (Wagner et al., 2006; Yochum et al., 2008). Additionally, when VPA is metabolized it is known that oxidative stress ensues, as measured by a decrease in cellular glutathione peroxidase and increased ROS (Jurima-Romet at al., 1996). This connection to oxidative stress makes VPA a prime model for examination of autism prevention strategies. That is, the hypothesis that antioxidant pretreatment may protect mice against VPA-induced oxidative stress may be tested.

**Antioxidant Treatment of Symptoms**

Improvement in behaviors following antioxidant administration to individuals with autism further elucidates the role of oxidative stress in contributing to the etiology of autism. Double-blind, placebo controlled trials have indicated the efficacy of high-dose vitamin C or carnosine on improving behaviors associated with autism (Chez et al., 2002; Dolske et al., 1993). Additionally, a three-week supplement of betaine and folinic acid administered to twenty children with autism who displayed low levels of GSH and cysteine showed an improvement in blood plasma levels of antioxidants (Roberts et al, 2010). Recent work has shown pretreatment of astaxanthin, an antioxidant with the
ability to cross the blood brain barrier, significantly improved behavioral deficits following prenatal VPA exposure in humans (Al-Amin et al., 2013). Additionally, sulforaphane, an antioxidant found in broccoli sprout extract, has been shown to induce NRF2 expression and to lessen the symptoms of autism in young boys (Singh et al., 2014). Taken together, these lines of evidence support the hypothesis that at least some children with autism exhibit enhanced oxidative stress and antioxidant pretreatment may lessen the severity of symptoms.

**Antioxidant Pretreatment: Prevention of Symptoms**

Previous work in rodents found that vitamin E treatment prior to VPA exposure during gestation reduced the likelihood of fetal toxicity (Deeb et al., 2000). Additionally, work in our lab has shown Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), a water-soluble vitamin E derivative, when used as the antioxidant pretreatment to MeHg challenge, protected mice against behavioral deficits induced by the MeHg. Specifically, Trolox effectively attenuated deficits in the mid-air righting reflex maturation, a behavior disrupted with MeHg challenge alone (Cheh et al., 2010). MeHg, like VPA, is known to exert its neural damage through oxidative stress. Further, work in our lab has shown vitamin E in a corn oil vehicle can also attenuate behavioral deficits associated with postnatal VPA treatment (Ming et al., 2008). Our lab has yet to examine the effectiveness of Trolox, a water-soluble form of vitamin E in a VPA model. There is also evidence of a therapeutic action of green tea extract following VPA exposure. Green tea extract ameliorated behavioral changes associated with VPA treatment including motor delays and anxiety as well as neutralization of free radicals and decreased lipid peroxidation levels following treatment (Banji et al., 2011). Collectively, these results
indicate a protective role of antioxidant pretreatment in both MeHg and VPA-induced behavioral deficits suggesting a role of oxidative stress in the development of behavioral deficits in both these animal models of autism.

**Animal Model of Autism**

An animal model has been developed in which mice are exposed to any of a number of toxicants early in life (i.e. pre- or early post-natal exposure) and are then assessed for their behavioral maturation as well as for neuropathological damage. The behavioral tasks assess the maturation of social, cognitive, emotional and motor skills; deficits are further classified as retardations (i.e. the toxicant slows and/or eliminates the development of a skill), regressions, (i.e. a skill matures at the same rate as control-treated pups but then the toxicant exposure results in a loss of those skills), or intrusions (i.e. the skills do mature on schedule but their appearance is overshadowed by stereotypic or self-injurious behaviors). Further, this model incorporates genetic alterations by assessment of toxicant-induced behavioral and neuropathological damage in knockout versus wild type mice. These mice are not autistic; rather the toxicants selected have been associated with human autism as are the genetic alterations. Thus, the use of this model allows for the demonstration of toxicant-induced deficits in neurobehavioral development with critical variables including time of toxicant exposure as well as genotype and sex of the littermate offspring (Furnari et al., 2014; Wagner et al., 2006; Yochum et al., 2008).

Previous work has indicated that toxicant exposure on postnatal day 14 (P14) results in a regression of behaviors such as the mid-air righting reflex. P14 was selected for postnatal treatment as the behavioral skills have now matured by that day and, hence,
it is possible to demonstrate regression. Further, as noted above, pretreatment with the antioxidant, Trolox, was shown to protect pups against methylmercury challenge (Cheh et al., 2010) and vitamin E pretreatment protected pups against VPA-induced regression (Ming et al., 2008). Both toxicants have been associated with autism and are known to generate oxidative stress. Finally, recent studies examined postnatal VPA administration delivered to P14 pups with an Nrf2 deletion resulting in enhanced sensitivity to the neurodevelopmental toxicity induced by the VPA (Furnari et al., 2014). This provides a convincing demonstration of the importance of NRF2 in managing toxicant-induced damage during early development.

Summary and hypotheses

The present study aims to determine whether NRF2 is necessary to maintain stable brain and behavioral development in the face of early postnatal challenge with VPA. If VPA stress induction shows a reliance on NRF2, this will show that NRF2 may be a critical regulator of neuroprotection in early postnatal development. Specifically, since developmental disorders including ASD have been linked to oxidative stress, we hypothesize that pups lacking NRF2 will not have sufficient antioxidant neuroprotection against physiological perturbations that result early postnatal challenge and this will result in a pronounced deficit in postnatal development of social, cognitive, and emotional behavior. The present study will assess mid-air righting, rotarod, social approach, Morris water maze, and Y maze performance. It is hypothesized that Nrf2<sup>-/-</sup> mice exposed to VPA will show the greatest impairments in the described tasks. Additionally, previous work in our lab has shown antioxidants (including Trolox) were fully effective against post-natal challenge with toxicants such as methylmercury but
effectiveness of Trolox is unknown in NRF2 deficient mice challenged with VPA. We predict that antioxidant pretreatment will protect mice against the behavioral deficits induced by VPA in the *Nrf2* mice and the magnitude of this difference will be most evident in the *Nrf2* knockout mice.
MATERIALS AND METHODS

**Subjects:** All animals were maintained under standard vivarium conditions with free access to food and water and a 12:12 hour light:dark cycle. All procedures were approved by the Animal Care Committee and our facility meets AAALAC standards. From our original colony of \(Nrf2^{+/}\) mice on a C57Bl/6J background (obtained from Dr. Tony Kong of Rutgers University) we have derived wildtype and knockout \(Nrf2\) female mice that were mated with male wildtype and knockout \(Nrf2\) mice generating litters that contain either +/+ or +/- genotypes. Breeding in this way allowed for adequate time for breeding and only produced necessary animals and removed the need for additional genotyping.

**Group Design:** One male (KO or WT) was placed with two receptive females (KO or WT) together for 8 days to breed KO and WT litters. Day of birth was noted as postnatal day 0 (P0). Pups were weighed and sexed on P13 and weighed daily until P19. On P14, one set of pups was injected with either saline or valproic acid (400 mg/kg) delivered in a saline vehicle in a volume of 1.0 ml/100 g body weight. In order to determine if a one-hour pretreatment with Trolox protected mice against the toxicity induced by the P14 VPA, a second set of mice was injected with 25 mg/kg of Trolox in a saline vehicle in a volume of 1.0 ml/100g body weight administered one hour before either the VPA or saline treatment on P14.

**Behavioral Tests:**

**Motor Tasks:** Although autism usually is not associated with severe motor disturbances, we and others have characterized deficits in children with autism such as delays in motor milestone development, disturbances in reach-to-grasp, deficiencies in gross and fine motor movement, and underdevelopment of postural control (Wagner et al., 2006; Ming
et al., 2007). In addition, stereotypic or repetitive motor behaviors often appear in autism. In mice, a wide variety of motor tests are available for assessing behavioral disturbances across development. Mid-air righting was utilized in this study. In this task animals are dropped dorsal side down from 18 cm above a padded surface and their ability to right themselves (land on all four paws) was assessed. The timing of this test is based on known developmental milestones, with mid-air righting taking place on P13 – P19. Previous research has shown that in postnatal VPA treatment produces significant deficits in mid-air righting on P15 in both NRF2 WT and KO animals (Furnari et al., 2014).

Additionally, previous work has demonstrated the effectiveness of vitamin E pretreatment on mid-air righting behavior in a MeHg model of autism (Ming et al., 2008). The present study sought to determine if pups lacking NRF2 treated with both Trolox and VPA on P14 will display fewer deficits than what is seen with VPA treatment alone. Performance was measured by the correct responses across three trials per day.

**Rotarod:** We tested mice on P25-27 and again between P90-200 for their performance on the rotarod. Mice were placed on the rotating rod for three test trials each day for three consecutive days and latency to fall was recorded, with a 60-sec maximum. The rotarod consists of a 6.0 cm diameter cylinder covered in textured material and run at 12 RPM.

**Spatial Learning and Memory:** The present study employed a scaled down Morris water maze for pups (MWM; Wagner et al. (2006)) to test for spatial memory. The water maze use was a circular galvanized steel tub 61 cm in diameter and 29 cm in height, filled ¾ of the way with room-temperature water, made opaque with non-toxic white paint powder. A white circular escape platform, measuring eight cm in diameter, was placed in one quadrant of the maze, two cm below the surface of the water, its position held constant.
The water maze is a learning paradigm in which an animal must make use of spatial cues to navigate the maze; this skill does not mature in rodents until about day 22 of life. Additionally, there were various extra-maze cues in the testing room to facilitate mapping of the spatial environment. Mice were tested on five consecutive days, beginning on postnatal day 30 and continuing through day 34. For acquisition, which occurred on days one through four, five trials were given each day. Each trial started from a randomly assigned quadrant of the maze and each mouse was allowed a maximum of 60 seconds to find the escape platform on each trial. For these studies, the hidden platform remained in the same location each day. If the animal did not escape, it was placed on the platform for a 15 second inter-trial interval (ITI). If the animal successfully found the platform, there was a 15 second ITI, during which the animal remained on the platform. At the completion of this phase of testing, we used one, 60 second probe trial (day 5) where there was no platform available. We monitored the performance of the subject for time spent in the proper quadrant (compared to time spent in other quadrants). Performance was measured by latency to find the platform, distance travelled, and mean speed.

**Social Approach:** Social deficits are a core sign of autism. Therefore, we assessed social interactions using our standard social measure. For this test, each set of mice were of same genotype, sex, and age (P40). We used an automated chamber and tracking software to quantify social approach in mice. There were two wire cylinder cage enclosures (11 cm diameter) on opposite sides of an open field (36.5 cm x 36.5 cm). This test consisted of three 10 minute phases. Phase one (stranger phase) acted as a period of habituation with both wire cages empty. In phase two, a target mouse (same treatment, age, sex, and genotype) was enclosed in one wire cage and an empty wire cage on the
opposite side of the open field. The test mouse was placed in the center of the open field and time spent with the stranger and empty cages were automatically recorded. This was followed by phase three (test phase) in which a new stranger (same treatment, age, sex, and genotype) were placed in the once empty wire cage. The test mouse was placed in the center of the open field and time spent with the novel and habituated stranger cages were automatically recorded.

**Y maze:** Perseverative behaviors are another core deficit in autism. Use of the Y-maze reversal task allowed examination of perseverative behaviors expected to be exhibited by VPA treated animals and attenuated by antioxidant pretreatment between P42-P60. This task employs a water filled Y shaped maze, which was made opaque using nontoxic white paint powder. Additionally, there were various extra-maze cues placed on the walls for animals to use to map the spatial environment. The Y maze consists of three arms, one of which is designated the start arm while the other two are target arms. Within the maze, there is a hidden escape platform (10 cm in diameter) placed in one of the target two arms. There were two phases of Y maze testing; acquisition and reversal. Each phase consisted of 6 trials per day with a 5-minute inter-trial interval. During acquisition, animals were placed in the start arm and allowed to make a choice between one of the two target arms. Once an animal had chosen an arm (defined by full body entrance into an arm), a guillotine was placed at the entrance of the chosen arm, forcing the animal to stay in the chosen arm. If this was the correct arm (arm with the hidden platform) animals were allowed 10 seconds on the platform to gather spatial cues before the trial ended. If the animal chose the incorrect target arm (arm without the hidden platform), this was considered an error and animals were allowed 10 seconds to swim the incorrect arm
before the trial ended. Completion of acquisition was defined as 11 correct responses in two consecutive days (ie. one error in two days of testing). Immediately following acquisition (following day) animals began reversal training. During reversal, the same protocol for acquisition was used except the hidden platform was switched to the opposite target arm. Completion of reversal is also defined by 11 correct responses in two consecutive days. Animals were considered to have “failed” the task if either acquisition or reversal lasted more than 10 days. Data collected consisted of performance on each trial as well as total number of errors and total number of days to both acquisition and reversal.

*Measures of NRF2:* To determine if postnatal VPA induces the expression of NRF2 in early postnatal brains, wild type males and females were administered VPA or saline on P14 or ~P90 and their brains were harvested two-hours post injection. Western blotting was used to measure NRF2 expression. Tissue samples were homogenized and protein extracted through NER-CER kits (Thermofisher) to separate cytosolic and nuclear protein samples. A protein assay was conducted to determine amount of protein in each sample using a Bradford coomassie protein assay kit (Pierce). Nuclear protein samples were run on SDS-PAGE Tris-glycine-SDS gels to separate protein samples, which were then transferred to a PDVF membrane (Bio-rad) for immunoblotting. The PDVF membrane was be incubated with primary antibodies for NRF2 and H3 (Cell Signal Technologies) with 1:1000 dilution. Data are presented as protein target ratio to general housekeeping gene.

**Data Analysis:** Behavioral tests required repeated measures ANOVAs or univariate ANOVAs followed by Tukey’s post hoc tests at p<0.05. Data was collapsed across sex
when no sex differences were observed. Similarly, if no differences were found between Trolox+saline and saline treatment, these groups were collapsed. Western blotting data required analysis by image-j software followed by t-tests at p<0.05.
RESULTS

Body Weight P13-19

Weight was measured daily from P13-19 and no effect of treatment was observed (F(2,36) =1.279, p =0.29) indicating there was no effect of VPA on body weight compared to saline-treated animals.

Body Weight P40

At postnatal day 40 there were still no observed differences in weight by genotype or treatment (F(2,55) =1.431, p=.248).

Mid-air righting

Ability to right in mid-air was examined on postnatal days 13 to 19. A two-way mixed ANOVA indicated a significant two-way interaction between day and treatment, F(12,468)=3.798, p<.01 (Fig. 1). All other interactions were found to be not statistically significant (p>.05). Additionally, there was a significant between subjects effect of treatment (F(2,72)= 10.539, p<.01) with further Tukey post hoc analyses revealing a significant difference between saline and VPA treated animals (p<.01) and saline and trolox+VPA treated animals (p=.02). Results warranted further investigation on a day by day basis in which a series of two way ANOVAs were run. There were no significant differences between treatment groups on P13 or 14 before the VPA administration (p>.05) while P15-P19 showed significant differences between the VPA treatment groups (p<.05).

Rotarod - Adolescence

The present study expected to examine performance of KO and WT animals treated with VPA or saline and/or pretreated with trolox on rotarod in adolescence. Issues
were encountered with the rotarod during the running of the pretreated animals and as a result, only data for VPA and saline treated KO and WT animals were analyzed. A two way repeated measures ANOVA (genotype x treatment) revealed a significant between subjects effect of treatment \((F(1,40)=3.858, p=.056)\) with VPA treated animals spending significantly less time on the rotarod compared to saline treated animals (Fig. 2).

**Rotarod - Adulthood**

Rotarod performance, a measure of motor ability, was averaged for each day across three consecutive days. A two way repeated measures ANOVA (genotype x treatment) was run on time spent on the rotarod and revealed no significant difference between groups on genotype \((F(1, 29)=1.152, p=.232)\) or treatment \((F(2,29)=.635, p=.639)\). Results are visualized in figure 3 which shows no difference in motor abilities across groups.

**Morris water maze**

Morris water maze acquisition (postnatal days 30 to 33) was assessed by the latency to find the platform, distance traveled, and swim speed (Fig. 4). A two-way mixed ANOVA was run to assess the latency to find the platform during acquisition trials. There were no statistically significant interactions \((p>.05)\). Results indicate a significant between subject effect of treatment on latency to find the platform \((F(2,72)=1.058, p=.003)\) (Fig. 4C). Further Tukey post hoc analyses revealed a significant difference between SAL and VPA-treated animals \((p=.028)\) with saline-treated animals finding the platform significantly faster than VPA-treated animals. There was no effect of genotype \((p>.05)\) on latency to find the platform. No significant
differences in acquisition were found for treatment or genotype for path length (Fig. 5) or swim speed ($p>.05$).

Performance on the probe test (postnatal day 34) was assessed by the number of entries, time spent in the target quadrant, and distance traveled in the target quadrant (the quadrant that contained the hidden platform during acquisition). A two way ANOVA for entries revealed no differences between groups on genotype ($F(1,72)=58.582, p=.012$) or treatment ($F(2,72)=.378, p=.687$). There were no differences in time spent in the target quadrant or distance traveled for treatment or genotype ($p>.05$) (Fig. 6).

**Social Approach**

Social approach behavior was quantified in two stages (Fig. 7). The first stage consisted of a stranger in one zone of the apparatus (stranger phase). Time spent in each of the zones was scored by a trained observer and results examined time spent in the “stranger 1 zone” compared to time spent in all other zones (Fig. 8A). A two way ANOVA revealed no significant difference between genotype ($F(1,63)=.190, p=.665$) or treatment ($F(2,63)=.884, p=.418$) with all groups spending more time with the stranger compared to the empty zones.

The second stage consisted of placing a second (novel) stranger into one of the previously empty zones (test phase). Videos were scored for time spent in “stranger 1 zone”, “stranger 2 zone” and the empty zone. This allowed for comparison of time spent interacting with the habituated stranger, the novel stranger and the empty zones (figure 8B). Preference between stranger 1 and stranger 2 was expected to diverge, with VPA-treated animals preferring the familiar animal and saline and trolox+saline-treated animals preferring the stranger animal. A two way ANOVA indicated no group
differences in time spent with the habituated stranger based on genotype \((F(1,63)=.206, p=.651)\) or treatment \((F(2,53)=1.213, p=.304)\). Additionally there were no differences in time spent with the novel stranger for genotype \((F(1,63)=.222, p=.639)\) or treatment \((F(2,63)=1.213, p=.304)\) indicating VPA treatment did not affect social behavior.

*Distance traveled*

Motor activity was monitored prior to social testing during a 5 minute habituation period (Fig. 8). A two-way ANOVA was run to assess differences in treatment or genotype on distance traveled. Results showed no effect of treatment \((F(2,63)=.080, p=.923)\) or genotype \((F(1,63)=.024, p=.879)\) indicating VPA exposure did not affect motor ability.

*Y maze*

Y maze data was examined for number of days to acquisition, errors to acquisition, days to reversal and errors to reversal (Fig. 9). A two-way ANOVA (treatment x genotype) yielded no significant differences between treatment groups or genotype in any measure of Y maze performance. Figure 10a shows no differences between groups on days to acquisition \((F(2,46)=.712, p=.496)\) indicating all animals acquired the task at the same rate. Figure 10b shows errors to acquisition in which there were no differences between groups \((F(1,46)=.443, p=.645)\). There were no differences between groups in days to reversal \((F(1,46)=.712, p=.496)\) or errors to reversal \((F(1,46)=.588, p=.560)\) as depicted in figure 10c and d respectively. This suggests there was no perseveration difference evident by genotype or treatment.

*NRF2 expression 2 hours post VPA exposure*
NRF2 expression was examined through Western blot analysis in which nuclear fractionations were probed for NRF2 and H3 (housekeeping) two hours following VPA exposure in both young animals and adult animals (Figure 10). Results indicated a trend towards increased nuclear NRF2 expression in P14 VPA treated animals compared to saline treated controls (N=4/group) ($t(7)=-1.923, p=.09$) (figure 11A). No differences between VPA and saline treated adult animals (~P90) were observed ($p>.05$) (Fig.11B). There were no differences in amount of protein in each sample ($p>.05$).
DISCUSSION

Although the etiology of autism is not well understood, environmental toxicants, genetics and infection have all been implicated in its development. More specifically, oxidative stress has been hypothesized as a potential mechanism underlying neurobehavioral development disruptions associated with autism (Edelson & Cantor, 1998). The results presented here suggest VPA, when administered early postnatally, may induce oxidative stress which in turn may cause the development of some autistic-like behaviors.

The present study was able to successfully replicate previous work by Wagner et al. (2006) to model autistic regression in which a skill matures at the same rate as control-treated pups but then the toxicant exposure results in a loss of those skills. Previous work, along with the present study, demonstrate postnatal VPA administration results in regression in ability to mid-air right with VPA treated animals performing significantly worse than saline treated controls following exposure. Similar to Wagner et al. (2006), there were no observed effects of VPA treatment on general motor behavior or body weight throughout testing as observed in the open field habituation phase of social testing, Morris water maze path length or swim speeds. Taken together these results indicate it is possible to model autistic regression with no adverse effects of VPA treatment on motor development or body weight.

VPA acts as a GABA agonist which when metabolized is known to induce oxidative stress, as observed through a decrease in endogenous regulatory antioxidant enzymes (Jurima-Romet et al., 1996). Not only is VPA associated with an oxidative stress response, its role as a GABA agonist may act as a mechanism to induce oxidative
stress. Recent work by Ben-Ari (2014) suggests GABA may shift from an excitatory action during fetal development and into early postnatal to inhibitory role around the third week of life in rodents. As such, by administering VPA at postnatal day 14 the present study targets the window of hypothesized excitatory GABA action. If GABA is indeed excitatory at this time, VPA administration would be expected to result in GABA buildup resulting in excitotoxicity during a critical developmental period.

The present study further extends previous findings on oxidative stress induction following VPA exposure through examination of NRF2 protein levels in VPA-treated WT animals. Reactive oxygen species (ROS), the byproduct of oxidative stress, are neutralized by endogenous antioxidant mechanisms, of which NRF2 is a master regulator (Wakabayashi et al., 2010; Sandberg et al., 2013). When NRF2, which is normally found bound to KEAP1 in the cytoplasm, is activated by ROS, it enters the nucleus and initiates downstream induction of antioxidant genes (Wakabayashi et al., 2010; Sandberg et al., 2013). As such, nuclear NRF2 protein expression through Western blotting analysis, was used as a measure of oxidative stress response as NRF2 observed in the nucleus can be thought of as “activated” NRF2. The present study observed increased nuclear NRF2 levels in VPA treated animals compared to controls two-hours post-exposure, demonstrating that VPA induces oxidative stress. Further, the present study examined VPA treatment on both young animals (P14) as a means to target the critical window of GABA shift in which GABA is hypothesized to be excitatory in comparison to adult animals (~P90) to target inhibitory GABA. Increased NRF2 was observed in the VPA-treated pups but the effect was not present in the VPA-treated adult animals, supporting the GABA shift hypothesis. Together, Western blotting results support the hypothesis
that VPA induces oxidative stress and that GABA has a shift in action from early postnatal life to adulthood.

*Nrf2* knockout mice have been shown to have enhanced susceptibility to various environmental toxicants as well as behavioral deficits following postnatal VPA exposure (Itoh et al., 1997; Liu et al., 2010; Ishii et al., 2005; Innamorato et al., 2010; Furnari et al. 2014). The present study examined mice lacking the NRF2 gene and results supported previous findings by Furnari et al (2014) further supporting oxidative stress as a mechanism for behavioral deficits observed following VPA treatment. Children with autism have been found to have increased body burdens of environmental toxicants associated with oxidative stress as well as increased levels of oxidative stress biomarkers indicated increased susceptibility to oxidative stress (Edelson & Cantor, 1998; Dietert & Dietert, 1997; Ming et al., 2005). This potential inability to manage high levels of oxidative stress following toxicant exposure compounded with genetic susceptibility may offer a potential mechanism for the development of autism.

The identified connection of oxidative stress to VPA exposure makes it a prime model for examining antioxidant pretreatment as a potential mechanism for protection from the previously identified deleterious effects of VPA. Previous work has indicated pretreatment of high levels of antioxidants reduces the behavioral effects associated with toxicant exposure (Cheh et al., 2010; Deeb et al., 2000). Specifically, previous work by Ming et al. (2008) suggests vitamin E can protect postnatally VPA treated animals from behavioral deficits while work by Cheh et al. (2010) suggests Trolox, a water-soluble form of vitamin E, can protect animals from MeHg challenge. The present study extended upon this work by examining Trolox as a pretreatment serving to protect
animals from VPA exposure. Results revealed VPA treated animals pretreated with Trolox were partially protected from behavioral deficits observed in mid-air righting and Morris water maze learning in VPA-alone treated animals. Collectively, these findings confirm VPA acts through the generation of ROS.

Although the present study provided evidence of autistic like regression as well as impaired learning in the Morris water maze, some expected behavioral deficits were not observed. A plausible explanation for the lack of evidence to support deficits in social approach behavior may be due to the fact that the social deficits associated with postnatal VPA treatment are subtle and our ability to measure social behavior may not have been sensitive enough to detect deficits. To further explore this, a follow up study is currently being conducted examining social play behavior in these animals. Social play is a basic measure of social behavior in which two previously isolated animals are placed in a chamber and allowed to interact for a 30-minute session on two consecutive days (Cheh et al., 2006). Videos are then scored by trained observers for a variety of social behaviors including crawl over/under, anogenital sniffs, allogrooming, self-grooming, chases, and aggression.

Additionally, no treatment or genotype differences were observed in Y maze testing for perseverative behaviors or rotarod testing in adulthood. Y maze testing is a novel measure for examining the effects of VPA treatment and Nrf2 genotype effects as well as Trolox pretreatment protection and as such a more basic measure such as marble burying may be warranted in further studies (Sungur et al., 2014). Differences between treatment and genotype were identified in VPA and saline-treated WT and KO animals in adolescence but pretreatment was not able to be examined at this time point. Rotarod
testing in adulthood revealed no difference between groups which may be due to the fact testing took place between 80 and 175 days post exposure.

To extend the present findings, further work should be conducted to examine VPA treatment at varying time points, particularly prenatal, P7, and P21 in hopes to identify the critical window of GABA shift and then replicated with Trolox pretreatment. Further, future Western blotting studies should examine the protective effects of Trolox pretreatment on nuclear NRF2 expression at P14 and extend the findings to the additional time points. It may also be beneficial to examine the effects of repeated VPA administration during early postnatal development and to build upon previously described behavioral deficits recently reported by Bath & Pimental (2017). Further, examining repeated VPA administration in combination with Trolox pretreatment may elucidate its efficacy over time.

The mechanism underlying neurobehavioral development disruptions associated with autism is far from understood but the results of the present study, at least in part, support the hypothesis that oxidative stress induction may result in behavioral abnormalities akin to autism. The use of VPA as a method to induce oxidative stress was successful in the present study as revealed by Western blot analysis following VPA exposure. Behavioral results indicated an effect of VPA treatment in mid-air righting ability, Morris water maze learning, and rotarod performance. Further, Trolox pretreatment showed protection in the mid-air righting task as well as MWM testing. No effects of treatment or genotype were observed in social approach or Y maze testing but the methods used for these behaviors may need modification for more subtle identification of deficits in future research. Collectively, the results of the present study
warrant further investigation into the link between oxidative stress and autism as well as antioxidants as a protective mechanism from behavioral deficits. Further, this study confirms postnatal VPA as a valid model of autistic regression as well as VPAs potential mechanism of action through oxidative stress induction and the ability of antioxidants to act as a protective mechanism.
References


FIGURE LEGENDS

Figure 1A: Mid-air righting ability from P13-19 as a line graph to depict trend. Pups received VPA following testing on P14. Values are mean correct responses per day.

* indicates significant difference between VPA and saline groups (p <.05)

Figure 1B: Mid-air righting ability from P13-19 depicted as a histogram to convey standard error. Pups received VPA following testing on P14. Error bars represent SEM.

* indicates significant difference between VPA and saline groups (p <.05)

Figure 2A: Rotarod performance from P25-P27 as a line graph to depict trend. Values are mean time spent on the rotarod.

Figure 2B: Rotarod performance from P25-P27 collapsed across genotypes. Values are mean time spent on the rotarod. Error bars represent SEM.

* indicates significant difference between VPA and saline groups (p <.05)

Figure 3A: Rotarod performance of adult animals (~P90-200) as a line graph to depict trend. Values are mean time spent on the rotarod.

Figure 3B: Rotarod performance of adult animals (~P90-200) depicted as a histogram to convey standard error. Values are mean time spent on the rotarod. Error bars represent SEM.
Figure 4A: Morris water maze acquisition as a line graph to depict trend. Values are mean latency (seconds) to find the platform across 5 trials per day.

Figure 4B: Morris water maze acquisition depicted as a histogram to depict trend. Values are mean latency (seconds) to find the platform across five 60 second trials per day. Error bars represent SEM.

Figure 4C: Morris water maze acquisition (day 1-4) collapsed across genotype. Values are mean latency (seconds) to find the platform across five 60 second trials per day. Error bars represent SEM.

* indicates significant difference between VPA and saline groups (p <.05)

Figure 5A: Path length to find the platform in acquisition phase of the Morris water maze task as a line graph to depict trend. Values are mean path length (m).

Figure 5B: Path length to find the platform in acquisition phase of the Morris water maze task as a histogram to depict error. Values are mean path length (m). Error bars represent SEM.

Figure 6: Time spent in the target quadrant during the probe trial (day 5). Values are mean time spent (sec) during a 60 second probe trial. Error bars represent SEM.
Figure 7A: Time spent interacting with stranger 1 in social approach testing. Values are mean time spent in proximity of the stranger animal compared to the areas without a stranger (empty) in a 10-minute test session. Error bars represent SEM.

Figure 7B: Time spent interacting with habituated stranger (stranger 1) and novel stranger as compared to time spent in empty zones across 10-minute test. Values are mean time spent in each zone. Error bars represent SEM.

Figure 8: Distance traveled (m) in open field across a 5 minute test. Values are average distance traveled. Error bars represent SEM.

Figure 9A: Average days to successfully complete acquisition in the Y maze task. Values are mean days. Error bar represent SEM.

Figure 9B: Average errors to successfully complete acquisition in the Y maze task. Values are mean errors. Error bar represent SEM.

Figure 9C: Average days to successfully complete reversal in the Y maze task. Values are mean days. Error bar represent SEM.

Figure 9D: Average errors to successfully complete reversal in the Y maze task. Values are mean errors. Error bar represent SEM.
Figure 10A: Example blot probed with NRF2 (68 kDa). Ladder labeled for nearest relevant band (60 kDa).

Figure 10B: Example blot probed with H3 (15 kDa). Ladder labeled for nearest relevant band (20 kDa).

Figure 11A: NRF2 expression levels 2 hours post VPA exposure in pups (P14) + indicates trend toward significant difference between VPA and saline (p=.09)

Figure 11B: NRF2 expression levels 2 hours post VPA exposure in adult animals (~P90)
Figure 1A: Mid-air righting trend lines

Correct Responses over Day of life for KO SAL, WT SAL, KO T+VPA, WT T+VPA, KO VPA, and WT VPA treatments.

Figure 1B: Mid-air righting performance from P13-19

Correct responses over Day of life for KO SAL, WT SAL, KO T+VPA, WT T+VPA, KO VPA, and WT VPA treatments.
Figure 2A: Rotarod performance from P25-27 trend lines

Figure 2B: Rotarod performance from P25-27 by treatment
Figure 3A: Adult rotarod performance curve

![Adult rotarod performance curve](image)

Figure 3B: Adult rotarod performance

![Adult rotarod performance](image)
Figure 4A: Morris water maze acquisition curves (Day 1-4)

![Figure 4A: Morris water maze acquisition curves (Day 1-4)](image)

Figure 4B: Morris water maze acquisition performance (Day 1-4)

![Figure 4B: Morris water maze acquisition performance (Day 1-4)](image)
Figure 4C: Morris water maze acquisition by treatment
Figure 5A: MWM acquisition path length trend

Figure 5B: MWM acquisition path length
Figure 6: Time spent in the target quadrant on probe trial

![Bar chart showing time spent in the target quadrant on probe trial.](chart.png)
Figure 7A: Social Approach behavior with stranger 1

Figure 7B: Social approach behavior in the test phase
Figure 8: Distance traveled in open field
Figure 9A: Days to acquisition in Y maze

![Graph showing days to acquisition in Y maze for different groups.]

Figure 9B: Errors to acquisition in Y maze

![Graph showing errors to acquisition in Y maze for different groups.]
Figure 9C: Days to reversal in Y maze

Figure 9D: Errors to reversal in Y maze
Figure 10A: Example NRF2 blot

Figure 10B: Example H3 blot
Figure 11A: P14 NRF2 expression 2 hours post VPA exposure

Figure 11B: Adult NRF2 expression 2 hours post VPA exposure