BEHAVIORAL, NEUROCHEMICAL, AND NEUROIMMUNE CHANGES IN NRF2 KNOCKOUT MICE FOLLOWING EARLY POSTNATAL EXPOSURE TO VALPROIC ACID

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ABSTRACT OF THE DISSERTATION

Behavioral, neurochemical, and neuroimmune changes in Nrf2 knockout mice following early postnatal exposure to valproic acid

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In the present study, two agents known to exert neurotoxic effects through the generation of reactive oxygen species were administered to genetically-altered mice lacking the Nrf2 gene. Nrf2 is a transcription factor that induces genes that protect against oxidative stress and Nrf2 knockout mice have been shown to be more sensitive to oxidative stress. The first agent, valproic acid, is a GABA (gamma amino butyric acid) agonist known to cause behavioral deficits and damage to the hippocampus and cerebellum in rodents when administered on postnatal day 14, a time when behavioral skills are first maturing. The second, amphetamine, is a stimulant that induces dopamine release leading to oxidative stress with long-term effects of dopamine depletion and cell death. In prior studies, it was shown that early exposure to one toxicant leads to increased sensitivity to other toxicants. Accordingly, in these studies, Nrf2 knockout mice were exposed to valproic acid early in life and treated with amphetamine as adults.

It was found that Nrf2 knockout mice were more sensitive to the toxic effects of valproic acid in development as seen through the open field activity test, rotorod, and Morris water maze. In addition, valproic acid-treated mice were found to be less social in the social chambers during adulthood. No differences were found in dopamine depletion between genotypes and postnatal day 14 pretreatment; all mice exposed to amphetamine had lower concentrations of dopamine compared to saline-treated counterparts. In conclusion, it appears that Nrf2 knockout mice are more sensitive to the behavioral toxicity cause by valproic acid during development but did not show enhanced sensitivity to amphetamine as adults. The Nrf2 knockout mice appear to be an excellent model to assess the effects of oxidative stress inducing neurotoxicants on behavioral development.

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INTRODUCTION

Oxidative Stress

Normally, there is a balance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms in the body. As more ROS are produced, there is an increase in the transcription and activity of antioxidant enzymes to detoxify the ROS and maintain the stable state called redox homeostasis (Droge et al., 2002). However, a state of oxidative stress can occur that is caused by the overproduction of reactive oxygen species (ROS) and electrophiles through endogenous mechanisms or exposure to xenobiotics, heavy metals, or ionizing radiation (Kaspar et al., 2009). ROS are produced during the normal respiration process in the mitochondria or endoplasmic reticulum (Droge, 2002). They are also formed during cytochrome P450 reactions as well as through other enzymatic reactions such as those involving cyclooxygenase, lipoxygenase, and monoamine oxidase. Reactive oxygen species include superoxide anion, hydrogen peroxide, and hydroxyl radical among others (Droge, 2002). Superoxide anion ($O_2^{\cdot -}$) is the product of the first reduction of molecular oxygen (O_2). Hydrogen peroxide (H_2O_2) is the two electron reduction product of molecular oxygen and can be formed either directly from molecular oxygen or by the dismutation of superoxide anion

(Fridovich, 1998). Hydroxyl radical (HO·) is easily produced and extremely reactive, as are the other ROS (Fridovich, 1998). One mechanism for the production of hydroxyl radical is the Fenton reaction in which hydrogen peroxide reacts with iron ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$). The hydroxyl radical initiates lipid peroxidation, which is the oxidative breakdown of lipids (McCord and Day, 1978). This process of lipid peroxidation can also occur under enzymatic control through the enzymes cyclooxygenase and lipoxygenase. The accumulation of ROS and oxidative stress can lead to modifications in DNA that can be mutagenic (Meneghini 1997). ROS can also cause damage to lipids, proteins, and carbohydrates, leading to cell and tissue damage (Kaspar et al., 2009). Oxidative stress has also been associated with a variety of disease states such as rheumatoid arthiritis, Parkinson's disease, Alzhemier's disease, ALS, cystic fibrosis, cancers and many others (Droge, 2002).

Cells have several lines of defense against oxidative stress. Superoxide dismutase is a protective enzyme that catalyzes the conversion of superoxide to hydrogen peroxide. Hydrogen peroxide dismutase, which is also known as catalase, catalyzes the conversion of hydrogen peroxide to molecular oxygen and water. Peroxidase enzymes can use either hydrogen peroxide or an organic hydroperoxide to form water and an oxidized substrate. NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1(HO-1), and glutathione S transferase (GST) are enzymes that are involved in detoxification of reactive oxygen species (Alam et al., 1999; Nguyen et al., 2000; Vengupol and Jaiswal, 1996). NAD(P)H: quinone oxidoreductase 1 (NQO1) catalyzes the 2 electron reduction of quinones, quinoneimines, nitroaromatics, and azo dyes (Ross, 2005). It uses NADH or NADPH as electron donors. It is extremely inducible in response to electrophilic metabolites and

oxidative stress (Ross, 2005). In addition to metabolizing xenobiotic quinones, NQO1 also metabolizes endogenous quinones such as ubiquinone and vitamin E quinone. These two quinones in particular need to be reduced in order to provide their potent antioxidant effects (Ross, 2005). NQO1 has also been found to function as a direct superoxide scavenging enzyme (Ross, 2005). Heme oxygenase-1 is an isoform of the heme oxygenase enzymes that catalyze the first step in heme catabolism (Alam et al., 1999). This first step involves the oxidative cleavage of b-type heme which is the most abundant form of heme found in hemoglobin, myoglobin and in the peroxidase family of enzymes (Ponka, 1999). The oxidative cleavage of heme produces iron, carbon monoxide, and biliverdin. The biliverdin is then converted to bilirubin by biliverdin reductase (Alam et al., 1999). Heme oxygenase-1 is induced by several agents that stimulate the production of reactive oxygen species or deplete gluthathione levels, including heavy metals, UV irradiation, inflammatory cytokines and its substrate, heme (Alam et al., 1999). In addition, heme oxygenase-1 is considered an antioxidant enzyme involved in cellular defense against oxidative stress because its substrate, heme, is a potent pro-oxidant, and its product bilirubin is a potent antioxidant (Alam et al., 1999). Glutathione S-transferases catalyze the conjunction of a number of substrates, such as quinones, α,β-unsaturated carbonyls, arene oxides, and others with glutathione (Hayes et al., 2005). GSTs metabolize cancer chemotherapeutics, carcinogens, insecticides, herbicides, and by-products of oxidative stress by catalyzing the addition of GSH (Hayes et al., 2005). Glutathione S-transferases are also involved in the synthesis of leukotrienes, prostaglandins, testosterone, and progesterone (Hayes et al., 2005). The synthesis of steroid hormones involves side-chain cleavage and oxidation of the 3beta-hydroxyl group

of the cholesterol metabolite 3beta-hydroxy-5-pregnene-20-one, resulting in a 3-ketoΔ^5-steroid (Hayes et al., 2005). The 3-ketoΔ^5-steroid is then converted to the 3-keto Δ^4-steroid isomer by cytosolic GST (Hayes et al., 2005).The glutathione S-transferases are involved in protection against endogenous oxidative stress. GSTs can reduce cholesteryl hydroperoxides and fatty acid hydroperoxides, as well as phospholipid hydroperoxides to stop the formation of epoxides and reactive carbonyls (Hayes et al., 2005). Oxidation of nucleotides and catecholamines yields toxic products that can then be detoxified by conjugation with glutathione by glutathione S-transferases (Hayes et al., 2005).

Valproic Acid

Valproic acid (VPA) is a branched short-chain fatty acid with a half life of 9 to 16 hours that is used clinically as an anticonvulsant agent (Chateauvieux et al., 2010). It is also used to treat a variety of other disorders including migraine headaches and schizophrenia. Valproic acid is widely used as a mood stabilizer in the treatment of bipolar disorder (Go et al., 2011). Valproic acid has been shown to induce GABA (gamma amino butyric acid). Valproic acid has also been shown to be an HDAC inhibitor leading to investigations into using valproic acid as a possible cancer therapy (Gottlicher et al., 2001; Kawai and Arinze, 2006). In addition to these effects, valproic acid has also been found to induce oxidative stress and to cause a variety of toxicities (Defoort et al., 2006; Tabatabaei and Abbott, 1999; Schneider et al., 2008; Na et al., 2003; Banji et al., 2011).

Valproic has been shown to alter the activity of GABA (gamma amino butyric acid) in the brain. GABA is the main inhibitory neurotransmitter in the central nervous

system (Ben-Ari et al., 2007). Valproic acid inhibits GABA transaminase, which inhibits the degradation of GABA (Fukuchi et al., 2009). It also increases GABA synthesis and decreases GABA turnover rate (Mesdjian et al., 1982). GABA released from neurons act at postsynaptic ionotropic $GABA_A$ receptors that are permeable to chloride (Diene et al., 2013). If the reversal potential for chloride is below the resting membrane potential (the concentration of intracellular chloride is low), chloride enters the cell and the inward flux hyperpolarizes the cell membrane (Bregestovski and Bernard, 2012). GABA has also been shown to play a role in neuronal migration (Behar et al., 2000). GABA has been shown to act as a motility-promoting signal and an acceleratory signal for migrating neuroblasts (Behar et al., 2000; Behar et al., 2001)

Valproic acid inhibits class I and class II HDACs (histone deacetylases) (Gottlicher et al., 2001). Acetlyation and deacetlyation of histones are important modulations that effect the expression of genes. DNA is wound around an octomere of histones to form chromatin (Chataeuvieux et al., 2010). This chromatin can be in a condensed, non-transcription form called heterochromatin, or it can be in a less condensed, active form called euchromatin (Chataeuvieux et al., 2010). When the histones are acetylated, they are in the euchromatin form and when they are deacetlyated, they no longer allow transcription (Chateauvieux et al., 2010). Acetlylation of histones is carried out by histone acetyltransferases (HAT), while deacetylation is done by histone deacetylases (HDACs) (Gottlicher et al., 2001). Valproic acid inhibits some HDACs and alters the expression of certain genes. Valproic acid relieves HDAC-dependent transcriptional repression, which induces gene transcription (Gottlicher et al., 2001). Only the S-4-yn-VPA stereoisomer of valproic acid is teratogenic, while both isomers have

low antiepileptic activity (Nau et al., 1999). When the isomers of valproic acid as well as the racemic mixture were evaluated, only the teratogenic isomer and the racemic mixture of valproic acid were found to be HDAC inhibitors (Gottlicher et al., 2001). The HDAC inhibitors also induce differentiation and or apoptosis of transformed cells without evidence of abhorrent repression. Valproic acid induces a type of differentiation characterized by reduced proliferation that is suggested to be caused by the HDAC inhibitory properties of valproic acid (Gottlicher et al., 2001). Valproic acid induces gene transcription through its HDAC inhibitory properties (Kawai and Arinze, 2006). Valproic acid increases the activity of $G\alpha_{i2}$, which is a signal transduction protein, by HDAC inhibition (Kawai and Arinze, 2006). This induction of $G\alpha_{i2}$ activity by HDAC inhibitor has been shown to be antioxidant sensitive. Valproic acid has been shown to increase the production of reactive oxygen species in cells and to increase $G\alpha_{i2}$ activity. When cells were pretreated with antioxidants, the valproic acid-induced $G\alpha_{i2}$ promoter activity was reduced by 80% (Kawai and Arinze, 2006).

Valproic acid has also been shown to have toxic effects on humans and other species. In humans, valproic acid has been linked to autism through prenatal exposures and experimentation in animals has shown toxicities that are similar to those found in autistic humans. Children exposed to valproic acid *in utero* have been described as having "fetal valproate syndrome" which is characterized by symptoms similar to autism including stereotypic and hyperexcitable behavior, language and communication deficits, and behavioral developmental delays (Moore et al., 2000; Koch et al., 1996). Neuroradiological studies have shown people with autism to have cerebellar hypoplasia (Hashimoto et al., 1995). A neuropathological study of brain tissue from autistic patients

also found reduced Purkinje cell number in all adult cases (Bailey et al., 1998). Differences have been found in the granule cells of the cerebellum and hippocampus in people with autism compared to healthy controls (Ingram et al., 2000; Bauman and Kemper 2005). Rats exposed to valproic acid have been shown to exhibit characteristics similar to the behavioral symptoms seen in autism such as lower sensitivity to pain, stereotypic hyperactivity, and decreased social behaviors (Schneider et al., 2008). Other studies have also shown cognitive deficits, motor deficits, and impairments in social development and attention in rodents after exposure to valproic acid *in utero* or postnatally (Chapman and Cutler, 1989; Wu and Wang, 2002). Sodium valproate administered postnatally has also been shown to enhance cell death of the granule cells in the cerebellum and hippocampus (Yochum et al., 2008). Exposure to valproic acid during a critical period when the neural tube closure occurs, results in fewer Purkinje cells in the cerebellar vermis and a reduced tissue volume in the cerebellum of rats (Ingram et al., 2000). Ingram et al (2000) note, that there is nothing about this time point that would predict fewer Purkinje cells or reduced tissue volume and that the only data that would predict this outcome come from human cases of autism. Valproic acid has also been shown to inhibit N-methyl-D-aspartate(NMDA)-evoked depolarizations in rats (Zeise et al., 1991). Rats administered a N-methyl-D-aspartate (NMDA) antagonist and the GABA subtype A agonists phenobarbitol from postnatal day 6 through postnatal day 10 showed reduced neurogenesis and cell migration in the dentate gyrus at postnatal day 15 (Stefovska et al., 2008). By six months of age these rats had fewer neurons in the dentate gyrus and performed worse than saline-treated littermates in the water maze (Stefovska et al., 2008). Another group found that when rats were exposed to valproic acid from

embryonic day 14 through embryonic day 19, the pups were found to have an increased number of hippocampal and cortical dysplasias at postnatal day 30 compared to saline-treated controls (Manent et al., 2007). Manent et al (2007) suggest that according to their findings these abnormalities were due to neuronal migration defects and neuronal cell death. GABA has also been shown to play a role in neuronal migration (Behar et al., 2000). GABA has been shown to act as a motility-promoting signal and an acceleratory signal for migrating neuroblasts (Behar et al., 2000; Behar et al., 2001).

In addition to these toxicities, valproic acid has also been reported to induce hyperammonemic encephalopathy. Hyperammonemia in the absence of abnormal liver function is common in the majority of people who take valproic acid, although it is typically asymptomatic (Hung et al., 2011). Several human cases have reported, however, serious symptomatic valproic acid-induced hypperammonemia with encephalopathy. The signs of valproic acid induced hyperammonemic encephalopathy include impaired consciousness, focal neurologic symptoms, and increased seizure frequency (Verotti et al., 2002). The mechanism by which this occurs is still unclear, but it has been suggested that the hyperammonemia could lead to an inhibition of glutamate uptake by astrocytes which could lead to neuronal injury and cerebral edema (Verotti et al., 2002). The increased glutamate could lead to increases in intracellular osmolarity, causing an influx of water that then leads to swelling of astrocytes, altering the astrocyte enegery metabolism and resulting in cerebral edema (Verotti et al., 2002). In the developing brain, hyperammonemia can cause irreversible damage that can lead to cortical atrophy, ventricular enlargement and demyelination, and cognitive impairment (Cagnon and Braissant, 2007).

It has been reported that the toxicity caused by valproic acid may be due at least in part to oxidative stress. Research *in vitro* has shown that valproic acid induced the generation of hydrogen peroxide in the presence of iron-induced rabbit microsomes (Tabatabaei and Abbott, 1999). It was suggested that valproic acid induced uncoupling of the cytochrome P450 cycle which allowed the release of hydrogen peroxide (Tabatabaei and Abbott, 1999). The addition of catalase, the enzyme responsible for detoxification of hydrogen peroxide, was found to prevent the valproic acid-induced lymphocyte toxicity (Tabatabaei and Abbott, 1999). Valproic acid was also shown to increase reactive oxygen species levels in embryoid bodies from murine ES cells which in turn inhibited cardiomyocyte differentiation (Na et al., 2003). The cardiomyogenic differentiation was restored upon treatment with vitamin E, a free radical scavenger (Na et al., 2003). Vitamin E has been shown to be protective in other valproic acid-induced toxicities. Vitamin E was shown to attenuate valproic acid-induced neural tube defects (Al Deeb et al., 2000). Another study found that valproic acid exposure induces reactive oxygen species levels and increases homologous recombination frequency in Chinese hamster ovary 3-6 cells (Defoort et al., 2006). Researchers also found that if they preincubated the cells with polyethylene glycol-conjugated (PEG)-catalase, the increase in ROS was attenuated and the increase in homologous recombination frequency was blocked, providing further proof of oxidative stress mechanism for neural tube defects (Defoort et al., 2006). Treatment with antioxidants has also been shown to ameliorate the behavioral deficits associated with valproic acid in animal models of autism. Green tea extract administered to mice on postnatal days 13 through day 40 and administered valproic acid on postnatal day 14 performed behavioral tests similarly to control animals (Banji et al.,

2011). It was also shown that green tea extract protected the Purkinje cells from valproic acid induced injury (Banji et al., 2011).

Another source of oxidative stress is excitotoxicity. In the developing neonate, GABA is an excitatory neurotransmitter and valproic acid increases the concentration of GABA which might be excitotoxic (Levav-Rabkin et al., 2010; Lombardo et al., 2005). GABA released from neurons act at postsynaptic ionotropic $GABA_A$ receptors that are permeable to chloride (Diene et al., 2013). The direction of the chloride determines if the cell will become depolarized or hyperpolarized. If the reversal potential for chloride is below the resting membrane potential (the concentration of intracellular chloride is low), chloride enters the cell and the inward flux hyperpolarizes the cell membrane (Bregestovski and Bernard, 2012). This is what normally occurs in the adult brain. The ability of GABA to depolarize and excite immature neurons is due to the higher intracellular concentrations of chloride in immature neurons (Tyzio et al., 2003; Ben Ari et al., 2007). This increase in intracellular chloride is thought to be caused by the delayed expression of the chloride extruder potassium- chloride cotransporter (KCCO2) and the high expression of the chloride loader sodium-potassium-2chloride cotransporter (NKCC1) in immature neurons (Kakazu et al., 1999; Yamada et al., 2004). As the brain matures, there is an upregulation of KCCO2 that reduces the intracellular concentration of chloride to mature levels and produces the switch of GABA responses from depolarizing to hyperpolarizing (Rivera et al., 1999). The mRNA of KCCO2 was barely detectable at postnatal day 0, but there was an increase in expression by postnatal day 5 and by postnatal day 9, expression of KCCO2 was at adult levels in the rat hippocampus (Rivera et al., 1999). In the rat hippocampus, GABA does not become strictly

hyperpolarizing until the end of the first postnatal week (Cherubini et al., 1991). In the mouse spinal cord the formation of cyto- and synaptic- architecture occurs prior to other brain regions during embryonic development and as such the shift in KCCO2 expression and the resulting GABA inhibitory responses occur much earlier in development (Gao and Ziskind-Conhaim, 1995; Sibilla and Ballerini, 2009). GABAergic fibers were detected in the ventral horn of the spinal cord by embryonic day 11 and in the dorsal horn after embryonic day 13 (Kosaka et al., 2012). The expression of KCCO2 is detected after embryonic day 13 in the ventral horn of the spinal cord and by embryonic day 17 in the dorsal horn (Kosaka et al., 2012). These results indicate that GABA may induce depolarization for several days until the expression of KCCO2 is abundant and the GABAergic synapses form (Kosaka et al., 2012). In the Purkinje cells of the cerebellum, the expression of KCCO2 also occurs during embryonic development. Purkinje cell precursors proliferate in the ventricular zone and migrate toward the developing external granular layer and once settled there they extend their dendrites to form excitatory and inhibitory synapses (Yuasa et al., 1996; Altman and Bayer, 1997). The Purkinje cells were shown to contain KCCO2 within their dendrites and somata after they settled in the Purkinje cell layer at embryonic day 15 and the vermis at embryonic day 17 (Takayama and Inoue, 2007). Takayama and Inoue (2007) suggest that GABA is excitatory in the developing neurons during proliferation and migration, but is inhibitory after forming the first synapses.

GABA has also been found to be excitatory in certain pathological conditions. The injury-mediated shift in GABA is due to the immediate influx of chloride and may also be caused by a shift in gene expression that reverts chloride homeostasis back to its

developmental state (van den Pol et al.,1996). GABA has been found to be excitatory in adult hippocampal slices from epileptic patients due to the low KCCO2 membrane expression contributing to the development of epileptiform discharges (Cohen et al., 2002). GABA was also found to be excitatory in neurodegeneration. Hippocampal slices from adult mice chronically deprived of NGF showed depolarizing and excitatory responses to GABA compared to age-matched controls (Lagostena et al., 2010). GABA has been found to be depolarizing and excitatory in other pathological conditions including axonal injury and trauma and osmotic shock (van de Pol et al., 1996).

Recently, the concept of excitatory GABA in the immature brain has been disputed. Some of the observations of GABA being excitatory are from research done with rodent brain slices. Ben-Ari et al (2007) found that the switch from excitatory to inhibitory GABA took place during the second postnatal week (P12-P13) in these rodent brain slices. Zilberter et al., (2010) dispute this conclusion and suggest that the neurons are energy deficient in glucose-perfused slices and that is why they show depolarizing action of GABA. This group found that GABA switched from excitatory to inhibitory in glucose perfused slices in the presence of additional Energy Substrates (ESs) such as ketone bodies metabolites lactate or pyruvate. Another group found that the slicing procedure damaged the surface neurons, but not the deep neurons, and that the damaged neurons in neonatal slices lead to the accumulation of intracellular chloride (Dzhala et al., 2012). This observation was further extended by Bregestovski and Bernard (2012) in their model they suggest the injured surface neurons have a high-energy requirement that is not met by glucose and that is what causes the excitatory action of GABA and the associated GDPs (Giant Depolarizing Potentials). While these groups do bring up

interesting findings to argue against the excitatory action of GABA in the developing brain, Ben-Ari et al, (2012) provide an extensive rebuttal of each point made in these experiments and point to the fact that these experiments were based on a small set of studies that have been invalidated and contradicted by a number of other groups.

Valproic Acid in Rodents

Valproic acid exposure *in utero* and postnatally to rodents has been used as a useful model for some of the features of autism. The similarities between rodents exposed prenatally to valproic acid and the clinical observations of autistic humans led to the proposal of valproic acid treatment as a model of autism (Rodier et al., 1997). A prenatal exposure period of embryonic day 13 corresponds to a time point when Purkinje cell generation is occurring in the mouse (Inouye and Murakami, 1980). Exposure to valproic acid at this time point results in developmental retardation, observed as delayed appearance of the ability to perform surface righting, mid-air righting, and negative geotaxis behavioral assays (Wagner et al., 2006). Postnatal day 14 corresponds with the period when hippocampal and striatal differentiation and migration are still occurring in the mouse brain and critical developmental behaviors mature or first appear in the BALB/c mouse (Wagner et al., 2006; Rice and Barone, 2000). In addition, cerebellar granule cells are also undergoing migration and differentiation at this time point (Rice and Barone, 2000). Mice exposed to valproic acid on postnatal day 14 had enhanced cell death in the cerebellum and hippocampus. The exposed mice had a 30-fold increase in TUNEL-positive cells over baseline controls in the cerebellum and a 10-fold increase in the hippocampus (Yochum et al., 2008). These TUNEL-positive cells were the granule

cells and in the cerebellum, these cells innervate the Purkinje cells (Yochum et al., 2008). Behavioral tests can be done that are associated with brain regions known to be affected by valproic acid. The behavioral assays that are associated with the cerebellum are mid-air righting, surface righting, and negative geotaxis, those that target the striatum are the locomotor activity and the visible platform water maze and the tests that are associated with the hippocampus include the passive avoidance test and the hidden platform water maze. The deficits observed on these behavioral tests can then be classified as retardations, regression, or intrusions reflecting the developmental components of autism (Wagner et al., 2006). It has been shown that *in utero* exposure to valproic acid results in developmental retardations reflected by delayed appearance of the ability to perform surface righting, mid-air righting, and negative geotaxis behavioral assays. Mice exposed to valproic acid prenatally had slower surface righting up to postnatal day 8 and also had a delay in the ability to mid-air right (Wagner et al., 2006). Postnatal exposure to valproic acid also resulted in retardations reflected by delays in the ability to perform the negative geotaxis assay and water maze performance. Valproic acid treated mice were found to perform worse than saline-treated controls in spatial learning in the water maze and in the grip strength test (Wagner et al., 2006). Postnatal exposure to valproic acid also resulted in regression of the previously acquired skills to perform mid-air righting (Wagner et al., 2006; Banji et al., 2011). Green tea extract given orally has been shown to ameliorate the behavioral deficits found in mice treated with valproic acid on postnatal day 14 (Banji et al., 2011). The mice dosed with green tea extract were able to perform the mid-air righting task successfully as did control mice not receiving valproic acid, while mice that only received valproic acid had deficits in this skill consistent with prior behavior studies

(Banji et al., 2011; Wagner et al., 2006). Green tea extract protected performance on the rotorod, the spatial memory water maze assay, as well as negative geotaxis (Banji et al., 2011). Valproic acid has also been implicated in increased anxiety, as demonstrated using the elevated plus maze (Schneider et al., 2008). The antioxidant treatment with green tea extract was found to ameliorate this increased anxiety induced by valproic acid (Banji et al., 2011).

Nrf2

Nuclear factor-erythroid 2 (NF-E2) related factor 2 (Nrf2) is a transcription factor that is involved in defense against oxidative stress (Baird and Dinkova-Kostova, 2011). Nrf2 is expressed in many tissues throughout the body including liver, kidney, skin, and gastrointestinal tract (Moi et al., 1994). Nrf2 is a member of the Cap 'n' Collar (CNC) family of regulatory proteins that has a basic leucine zipper DNA binding domain (Moi et al., 1994). This basic leucine zipper helps to facilitate dimerisation and DNA binding (Moi et al., 1994). Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1) is a negative regulator of Nrf2 (Itoh et al., 1999). Keap1 has a BTB protein interaction domain (Broad-Complex, Tramtrack, and Bric a Brac) and a Kelch domain that binds to Nrf2 (Itoh et al., 1999; Baird and Dinkova-Kostova, 2011). Under normal conditions, Keap1 sequesters Nrf2 in the cytoplasm, eventually leading to its degradation. The BTB domain interacts with Cullin3 (Cul3)-based E3 ubiquitin ligases as a substrate adaptor allowing for the ubiquination and degradation of Nrf2 (Kobayashi et al., 2004). One Nrf2 molecule is bound to a dimer of Keap1 with each of the Keap1 Kelch domains binding to one of two keap1-binding sites, the DLG or ETGE binding

motifs within the NEh2 domain of the Nrf2 molecule (Itoh et al,1999; McMahon et al., 2006). This hinge and latch model suggests that the hinge is the binding at the ETGE motif and the latch is the binding at the DLG motif that holds the Nrf2 allowing for interactions with ubiquitin and degradation during normal conditions (McMahon et al., 2006). Once the cell is exposed to oxidative stress, these inducers react with cysteine residues in Keap1 which releases the Nrf2 at its DLG binding site causing Nrf2 to no longer be a target for ubiquination (McMahon et al., 2006). The Nrf2 is no longer being degraded, but is however still attached to Keap1 at its EGTE binding motif, and therefore, any new Nrf2 will accumulate in the cell and be free to translocate into the nucleus and induce transcription of its target genes (McMahon et al., 2006).

This model suggests that Nrf2 doesn't dissociate from Keap1, however, there is evidence that in response to certain inducers like cadmium and arsenic, Nrf2 does dissociate, and it is, therefore, likely that the mechanism of control is different depending on the inducer (He et al., 2008; He et al., 2006). Nrf2 may also be able to interact with inducers directly which suggests a possible Keap1-independent model of Nrf2 control. Nrf2 has putative nuclear import and export (NES) signals, one of which is located in the Neh5 transactivation domain (Li et al., 2006). This NES has been shown to be sensitive to reactive oxygen species and has been suggested to regulate Nrf2 (Li et al., 2006). Under normal conditions, the export signals (NES) overcome the import and Nrf2 is found in the cytoplasm not the nucleus. Oxidative stress or other inducers cause the export signals to inactivate and Nrf2 is able to translocate into the nucleus and induce the transcription of antioxidant genes (Li et al., 2006). This model does, however, conflict with some previous reports. The concentration of inducers required for the activity of the

Nrf2 NES is much higher than those to inactivate Keap1 (McMahon et al., 2003). There is also evidence that keap1-knockout mice show levels of Nrf2 target genes are constitutively upregulated and cannot be induced, providing support that Keap1, and not Nrf2, is responsible for inducer sensitivity (Wakabayashi et al., 2003).

Nrf2 induces enzymes and other proteins that protect against oxidative stress through transcriptional activation of their genes. The genes that Nrf2 upregulates include NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1(HO-1), and glutathione S transferase (GST), which are all involved in detoxification of reactive oxygen species (Alam et al., 1999; Nguyen et al., 2000; Vengupol and Jaiswal, 1996). The genes that Nrf2 induces contain the antioxidant responsive element (ARE) that allows interactions for basal and inducible expression (Rushmore et al., 1991). When activated by oxidative stress, Nrf2 binds to the ARE of these genes and induces their expression (Alam et al., 1999; Nguyen et al., 2000; Vengupol and Jaiswal, 1996). Nrf2 has been shown to bind to the ARE with a high affinity only when it is in a heterodimer with a small Maf protein (Itoh etal., 1997).

Nrf2 and its induction of phase II enzymes have been shown to be neuroprotective in several models of neurological diseases. Nrf2 has a role in protection following traumatic brain injury (TBI). Following TBI levels of Nrf2 and its target gene product enzymes NQO1 and HO-1 are significantly increased (Yan et al., 2009). In addition to this, Nrf2 knockout mice have been shown to have increased deficits in neurologic function and oxidative damage, as well as increased severity of edema following TBI (Hong et al., 2010; Jin et al., 2009). Nrf2 activation and induction of its downstream antioxidant proteins have been shown to be enhanced after middle cerebral artery

occlusion in the peri-infarct region (Tanaka et al., 2011). Nrf2 knockout mice have been shown to have larger infarct size following middle cerebral artery occlusion and have been found to produce more ROS in response to brain injury (Shah et al., 2007; Zhao et al., 2007). In a model of Alzheimer's disease, over-expressing Nrf2 through genetic manipulation or increase Nrf2 activity by tBHQ was found to protect against amyloid-beta$_{1\text{-}42}$ – induced neuronal cell death of cultured hippocampus (Kanninen et al., 2008). Nrf2 has also been implicated as neuroprotective in genetic models of familial Parkinson's disease. Over-expression of Nrf2, or down-regulating its negative regulator Keap1 was found to restore locomotor activity in genetic model of familial PD (Barone et al., 2011). In MPTP models of Parkinson's disease, Nrf2 knockout mice have been shown to have a greater loss of dopamine transporters in the striatum and wild type mice treated with the Nrf2 inducer D3T were protected from MPTP toxicity (Burton et al., 2006).

Dietary phytochemicals alter the activity of Nrf2 and its downstream genes involved in the defense against reactive oxygen species. Dietary phytocehmicals are non-nutritive components in plant-based diets containing antioxidant/polyphenols that have been associated with disease prevention (Surh, 2003). However, these compounds can under biochemical transformations that affect their bioavailability (Rahman et al., 2006). Epigallocatechin gallate (EGCG) is an antioxidant polyphenol found in green tea (Surg, 2003). Green tea polyphenols can cross the blood brain barrier as is evidenced by its ability to reduce striatal dopamine depletion in mice exposed to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Hong et al., 2000). EGCG has been shown to induce phase II enzymes through the antioxidant response element (ARE) by activating MAPK pathways

that are upstream regulators of Nrf2 (Yu et al., 1997). When reseveratrol, a grape extract, was given orally at a 25 mg dose, 70% was absorbed but its bioavailability was only 1% due to rapid hepatic glucuronidation and sulfation in humans (Walle et al., 2005). Resveratrol has also been shown to stimulate Nrf2 in through MAPK pathways in PC12 cells (Chen et al., 2005). 6-methylsulphinylhexyl isothiocyanate (6-HITC) is a GST inducer found in Japanese horseradish, wasabi (Morimitsue et al., 2002). Oral administration of 6-HITC was found to induce phase II enzymes in the liver, while this induction was lost in Nrf2 knockout mice (Morimitsue et al., 2002). Curcumin is a yellow pigment in the rhizome of turmeric (Surh, 2003). Approximately 40-85% of ingested curcumin is unaltered in the GI tract, while most of the absorbed curcumin is metabolized in the intestinal mucosa and the liver by glucuronidation (Wahlstrom and Bennow, 2002). While curcumin does have low bioavailability, it has been found to decrease the low-density lipoprotein oxidation and decrease free radicals that cause deterioration of neurons in dendritic cells (Kim et al., 2005) Caffeic acid phenethyl ester (CAPE) is found in propolis, a product produced by the honeybee (Russo et al., 2002). Curcumin and CAPE have both been found to increase Nrf2 expression by inactivating the Nrf2-Keap1 complex in mice renal epithelial cells (Balogun et al., 2003). Another group found that curcumin increased translocation of Nrf2 into the nucleus as well as DNA binding activity at the antioxidant response element in the glutamate-cysteine ligase gene (Dickinson et al., 2003).

The Cap ‘n’ Collar (CNC) family of regulatory proteins includes not just Nrf2, but Nrf1 and Nrf3 among others. Nrf3 is expressed high levels in humans in the placenta and in intermediate to low levels in the heart, lung, kidney, pancreas, colon, spleen, and

brain (Kobayashi et al., 1999). In the mouse, Nrf3 is expressed at high levels in the thymus, brain, lung, stomach, uterus, placenta, adipose tissue, and testis (Derjuga et al., 2004; Fumatsu et al., 2004; Chevillard et al., 2010). Nrf3 has been shown to transcriptionally activate beta-globin gene expression in QT6 cells, and NQO1 gene expression in COS-1 cells (Zhang et al., 2009). Overexpression of Nrf3 has also been shown to repress NQO1 gene expression in human Hep-G2 cells and mouse embryonic stem cells (Sankaranarayanan and Jaiswal, 2004; Pepe et al., 2010). Nrf1, like Nrf2 is essential for the protection against oxidative stress and it induces phase II enzymes. Nrf1, however is not regulated by Keap1 and rather than localized in the cytoplasm, Nrf1 is localized in the endoplasmic reticulum (Zhang et al., 2006). Nrf1 is expressed ubiquitously, however the highest levels are found in the heart, muscle, liver, and kidney (Biswas and Chan, 2010). Nrf1 knockdown studies have shown cells lacking Nrf1 are more sensitive to oxidative stress (Ohtsuji et al., 2008). It has been suggested that Nrf2 responds to inducible oxidative stress, while Nrf1 responds to constitutive forms of oxidative stress (Ohtsuji et al., 2008). Some triple knockout mice lacking Nrf2, p45, and Nrf3 were found to survive to adulthood indicating that Nrf1 and other factors may be able to compensate for the absence of these genes (Derjuga et al., 2004).

Nrf2 Knockout Mice

Nrf2 knockout mice have enhanced susceptibility to various toxicities due to xenobiotics or the environment. They have reduced basal or lower induced levels of cytoprotective genes in various organs including liver (Itoh et al., 1997; Liu et al., 2010), gastrointestinal tract (Itoh et al., 1997), lung (Ishii et al., 2005), and brain (Innamorato et

al., 2010). In Nrf2 knockout mice, induction of many phase II enzymes was found to be near gone in the liver and GI tract, indicating that Nrf2 is needed for induction of phase II enzyme genes (Itoh et al., 1997). This suggests that the Nrf2 knockout mice would be highly susceptible to neoplastic transformation (Itoh et al., 1997). Nrf2 null mice have been shown to have a greater incidence of forestomach tumors and benzo[a]pyrene-induced DNA adducts compared with wild type control mice (Ramos-Gomez et al., 2003). It has been shown that Nrf2 knockout mice have a greater susceptibility to 1-bromopropane induced hepatoxicity due to low expression levels of antioxidant enzymes compared to wild type mice (Liu et al., 2010). Nrf2 knockout mice are also more susceptible to hepatotoxicity induced by acetaminophen. When exposed to acetaminophen, Nrf2 knockout mice have decreased survival rates, increased serum alanine aminotransferase activity and increased centrilobular necrosis (Chan et al., 2001). These results are thought to be caused by lower cellular thiol levels and decreased expression of detoxifying enzymes. Several studies have provided evidence of the importance of Nrf2 in the susceptibility to numerous toxins in the respiratory system. Nrf2 knockout mice were used to study emphysema and were found to be more susceptible to elastase-induced emphysema and have lower levels of antioxidant and antiprotease gene expression in alveolar macrophages compared to wild type counterparts (Ishii et al., 2005). Nrf2 deficient mice have been found to have increased DNA adduct formation after exposure to diesel exhaust compared to wild type mice (Aoki et al., 2001). It has also been shown that Nrf2 knockout mice had a 6.1 fold increase in mutation frequency in the lungs after exposure to benzo[a]pyrene compared with untreated Nrf2+/- mice (Aoki et al., 2007). Nrf2 has been shown to play a significant role

in protecting against pulmonary hyperoxia injury in mice. After 72 hour hyperoxia exposure, Nrf2 knockout mice had 7.6-fold greater pulmonary hyperpermeability, 47% more macrophage inflammation and 43% greater epithelial injury than did wild type control mice with the same exposure (Cho et al., 2002). It was also found that the Nrf2 knockout mice had significantly lower induction of mRNA levels of NAD(P)H: quinone oxidoreductase(NQO1), glutathione S-transferase (GST)-Ya and Yc subunits, UDP glycosyl transferase (UGT), glutathione peroxidase-2 (GPx2) and heme-oyxgenase-1(HO-1) compared with wild type controls (Cho et al., 2002). The Nrf2 knockout mice have been used to study Parkinson's disease and have been shown to be more susceptible to MPTP and show more inflammation compared to wild type animals (Innamorato et al., 2010). The neurotoxin 6-hydroxydopamine is a reactive oxidative stressor and complex I inhibitor used to model Parkinson's disease. It activates ARE-regulated genes (Jakel et al., 2007). Cultured cells of Nrf2 knockout embryos exposed to 6-hydroxydopamine had more apoptotic cells compared to controls (Jakel et al., 2007). Furthermore, 6-hydroxydopamine injections into the striata of Nrf2 knockout mice produced lesions double the size of those seen in wild type mice (Jakel et al., 2007). Traumatic brain injury (TBI) such as weight-drop head injury can result in secondary brain injuries. TBI-induced secondary brain injuries were found to be more severe in Nrf2 knockout mice compared to wild type mice. After weight-drop impact head injury, the Nrf2 knockout mice had increased severity of neurological deficit, apoptosis and brain edema 24 hours later (Jin et al., 2009). This increased injury was found to be associated with increased mRNA expression and protein expression of inflammatory cytokines and decreased mRNA

expression and enzymatic activity of NQO1 and GST-alpha1 compared with wild type controls (Jin et al., 2009).

Behavioral Tests

A variety of behavioral tests can be used to evaluate the health and development of mice as well as the impact of toxicants. Many behavioral tests are used to evaluate adult mice; however there are also tests that evaluate developing mice. Some of the early neonatal experiments include surface righting, mid-air righting, negative geotaxis, and hanging wire grip strength. The surface righting reflex is used to evaluate normal development in mice. Mice are placed on their backs and the time to turn over is recorded. Normal mice will immediately right themselves (Crawley and Paylor, 1997). The mid-air righting test evaluates another reflex in rodents that reflects the development of dynamic postural adjustments (Iezhitsa et al., 2001). Mid-air righting is the ability of the mouse to turn over in the air such that it lands on all four paws after being released from a supine position. Negative geotaxis is the ability of the mouse to orient itself on an inclined plane such that its head is facing the high end of the incline. This test is considered to evaluate vestibular or proprioceptive function (Frankel and Gunn, 1968). The righting reflexes and negative geotaxis have been linked to cerebellum development (Petrosini et al., 1990). Rats with a hemicerebellectomy show a delay in the development of these righting reflexes as well as negative geotaxis reactions, although recovery was found to be almost complete (Petrosini et al., 1990).Neuromuscular abnormalities can be evaluated with the hanging wire grip strength test. The mouse's two front paws are placed on a wire 30 cm above a padded receptacle and the latency for the mouse to fall

from the wire is recorded. This particular skill is acquired in mice around postnatal day 14. Normally a mouse can hang for several minutes, but a 60 second maximum is standard for a test session (Crawley, 2000).

Locomotor activity can be evaluated with the open field chamber. The mouse is placed in an open field, an empty cage, with photocell sensors placed throughout the chamber. The number of times the mouse breaks a beam can quantify the horizontal activity to determine total overall locomotion. In this behavioral test, the vertical activity and center distance could also be quantified. The ratio of center distance to total distance could also be used to evaluate anxiety, whereby highly anxious mice would avoid the center of the open field (Crawley and Paylor, 1997).

Motor coordination and balance can be evaluated using the rotorod. The rotorod assess the ability of the mouse to maintain balance and walk on a rotating rod. The latency to fall during a 60 second trial quantifies the length of time the mouse can balance. Mice that have motor coordination deficits, perhaps due to abnormalities in the cerebellum, have difficulty staying on the rotarod even at low speeds (Barlow et al., 1996; Sango et al., 1995).

The elevated plus maze evaluates anxiety in rodents. The basis for this evaluation is that rodents have a natural tendency to explore a new environment, but are averse to brightly lit, open areas and to height (Lister, 1987). The maze is a plus shaped maze with two long arms with walls and two short open arms with no walls. The mouse is observed for several minutes and the number of crosses made into either arm is recorded. Normally the mice will make more crosses into the closed arm rather than the open arm (Lister, 1987). In addition to detecting anxiogenic or anxiolytic effects, the total number of

entries into an arm gives a control measure of general hyperactivity or sedation (Crawley, 1999).

The Morris water maze evaluates learning and memory in rodents. The basis for the test is that rodents are highly motivated to escape from water by the fastest route possible (Crawley, 1999). The water maze is a circular tub filled with water made opaque by white non-toxic latex paint. Visual cues are strategically placed around the maze. There is a visible platform and a hidden platform paradigm of this test. In the visible platform test, the platform is a black platform that stands above the water level. This tests the visual ability of the animal to see the room cues and the motor ability of the mice to swim (Crawley, 1999). In the hidden platform test, the platform is white and stands below the water level. This tests the spatial learning and memory of the mice (Crawley, 1999). Diminishing latency to find the platform indicates learning. Normal performance on the visible platform paradigm, but impaired performance on the hidden platform paradigm is interpreted as an indication of learning and memory deficits. Hippocampal lesions impair acquisition of this task in rats and mice (Morris et al., 1982; Logue et al., 1997). If swimming speed is slower or if the mouse fails to swim at all, this may indicate dysfunction in visual neurons, motor neurons, spinal cord, and cerebellum, rather than a deficit in learning and memory (Crawley, 1999).

Evaluation of social behavior can be done with a variety of experiments including the social chambers. Social behavior is a complex behavior that is associated with several brain regions including the mesolimbic areas, the parietal cortex, and the cerebellum (Critchley et al., 2000). The mesolimbic areas include the ventromedial prefrontal cortex, the medial temporal lobes, the striatum and the limbic thalamus (Critchley et al., 2000).

Rodent studies have found that when two hypothalamic neuropeptides, oxytocin and vasopressin were altered social behavior was also altered. Oxytocin knockout mice display deficits in social recognition and social memory (Winslow et al., 2000). Vassopressin receptor subtype 1b knockout mice have reduced social motivation and aggression, but have normal olfactory function (Wersinger et al., 2004; Wersinger et al., 2006). While the cerebellum has typically been solely associated with motor activity, recent work in humans have shown cerebellum function to play a role in attention tasks that may provide a link to the social and cognitive deficits seen in autism (Allen et al., 2004). The social chambers evaluate social behavior in a social approach measure. Within the chamber are 2 cylinders. A mouse in placed in one cylinder and the other cylinder remains empty. The mouse being evaluated for social level is then placed between those cylinders and observed. The number of times the mouse touches either cylinder and the length of time spent touching the cylinder are recorded. This test is based on the natural tendency of mice to explore novel objects and to engage in social behavior (Crawley et al., 2000). A decrease in number of contact or a decrease in amount of time spent with the cylinder containing a mouse would indicate a deficit is social behavior (Crawley et al., 2000). Aside from the brain regions involved in social behavior, one concern for social behavioral testing is the olfactory function of the mice. A mouse with olfactory dysfunction could fail social tasks based on detection of odors (Crawley et al., 2000). This particular social test however can overcome the olfactory limitation because it allows for visual, olfactory, auditory and tactile contact between the mice.

The target biting chambers are used to evaluate defensive aggression in mice. The brain regions associated with aggressive behavior include the hypothalamus and midbrain

periaqueductal gray, and the regions of the brain associated with modulating functions include the amygdala, hippocampus, and prefrontal cortex (Siegel et al., 2007). Typical treatments for aggressive behavior in humans are serotonergic compounds and GABAergic compounds because the neurotransmitters GABA and serotonin that act through the 5-HT1 receptor inhibit aggressive behavior (Siegel et al., 2007). Fluprazine, a serotonin agonist has been shown to reduce aggressive behavior in the target biting chambers (Carelli and Wagner, 1988). For the target biting chambers, the mouse is restrained in a tube with its tail taped to two brass bar electrodes. The plastic tube containing the mouse is then placed into the chamber where a "target" cable tie is mounted on an omnidirectional switch. The tube has a small hole that allows access to the cable tie. The test consists of ten two minute trials. The two minute trials were divided into eight 15 second bins during which the mice had access to a target cable tie. Immediately following bin 8, and just prior to bin 1, the mice received a 2mA tail shock. The number of times the animal bit the target cable tie was recorded per bin. The number of bites to the target indicates aggression level, where a very low number of bites would indicate a lack of aggression and a very high number of bites would indicate a high level of aggression.

Nrf2 and Valproic Acid

There is evidence to suggest a link between valproic acid and the redox sensitive transcription factor Nrf2. As previously discussed, valproic acid has been shown to increase levels of reactive oxygen species. This increase in reactive oxygen species has been implicated in the toxicity caused by valproic acid, including neural tube

defects, behavioral deficits, and hippocampal and cerebellar abnormalities (Defoort et al., 2006;Wagner et al., 2006; Yochum et al 2008; Banji et al., 2011; Schneider et al., 2008). When reactive oxygen species accumulate in a cell, Nrf2 is activated and translocates into the nucleus, where it binds to the ARE on its target genes and upregulates their transcription (McMahon et al., 2006; Rushmore et al., 1991). Nrf2 induces the expression of genes that contain an ARE, including HO-1, NQO1, and GST. Valproic acid has been shown to induce gene transcription in an ARE-dependent manner for these same genes (Kawai and Arinze, 2006). Furthermore, the valproic acid induced -transcription was not found to be sensitive to antioxidants in the absence of the ARE sequence (Kawai and Arinze, 1999). Valproic acid has been shown to increases the activity of $G\alpha_{i2}$, a redox-sensitive transcription factor (Kawai and Arinze, 2006). This induction of $G\alpha_{i2}$ activity by HDAC inhibition has been shown to be antioxidant sensitive. Valproic acid has been shown to increase the production of reactive oxygen species in cells and to increase $G\alpha_{i2}$ activity. When cells were pretreated with antioxidants, the valproic acid-induced $G\alpha_{i2}$ promoter activity was reduced by 80% (Kawai and Arinze, 2006). Nrf2 is another redox-sensitive transcription factor. Kawai and Arinze (2006) provide evidence of a link between the valproic acid-induced elevated levels of reactive oxygen species and the activation of the transcription factor Nrf2 and the subsequent induction of ARE containing genes. Given this information, Nrf2 should be induced by valproic acid to subsequently induce transcription of ROS detoxifying enzymes. In the absence of Nrf2, such as in Nrf2 knockout mice, there may be and increased sensitivity to the toxicity induced by valproic acid. The Nrf2 knockout mice are lacking the ability to upregulate the genes induced by Nrf2 that protect against oxidative stress. Valproic acid has been

shown to induce behavioral and neuropathological defects in mice (Wagner et al., 2006; Yochum et al 2008; Banji et al., 2011; Schneider et al., 2008) Valproic acid administered to these mice should create deficits in behavior as well as increased brain injury caused by increased levels of reactive oxygen species because they lack Nrf2 and the ability to protect them against oxidative stress. There is no evidence to suggest that the Nrf2 knockout mice will have deficits in surface righting, mid-air righting, hanging wire grip strength, or negative geotaxis because developmental behavioral assays have not yet been done in these mice, nor has anyone reported abnormalities in Nrf2 knockout mice in the brain regions associated with these tests. There have also not been any reports of Nrf2 knockout mice exhibiting dysfunction in the brain regions associated with the water maze, rotorod, motor activity, social chambers, or target biting, therefore, the knockout mice should perform similarly to the wild type mice in these tests. It should be noted that there is not much behavioral research available on the Nrf2 knockout mice. That being said, there is an abundance of literature that suggests Nrf2 knockout mice are more susceptible to toxicity caused by known ROS-inducing toxicants (Itoh et al., 1997; Liu et al., 2010 Ishii et al., 2005; Innamorato et al., 2010; Liu et al., 2010; Chan et al., 2001). Valproic acid is a toxicant that has been shown to induce ROS and behavioral deficits (Wagner et al., 2006; Yochum et al 2008; Banji et al., 2011; Schneider et al., 2008). Therefore, it is likely that the Nrf2 knockout mice will be more sensitive to the damage caused by valproic acid and will therefore show enhanced deficits in the behaviors that have previously been reported in wild type mice exposed to valproic acid.

Amphetamine

Amphetamines are central nervous system stimulants that can cause long-lasting damage to the brain. The compounds in the class of amphetamines all have a similar structure and function, and are sometimes called sympathomimetic agents due to their actions mimicking those of epinephrine (Julien et al., 2005). Compounds in the amphetamine class have been used clinically to treat a variety of disorders. Amphetamine, dextroamphetamine, and methamphetamine are three such compounds, employed today in the treatment of ADHD (Julien et al., 2005). These drugs are also widely abused by people all over the world. The pharmacologic effects of these drugs include vasoconstriction, tachycardia, tremor, increased motor activity, insomnia, loss of appetite, and feelings of euphoria (Julien et al., 2005). People who chronically abuse amphetamines also experience psychosis and stereotypical behaviors, such as repetitive movements and sudden acts of aggression (Julien et al., 2005).

The pharmacological effect of amphetamine is believed to result, in part, by increasing the release of dopamine, norepinephrine, and serotonin and blocking their reuptake (Laruelle et al., 1995). Dopamine is a catecholamine, which is a neurotransmitter derived from the amino acid tyrosine. The other catecholamines are epinephrine and norepinephrine. Tyrosine is brought into the central nervous system and then hydroxylated by tryosine hydroxylase to L-DOPA (Stahl, 2000). Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of these catecholamines. L-Dopa can then become dopamine by the actions of the enzyme aromatic amino acid decarboxylase (Stahl, 2000). Through the actions of the enzyme dopamine hydroxylase, dopamine becomes norepinephrine and by phenylethanolamine N-methyltransferase,

norepinephrine can become epinephrine (Stahl, 2000). Serotonin or 5 hydroxytryptamine (5-HT) is a monoamine neurotransmitter synthesized from tryptophan (Huot et al., 2011). The rate limiting step in this pathway involves tryptophan hydroxylase which converts tryptophan to 5-hydroxy-L-tryptophan (5-HTP) (Huot et al., 2011). 5-HTP is converted to 5-HT by the enzyme 5-hydroxytryptophan decarboxylase (Huot et al., 2011).

Amphetamine acts on the transporters that are involved in the uptake of dopamine, norepinephrine, and serotonin. The amphetamines are taken into neurons by the transporters and once there, they induce the release of the biogenic amines from the cytoplasm into the synaptic cleft causing an increased neurotransmission of dopamine, norepinephrine, and serotonin (Yamamoto et al., 2010). Amphetamine has a high affinity for the dopamine transporter (DAT) (Yamamoto et al., 2010). The K_I value of amphetamine to inhibit DAT has been reported to be approximately 0.6μM (Han and Gu, 2006). This study found the K_I value which includes the affect of amphetamine induced substrate release and is therefore, not exactly the same as the dissociation constant K_D, a true measure of affinity (Han and Gu, 2006). The K_I value, measured from cultured cells, reflects the apparent affinity of the drug to the transporter (Han and Gu, 2006).

The dopaminergic neurons that are affected by amphetamines are located in the midbrain in the ventral part of the mesencephalon (Chinta and Anderson, 2005). One group of neurons projects from the substantia nigra to the striatum, which is part of the basal ganglia (Boron and Boulpaep, 2005). The substantia nigra, which is made up of two regions – the pars compacta and the pars reticula, is located in midbrain. The pars compacta is made up of densely packed neurons that send their axons to the striatum where they release the neurotransmitter dopamine. The striatum is made up of the caudate

nucleus and the putamen. The neurons of the substantia nigra project to the putamen, which then projects to the premotor and supplementary motor areas of the cortex. Thus the nigrostriatal pathway is involved in the planning and modulation of movement. Another pathway affected by amphetamine is the ventral tegmental pathway, where the neurons of the ventral tegmental area project to the prefrontal cortex and parts of the limbic system (Boron and Boulpaep, 2005). The limbic system is a group of structures in the brain, including the hippocampus, amygdala, anterior thalamic nucleui, and the septum. The limbic system supports a variety of functions including emotional behavior as well as motivation, memory and olfaction (Berne et al., 2008). These neurons are involved in the reward aspect of drug addiction. The dopaminergic neurons of the olfactory bulb, another part of the limbic system, have also been shown to be affected by amphetamine (Atianjoh et al., 2008).

The long term toxic effects of amphetamine are dopamine depletion and cell death. Amphetamine increases the release of dopamine and blocks its reuptake. Dopamine has been shown to have a role in the neurochemical deficits caused by amphetamine (Gibb et al., 1997). The increase of dopamine in the synaptic cleft is believed to enhance the production of reactive oxygen species which cause neurotoxicity (De Vito and Wagner, 1989). Dopamine can spontaneously oxidize in the presence of transition metal ions or can become oxidized through enzymatic catabolism involving monoamine oxidase (MAO) (Chinta and Anderson, 2005). Several studies support the relationship between oxidative stress and the damage to the dopamine neurons after exposure to amphetamine. Cubells et al., (1994) found that when cell cultures of the ventral midbrain dopamine neurons were treated with amphetamine, there was an

increase in intracellular oxidation. Antioxidants and free radical scavengers have been shown to protect against the amphetamine-induced reduction in striatal dopamine (Devito and Wagner, 1989; Wagner et al., 1985). The activity of superoxide dismutase (SOD) in the brain can be decreased by diethyldithiocarbamate (DDC). Pretreatment with DDC has been shown to greatly enhance the striatal dopamine depletion caused by amphetamine (Devito and Wagner, 1989). The amphetamine induced-depletion of striatal dopamine was also shown to be increased in BALB/c mice following pretreatment with DDC (Kita et al., 1998). In addition, mice that over express SOD are protected against amphetamine induced toxicity (Cadet et al., 1994). Further evidence supporting the role of oxidative stress in amphetamine induced neurotoxicity was found by Kita et al., (2000). The expression of cyclooxygenase 2 (COX2), a marker of oxidative stress and inflammation, was found to be increased in the striatum following amphetamine exposure (Kita et al., 2000). Two other mechanisms of amphetamine toxicity are excitoxicity and mitochondrial dysfunction, although both of these also involve oxidative stress (Yamamoto et al., 2010). The excitotoxicity is characterized by numerous actions such as enhanced glutamate release and activation of glutamate receptors, which then increase the level of calcium in the cell, eventually leading to an increase in free radicals and nitric oxide (Yamamoto et al., 2010). The nitric oxide then causes proteins involved with dopamine and serotonin to become nitrated (Yamamoto et al., 2010). Methamphetamine has been shown to cause a release of glutamate in rat striatum through the nigrostriatal pathway (Yamamamoto et al., 2010). It has also been shown that inhibition of the release of glutamate protected methamphetamine-induced injury to the striatal terminals (Yamamoto et al., 2010). Mitochondrial dysfunction occurs because the amphetamine

disrupts the electron transport enzyme complexes in the dopamine neurons (Yamamoto et al., 2010). Methamphetamine at toxic doses have been shown to inhibit mitochondrial electron transport chain enzyme complexes, complex I, complex II-III, and complex IV in the striatum and other dopamine-containing brain areas (Burrows et al., 2000; Brown et al., 2005; klongpanichapak et al., 2006). In addition, administration of energy substrates was found to attenuate the amphetamine-induced neurotoxicity to dopamine and serotonin nerve endings (Stevens et al., 1998). Yamamoto et al. (2010) reactive oxygen species, reactive nitrogen species, and increased calcium in the cell could contribute to the mitochondrial dysfunction induced by amphetamine exposure (Yamamoto et al., 2010). While the immediate effects caused by amphetamine are short term, they result in long-term, long-lasting neurotoxicity. Positron emission tomography studies revealed that patients who had abstained from amphetamine use for approximately 3 years had significantly decreases in DAT density in the caudate nucleus and putamen compared to controls (McCann et al., 1998). In rhesus monkeys, concentrations of dopamine and serotonin in the caudate were found to be below control levels four years after their last injection with methamphetamine (Woolverton et al., 1989). Rats and mice have also been shown to have long-lasting neurotoxicity following amphetamine exposure (Friedman et al., 1998; Halladay et al., 2003).

Amphetamine Toxicity in Rodents

Amphetamine has been shown to induce stereotypic and self-injurious behavior (SIB) in rodents. Stereotypic and self-injurious behaviors are observed in many neurological disorders including autism and Tourette's syndrome among others (Luchins,

1990; and Sandyk, 1988). The stereotypic behaviors are characterized by restricted patterns of interest or behavior, inflexible adherence to routines, repetitive motor mannerisms such as hand flapping or twisting, and persistent preoccupation with parts of objects (DSM-IV-TR, 2000). In their most severe form, the stereotypic behaviors escalate into repetitive self-injury (Lecavalier 2006). The cause of these behaviors has been linked to the dopamine pathways in the brain and treatments for self-injurious behavior are dopamine antagonists (Luchins, 1990). Breese et al., (1984) found that neonatal rats treated with 6-hydroxydopamine (6-OHDA) and then administered dopamine agonists were a useful model of self-injurious behavior. 6-OHDA has been shown to destroy catecholamine containing neurons in adult and developing rats (Breese et al., 1984). The treatment with 6-OHDA to the neonatal animal is thought to produce a hypersensitive dopamine system in the adult mouse (Breese et al., 1984). The rats treated with 6-OHDA as neonates exhibited L-dopa dose-related (10, 30, and 100 mg/kg L-dopa) increases in self-biting and self-mutilation behavior at P22 and P24. Methamphetamine has also been shown to induce stereotypic and self-injurious behaviors in rodents (Kita et al., 2000). The neurotoxicity caused by the methamphetamine is believed to be caused by excess dopamine release, which in turn produces ROS through auto-oxidation and the enzymatic catabolism of dopamine by monoamine oxidase (Kita et al., 2000; Devito and Wagner, 1989). The dopamine transporter is necessary for the striatal dopaminergic neurotoxicity caused by methamphetamine (Fumagalli et al., 1998). In rats, this neurotoxicity has been found to be long lasting similar to the long-term loss of dopamine transporters seen in human methamphetamine abusers (Friedman et al., 1998; McCann et al., 1998). Further support for the dopaminergic basis for the methamphetamine induced

neurotoxicity has been shown using dopamine receptor blockers. Wagner et al. (2004) found that risperidone, a 5-HT_2 and D_2 receptor blocker, protected against the self-injurious behavior induced by amphetamine. They also found that the dose of risperidone that ameliorated the self-injurious behaviors also reversed the amphetamine induced increase in serotonin, bringing the induced levels back to those seen in control animals (Wagner et al., 2004).

Early developmental exposure to a toxicant can cause sensitization to toxicants such as amphetamine later in adulthood. Specifically, early treatment with methymercury in rodents has been associated with altered sensitivity to dopamine agonists and antagonists in adults (Archer and Frederiksson, 1992; Gimenez-Llort et al., 2001; Ramussen and Newland, 2001). It has been shown that BALB/c mice exposed to methylmercury at an early postnatal time point had an increased sensitivity to amphetamine (Wagner et al., 2007). Both 2 and 4 mg/kg resulted in similar results following amphetamine treatment; however a higher dose of MeHg at postnatal day 13 did not result increased sensitivity. This finding lead the investigators to suggest that the increased sensitivity to amphetamine induced by MeHg was not dose dependent, and that the time point at which the pre-treatment is given is the critical factor in MeHg induced sensitivity to amphetamine (Wagner et al., 2007). The mice that received methylmercury during the critical period between postnatal day 3 and 13 showed increased incidence of self-injurious behavior following amphetamine exposure compared with mice treated with vehicle as neonates (Wagner et al., 2007). The same results were found for stereotypic behaviors. In addition to the behavioral differences, neurochemical differences were also found in this study. Mice pretreated with methylmercury and then

exposed to amphetamine had significantly increased serotonin concentrations as well as serotonin metabolites (Wagner et al., 2007). Amphetamine induced changes in the DOPAC/DA ratio were significantly reduced in the methylmercury pretreated mice as were the amphetamine induced changes in the HVA/DA turnover in the striatum (Wagner et al., 2007). Rats exposed to quinpirole from postnatal day 1 through postnatal day 11 were found to have increased horizontal motor activity following amphetamine exposure on postnatal day 60 compared to saline-pretreated controls (Cope et al., 2010). In addition, the rats pre-treated with quinpirole were found to have a 500% increase in accumbal dopamine overflow compared to control rats administered amphetamine (Cope et al., 2010). Exposure to the Gram (-) bacterial toxin, lipopolysaccharaide (LPS) at embryonic day 10.5 has also been shown to increase sensitivity to the dopaminergic toxicity caused by amphetamine. Animals exposed to LPS were found to have 33% fewer dopamine neurons compared to controls (Ling et al., 2004). These data support the hypothesis that early life exposure to a toxicant may enhance sensitivity to the same or a different toxicant later in life.

Nrf2 and Amphetamine

Given that Nrf2 is involved in the defense against oxidative stress and that one mechanism by which amphetamine induces toxicity is by the production of ROS, there is a potential link between Nrf2 status and amphetamine toxicity. Nrf2, as previously discussed, is a transcription factor that upregulates the transcription of genes that protect against oxidative stress. Nrf2 knockout mice lack this transcription factor and therefore, have increased sensitivity to reactive oxygen species. Amphetamine, as previously noted,

increases the release of dopamine, which in turn produces oxidative stress and causes neurotoxicity. Therefore, these knockout mice should be more sensitive to the neurotoxic effects of treatment with amphetamine. In addition, pretreatment with methylmercury has clearly been shown to enhance the sensitivity to amphetamine. We propose that pretreatment with the oxidative stress inducing neurotoxicant valproic acid may also enhance sensitivity to amphetamine. In addition, the Nrf2 knockout mice, pretreated with valproic acid could, therefore, have a potentially enhanced sensitivity to amphetamine treatment compared with saline-preteated Nrf2 knockout and wild type mice and compared with valproic acid-pretreated wild type mice.

EXPERIMENT ONE

VALPROIC ACID MODEL OF AUTISM: DIFFERENTIAL EFFECTS OF VPA ON NRF2 KNOCKOUT MICE IN DEVELOPMENTAL BEHAVIORAL ASSAYS

Rationale:

Previously, our lab has shown that treatment with valproic acid at an early postnatal time point results in deficits in behavioral tests that model some features of autism. Mice treated with 400 mg/kg of valproic acid by subcutaneous injection have been shown to have increased apoptosis in the cerebellum and hippocampus as seen in human autism data. The behavioral deficits found in mice following treatment with valproic acid were seen in tests that are associated with the cerebellum and the hippocampus. The 400 mg/kg dose results in a valproic acid concentration in the brain calculated to be approximately 6-10 µg/mL which is similar to clinical valproic acid concentration of the human brain at 7.5-20.8 µg/mL (Go et al., 2011). This single exposure to 400 mg/kg valproic acid on postnatal day 14 has previously been shown to produce motor and cognitive deficits in mice (Wagner et al., 2006). In addition, this single exposure on postnatal day 14 has been shown to cause granule cell death and cerebellar hypoplasia as well as cause damage to the Purkinje cell layer (Yochum et al., 2008; Banji et al., 2011). Postnatal day 14 corresponds with the period when hippocampal and striatal differentiation and migration are still occurring in the mouse brain and critical developmental behaviors mature or first appear in the BALB/c mouse (Wagner et al., 2006; Rice and Barone, 2000). In addition, cerebellar granule cells are

also undergoing migration and differentiation at this time point (Rice and Barone, 2000). As previously discussed, it is thought that the damage caused by valproic acid in the developing mouse is at least in part due to oxidative stress. The Nrf2 knockout mice have been shown to be more sensitive to oxidative stress because they are lacking the Nrf2 gene that induces transcription of some anti-oxidant defense mechanisms. Given this information, the Nrf2 knockout mice may prove to be more sensitive to the detrimental effects of valproic acid because they lack the appropriate defense mechanisms. The following experiment investigated the effect of valproic acid both in wild type and Nrf2 knockout mice in a battery of developmental behavioral tests.

Hypothesis: A single treatment with valproic acid, at 400 mg/kg subcutaneous injection on postnatal day 14, will result in functional and neuropathological deficits and Nrf2 knockout mice will be more sensitive compared to wild type mice.

Specific Aims:

- **Assess the behavioral and developmental differences between Nrf2 knockout mice and C57BL/6J wild type mice.**
- **Assess the behavioral consequences following P14 treatment with valproic acid in Nrf2 knockout mice and C57BL/6J mice.**

Methods:

Animals

Four female and two male C57BL/6J mice were purchased from Jackson Laboratories (Jackson Laboratories; Bar Harbor, ME) to begin the wild type colony. Wild type females were bred to wild type males to produce our wild type subjects. Four female and two male Nrf2 knockout mice were provided by Dr. Kong's lab at Rutgers University. Nrf2 knockout mice F8 were created by backcrossing with C57BL/6J, they were genotyped and used as previously described (Moi et al., 1994; Chan et al., 1996; Shen et al., 2005). Three primers were used for genotyping: (1) 3' primer: 5' GGA ATG GAA AAT AGC TCC TGC C 3'; (2) 5' primer: 5' GCC TGA GAG CTG TAG GCC C 3'; (3) lacZ primer, 5' GGG TTT TCC CAG TCA CGA C 3'. Nrf2 knockout mice-derived PCR products showed only one band of 220 bp. Nrf2 wild type mice derived PCR products showed a band of 260 bp.

Knockout females were bred to Nrf2 knockout males to produce our Nrf2 knockout subjects. The breeders were housed in individual shoebox cages with woodchip bedding and with free access to food and water in a temperature and humidity regulated room with a 12 hr light/dark cycle. During breeding, the females were placed into the males' cage and monitored for weight changes twice a week. Once the females were determined to be pregnant, they were returned to their home cage for the duration of the study until weaning of the pups at postnatal day 25.

Day of birth was considered postnatal day 0. Behavioral testing began on postnatal day 5 of life and continued until post natal day 25 at which time the pups were weaned. Mice were weighed twice a week between postnatal day 5 and postnatal day 24.

All behavioral testing was done in the morning approximately 2 hours following the beginning of the light cycle in the colony room. All weights were taken just prior to behavioral testing.

A minimum of 10 mice per genotype per treatment group per sex were evaluated in the behavioral experiments. An N of 10 is sufficient for meaningful statistical interpretations of behavioral experiments (Crawley, 2000).

Postnatal Sodium Valproate Administration

The litters from these breeding pairs were randomly assigned to either valproic acid treatment group or saline/no treatment group. The day of birth was considered postnatal day 0. On postnatal day 14, the pups received either saline or 400 mg/kg of valproic acid by subcutaneous injection.

Surface Righting

Pups were tested on postnatal day 5 through postnatal day 9. This time period was prior to treatment with valproic acid or saline. 61 total mice were evaluated: 15 wild type males; 15 wild type females; 15 Nrf2 knockout males and 16 Nrf2 knockout females. Each mouse was placed on its back and gently held with all four limbs extended outward at which time it was released. Time to right so that all four paws were touching the surface was recorded. A maximum score of 30 seconds was recorded if the mouse failed to right in that time period.

Mid-Air Righting

The mice were exposed to valproic acid or saline after the second day of this test on postnatal day 14. 106 mice were evaluated in this test: 15 male wild type saline-treated; 13 male wild type valproic acid-treated; 12 female wild type saline-treated; 14 wild type valproic acid-treated; 16 male knockout saline-treated; 14 male knockout valproic acid treated; 10 female knockout saline-treated; and 12 female knockout valproic-acid treated. The ability to right mid-air was assessed on postnatal day 13 through postnatal day 19 by holding the mouse by the scruff of the neck, ventral side up, with all four paws extended upward, 30 cm above a padded surface. Ability to right was scored positive if the mouse landed on all four paws. A score of 2 out of three successful mid-air righting attempts was recorded as ability to right on each day.

Hanging Wire Grip Strength

106 mice were evaluated in this test: 15 male wild type saline-treated; 13 male wild type valproic acid-treated; 12 female wild type saline-treated; 14 wild type valproic acid-treated; 16 male knockout saline-treated; 14 male knockout valproic acid treated; 10 female knockout saline-treated; and 12 female knockout valproic-acid treated. On postnatal day 13 through postnatal day 19, mice were placed on a wire, 30 cm above a padded surface. Latency to fall was recorded with a maximum of 30 seconds for each trial.

Negative Geotaxis

106 mice were evaluated in this test: 15 male wild type saline-treated; 13 male wild type valproic acid-treated; 12 female wild type saline-treated; 14 wild type valproic acid-treated; 16 male knockout saline-treated; 14 male knockout valproic acid treated; 10 female knockout saline-treated; and 12 female knockout valproic-acid treated. Negative geotropism was tested on postnatal day 13 though postnatal day 19. The mouse was placed on a grid wire surface (30 cm x 18 cm) on a 45 degree incline facing downward. The latency to turn 180 degrees such that the head was facing upward along the incline was recorded with a maximum of 30 seconds for each trial.

Rotorod

On postnatal day 20 through postnatal day 22, each mouse was placed on the rotorod with a circumference of 6 inches, rotating at 12 revolutions per minute. The rotorod was 60 inches above a padded receptacle. The latency to fall from the rotorod was recorded for each mouse for three trials, with each trial lasting no more than 60 seconds. 102 mice were evaluated in this test: 15 male wild type saline-treated; 12 male wild type valproic acid-treated; 11 female wild type saline-treated; 14 wild type valproic acid-treated; 16 male knockout saline-treated; 14 male knockout valproic acid treated; 10 female knockout saline-treated; and 10 female knockout valproic-acid treated.

Open Field Activity

On postnatal day 20 through postnatal day 25, mice were assessed for motor activity. Mice were taken out of their home cages and placed in activity chambers

consisting of a Plexiglas box (42 cm x 22 cm x 14 cm) with six photocell sensors placed 7 cm apart and 2.5 cm above the floor of the chamber. The number of times the mouse broke the photocell beams was recorded every 2 minutes for a total of 10 minutes a day. 102 mice were evaluated in this test: 15 male wild type saline-treated; 12 male wild type valproic acid-treated; 11 female wild type saline-treated; 14 wild type valproic acid-treated; 16 male knockout saline-treated; 14 male knockout valproic acid treated; 10 female knockout saline-treated; and 10 female knockout valproic-acid treated.

Morris Water Maze

The maze was a circular tub measuring 71 cm in diameter and 29 cm in height. The tub was painted white on the interior, filled ¾ full with water maintained at 23-26 ^{o}C and made opaque with white non-toxic latex paint. A starting point was determined randomly from one of four equally spaced quadrants. In the visible platform paradigm of the water maze, the water was only allowed to fill enough so that the platform sat 1.5 cm above the surface. Each mouse was given one 60 second trial to find the visible platform in each of 5 different positions in the water maze. In the hidden platform paradigm, done on postnatal day 21-24, a platform that was painted white and sat below the surface of the water was placed in one quadrant and the mouse was given 5 trials a day starting at different locations in the maze to find the hidden platform. Again, a maximum of 60 seconds was given for each trial in both the hidden or visible platform versions. 102 mice were evaluated in this test: 15 male wild type saline-treated; 12 male wild type valproic acid-treated; 11 female wild type saline-treated; 14 wild type valproic acid-treated; 16

male knockout saline-treated; 14 male knockout valproic acid treated; 10 female knockout saline-treated; and 10 female knockout valproic-acid treated.

Statistical Analysis

The behavioral tests and weights were analyzed using an ANOVA or repeated measures ANOVA, where appropriate, with the exception of mid-air righting, which was analyzed using X^2. When significant main effects were found, differences between groups were determined using Fisher's PLSD post-hoc tests. If an effect of sex was found, the males and females were then evaluated independently.

Results:

Weight (P5-P24)

A repeated measures ANOVA was used to analyze the average weight from postnatal day 5 through postnatal day 24, the age range of the developmental behavioral tests. 114 mice were weighed between P5 and P24 consisting of 17 male wild type saline-treated mice, 16 male wild type valproic acid-treated mice, 11 wild type female saline-treated mice, 19 female wild type valproic acid-treated mice, 16 male knockout saline-treated mice, 14 male knockout valproic acid-treated mice, 10 female knockout saline-treated mice, and 11 female knockout valproic acid-treated mice. A significant effect of genotype[$F(1,110)=12.785$, $p=0.0005$], a significant effect of treatment [$F(1,110)=37.001$, $p<0.0001$], and a significant effect of postnatal day [$F(5, 550)=1429.292$, $p<0.0001$] were found, as well as a significant interaction of postnatal

day by treatment [$F(5, 550)=43.736$, $p<0.0001$] and a significant interaction of postnatal day by genotype by treatment [$F(5,550)=5.740$, $p<0.0001$]. *Post hoc* tests revealed that the knockout mice ($p=0.0001$) weighed significantly more than the wild type mice overall; the saline-treated mice ($p<0.0001$) weighed significantly more than the valproic acid-treated mice overall. The weight at each postnatal day significantly changed compared to every other day ($p<0.0001$), except that postnatal day 13 and 16 were not found to be significantly different from each other. [Figure 1]

Significant differences in weight were found between the saline-treated wild type mice and the valproic acid-treated wild type mice. Prior to treatment on postnatal day 14, the wild type mice that would be in the saline-treatment group and the wild type mice that would eventually be in the valproic acid-treatement group did not have significantly different body weights. However, on postnatal day 9, the mice that would eventually be in the valproic acid-treated group had an average weight 4.022 g and the mice would eventually be in the saline-treated group had an average weight of 4.369 g. This 8% difference in body weight was found to be significant ($p=0.0032$). Following the postnatal day 14 injection, the valproic acid-treated wild type mice weighed significantly less compared to the saline-treated mice. The average weight of the saline-treated group on postnatal day 16 was 5.836 g, while the average weight valproic acid-treated was 4.751. This 20% difference in weight was found to be significant ($p<0.0001$). The average weight of the saline-treated group on postnatal day 20 was 6.396 g, while the average weight of the valproic acid-treated group was 5.446 g. This 15% weight difference was found to be significant ($p=0.0001$). The average weight of the saline-treated group on postnatal day 24 was 7.793 g, while the average weight of the valproic

acid-treated group was 6.897 g. This 11% difference in average weight was found to be significant ($p=0.0036$).

Significant differences in weight were found between the saline-treated Nrf2 knockout mice and the valproic acid-treated Nrf2 knockout mice. Prior to exposure to valproic acid on postnatal day 14, the Nrf2 knockout mice that would eventually be in the saline-treatment group did not have any significant difference in weight compared to the mice that would eventually be in the valproic acid-treatment group. Following valproic acid and saline exposure, the valproic acid-treated knockout mice weighed significantly less than the saline-treated knockout mice. On postnatal day 16, the average weight of the saline-treated mice was 6.365 g, while the valproic acid-treated mice weighed 5.264 g. This 17% difference in average body weight was found to be significant ($p<0.0001$). On postnatal day 20 the average weight of the saline-treated mice was 7.231 g, while the average weight of the valproic acid-treated mice was 5.476 g. This 24% difference in weight was found to be significant ($p<0.0001$). On postnatal day 24 the average weight of the saline-treated mice was 8.331 g, while the average weight of the valproic acid-treated mice was 6.816g. This 18% difference was found to be significant ($p<0.0001$).

Significant differences in weight were found between wild type and knockout mice within treatment groups. A significant difference between Nrf2 knockout mice that would be treated with valproic acid and wild type mice that would be treated with valproic acid was found prior to the postnatal day 14 exposure. The knockout mice were found to be significantly heavier than the wild type mice. On postnatal day 5 the knockout mice in the valproic acid group weighed 2.745 g, while the wild type mice weighed 2.515 g (an 8% difference), which was found to be significant ($p=0.0064$). On

postnatal day 9 the knockout mice in the valproic acid group weighed 12% more than the wild type mice. The knockout mice weighed 4.546 g, while the wild type mice weighed significantly less at 4.022 g ($p<0.0001$). On postnatal day 13 the knockout mice still weighed 12% more than the wild type mice. The knockout mice weighed an average of 5.808 g and the wild type mice weighed significantly less ($p<0.0001$) with an average weight of 5.126 g. Following exposure to valproic acid on postnatal day 14, the knockout mice were still significantly heavier, although now with a 10% difference, with an average weight of 5.264 g on postnatal day 16 ($p=0.0002$) compared to the wild type mice with an average weight of 4.751 g. On postnatal day 20 and 24, the valproic acid-treated mice of both genotypes did not show differences in body weight. The Nrf2 knockout mice treated with saline were also found to weigh significantly more than the wild type mice on postnatal day 9 ($p=0.0332$), postnatal day 13 ($p=0.0012$), postnatal day 16 ($p=0.0101$), and postnatal day 20 ($p=0.0004$). The average body weight of the Nrf2 knockout mice treated with saline was 4.691 g on postnatal day 9, while the wild type mice had an average weight of 4.369 g, which was a 7% difference. On postnatal day 13 the knockout mice had an average weight of 5.977 g, while the wild type mice had an average weight of 5.368 g, which was a 10% difference. The average body weight of the knockout mice on postnatal day 16 was 6.365 g, while the wild type mice had an average weight of 5.836, which was a 9% difference. On postnatal day 20 the knockout mice had an average weight of 7.231 g, while the wild type mice had an average body weight of 6.396 g, which was a 12% difference. By postnatal day 24, the wild type mice and the knockout mice did not have differences in body weight.

Surface Righting

A repeated measures ANOVA was used to analyze the average latency for the mouse to turn onto its stomach over 5 days of testing. A significant effect of day of testing was found [$F(4,280)=80.802$, $p<0.0001$] where the mice were faster to turn over on each day. On postnatal day 5 the mice took an average of 22.882 seconds to turn over. By postnatal day 9 the mice took an average of 2.416 seconds to turn over. No significant difference between wild type mice and Nrf2 knockout mice was found. [Figure 2]

Mid-Air Righting

X^2 analysis revealed no difference between the saline-treated wild type and saline-treated knockout mice ($X^2(6)=2.74$, $p=0.840$) and no difference between the valproic acid-treated wild type mice and the valproic acid-treated knockout mice ($X^2(6)=9.26$, $p=0.159$). The X^2 analysis did reveal an overall significant effect of p14 valproic acid treatment on mid-air righting in the wild type mice ($X^2(6)=17.1$, $p=0.009$) and in the Nrf2 knockout mice ($X^2(6)=52.4$, $p<0.0001$). Fisher's exact test further revealed that there was a significant impairment of valproic acid-treated mice on P15 ($p=0.0032$). On postnatal day 15 88.67% of the saline-treated mice were able to mid-air right, while only 24.52% of the valproic-acid treated mice were able to mid-air right. On postnatal day 16, 98.11% of the saline-treated mice were successful, while only 66.03% of the valproic-acid treated mice were successful. On postnatal day 17 and 18, 98.11% of the saline-treated mice were successful, while only 73.58% and 88.6% of valproic acid-treated mice were successful. On the last day of testing 100% of the saline-treated mice

were able to mid-air right, while only 81.13% of the valproic acid-treated mice werem successful. [Figure 3]

Negative Geotaxis

A repeated measures ANOVA was used to analyze the average latency to turn 180 degrees on a 45 degree inclined plane over seven days of testing. An overall effect of genotype [$F(1,102)=5.389$, $p=0.0223$] was found as was an overall effect of day of testing [$F(6,612)=2.195$), $p=0.0419$] and a day by treatment interaction [$F(6, 612)=2.982$, $p=0.0070$]. [Figure 4] *Post hoc* tests revealed that the valproic acid-treated wild type mice performed significantly worse than the valproic acid-treated knockout mice ($p=0.0107$), while the saline-treated wild type mice and the saline-treated Nrf2 knockout mice performed similarly ($p=0.3192$). The valproic acid-treated wild type mice had a total average latency to turn 180 degrees of 5.213 seconds, while the valproic acid-treated Nrf2 knockout mice had an average of 4.323 seconds. The valproic acid-treated wild type mice and saline-treated wild type mice performed similarly ($p=0.9648$) as did the valproic acid-treated Nrf2 knockout mice and the saline-treated Nrf2 knockout mice ($p=0.3972$). [Figure 5]

Hanging Wire Grip Strength

A repeated measures ANOVA was used to analyze the average latency to fall from a wire over seven days of testing. An overall effect of sex was found [$F(1,98)=4.385$, $p=0.0388$] and therefore, the data was analyzed separately for males and females.

Males

A repeated measures ANOVA was used to analyze the average latency to fall from a wire over seven days of testing for only male subjects. No effect of genotype [$F(1,54)=0.259$, $p=0.6131$], no effect of treatment [$F(1,54)=1.073$, $p=0.3049$], and no genotype by treatment interaction was found [$F(1,54)=2.396$, $p=0.1275$]. The only significant effect found was a significant effect of day of testing [$F(6,324)=38.941$, $p<0.0001$] whereby the mice improved each day and were able to hang onto the wire longer each consecutive day. On postnatal day 5 the average latency to fall from the wire was 5.123 seconds, while on postnatal day 19 the average latency to fall from the wire was 19.916 seconds. [Figure 6]

Females

A repeated measures ANOVA was used to analyze the latency to fall from the wire for only female subjects. No overall effect of genotype [$F(1,44)=0.115$, $p=0.7365$]was found, nor was there a genotype by treatment interaction [$F(1,44)=1.822$, $p=0.1840$]. An overall effect of treatment [$F(1,44)=3.995$, $p=0.0518$] was found. *Post hoc* tests revealed that the valproic acid-treated mice performed significantly worse compared with the saline-treated mice ($p=0.0395$). On average the valproic-acid treated mice had an average latency to fall from the wire of 14.758 seconds, while the saline-treated mice had an average latency to fall from the wire of 17.963 seconds. [Figure 7]

Open Field Activity

A repeated measures ANOVA was used to analyze the average total beam breaks in the open field activity assay. An overall effect of treatment [$F(1,94)=33.236$, $p=<0.0001$], an interaction between genotype and treatment [$F(1,94)=5.594$, $p=0.0201$] and an overall effect of sex [$F(1,94)=5.768$, $p=0.0183$] were found to be significant. Therefore, the data was analyzed separately for males and females.

Males

A repeated measures ANOVA was used to analyze the total average beam breaks for males. An overall significant effect of treatment [$F(1,53)=10.108$, $p=0.0025$], an overall significant effect of day of testing [$F(4,212)=3.641$, $p=0.0068$] and an overall significant interaction of day by genotype [$F(4,212)=3.587$, $p=0.0075$] was found. *Post hoc* tests revealed that the valproic acid-treated mice were less active compared to the saline-treated mice ($p=0.0018$) and that activity on postnatal day 20 was significantly different compared to postnatal day 21 ($p=0.0003$), postnatal day 22 ($p=0.0058$), postnatal day 23 ($p=0.0029$), and postnatal day 24 ($p=0.0359$). The saline-treated mice made an average of 712.819 beam breaks, while the valproic acid-treated mice made an average of 395.054 beam breaks. On postnatal day 20, the first day of testing, the mice made an average of 668.193 beam breaks, while the made 508.737 beam break, 547.667 beam breaks, 537.842 beam breaks, and 576.930 beams breaks on postnatal day 21, 22, 23, and 24 respectively. Further *post hoc* tests revealed that the wild type mice treated with valproic acid were not significantly different compared to the wild type mice treated with saline ($p=0.4203$), while the Nrf2 knockout mice treated with valproic acid were

significantly less active compared to the Nrf2 knockout mice treated with saline ($p=0.0004$). The Nrf2 knockout mice treated with valproic acid made an average of 298.957 beam breaks, while the Nrf2 knockout mice treated with saline made an average of 788.1 beam breaks. The difference over days of testing was also only found to be significant for the Nrf2 knockout mice. [Figure 8]

Females

A repeated measures ANOVA was used to analyze the average total beam breaks for females. An overall significant effect of treatment [$F(1,41)=22.398$, $p=0.0001$] and an overall significant effect of day of testing [$F(4,164)=2.841$, $p=0.0260$] was found. *Post hoc* tests revealed that the valproic acid-treated mice were significantly less active compared with the saline-treated mice ($p<0.0001$). The saline-treated mice made an average of 1028.514 beam breaks, while the valproic-acid treated mice made an average of 474.633 beam breaks. Activity on postnatal day 20 was found to be significantly different compared to postnatal day 21 ($p=0.0375$), postnatal day 22 ($p=0.0308$), postnatal day 23 ($p=0.0038$), and postnatal day 24 ($p=0.0273$). On postnatal day 20 the mice made an average of 816.8 beam breaks, while on postnatal day 21 they made an average of 723.8 beam breaks, on P 22 they made 720.244 beam breaks, on P23 they made 686.622 beam breaks, and on postnatal day 24 they made an average of 718.089 beam breaks. [Figure 9]

Rotorod

A repeated measures ANOVA was used to analyze the average latency to fall from the rotorod over three trials a day for three days. An overall significant effect of genotype [$F(1,97)=8.297$, $p=0.0049$], an overall significant effect of treatment [$F(1,97)=62.442$, $p<0.0001$], and a significant genotype by treatment [$F(1,97)=5.122$, $p=0.0259$] effect was found. In addition, a significant effect of day of trial [$F(2,194)=168.230$, $p<0.0001$] and a significant day of trial by treatment interaction [$F(2,194)=20.003$, $p<0.0001$] was found. *Post hoc* tests revealed that, overall, the wild type mice performed significantly better than the Nrf2 knockout mice ($p=0.0101$), the saline-treated mice performed significantly better than the valproic acid-treated mice ($p<0.0001$), and that the latency to fall was significantly higher each day compared to the day before ($p<0.0001$). The saline-treated wild type mice performed significantly better than the valproic acid-treated wild type mice ($p=0.0013$) and the saline-treated Nrf2 knockout mice performed significantly better than the valproic acid-treated Nrf2 knockout mice ($p<0.0001$). The saline-treated wild type mice had an average latency to fall from the rod of 40.533 seconds, while the valproic acid-treated wild type mice had an average latency to fall from the rod of 26.387 seconds. The saline-treated Nrf2 knockout mice had an average latency to fall from the rod of 38.985 seconds, while the valproic acid-treated Nrf2 knockout mice had an average latency to fall from the rod of 13.484 seconds. The saline-treated wild type mice and the saline-treated Nrf2 knockout mice were found to perform similarly, while the valproic acid-treated knockout mice performed significantly worse than the valproic acid-treated wild type mice ($p=0.0043$). [Figure 10]

Morris Water Maze

Visible Platform

A repeated measures ANOVA was used to analyze the visible platform paradigm of the water maze looking at the average latency to find the visible platform (seconds) over 5 trials. An overall significant effect of genotype [F (1,98)=8.596, p=0.0043] and an overall significant effect of treatment [F(1,98)=49.171, p<0.0001] was found. In addition, a significant effect of trial [F(4,392)=23.583, p<0.0001], a significant interaction of trial by genotype [F(4,392)=2.426, p=0.0475], and a significant interaction of trial by treatment [F(4,392)=4.079, p=0.0030] was found. The saline-treated wild type mice had an average latency to reach the visible platform of 28.369 seconds, while the valproic acid-treated wild type mice had an average latency of 45.173 seconds. The Nrf2 knockout mice treated with saline had an average latency to reach the visible platform of 35.762 seconds, while the valproic acid-treated knockout mice had an average latency of 51.295 seconds. *Post hoc* tests revealed that the wild type mice performed significantly better than the Nrf2 knockout mice overall (p=0.0063) and that the saline-treated mice performed significantly better compared to the valproic acid-treated mice overall (p<0.0001). The saline-treated wild type mice performed significantly better than the saline-treated knockout mice (p=0.0353). Although the wild type valproic acid-treated mice performed better than the Nrf2 knockout valproic acid-treated mice, the difference only approached significance (p=0.0532). [Figure 11]

Hidden Platform

A repeated measures ANOVA was used to analyze the hidden platform paradigm of the water maze looking at the average latency to find the hidden platform (seconds) over 4 days of testing with 5 trials per day. An overall significant effect of treatment [$F(1,98)= 105.434$, $p<0.0001$] and an overall genotype by treatment effect [$F(1,98)=26.050$, $p<0.0001$] was found. In addition, an overall significant effect of day of study [$F (3,294)=67.574$, $p<0.0001$], an overall significant interaction of day by genotype [$F=(3,294)=5.226$, $p=0.0016$], and a significant day by genotype by treatment interaction was found [$F(3,294)=2.819$, $p=0.0393$]. The wild type mice treated with saline had an average latency to find the hidden platform of 20.738 seconds, while the valproic acid-treated wild type mice had an average latency of 32.881 seconds. The Nrf2 knockout mice treated with saline had an average latency to find the hidden platform of 32.881 seconds, while the valproic acid-treated knockout mice had an average latency of 49.451 seconds. *Post hoc* tests revealed that valproic acid-treated mice performed significantly worse than saline-treated mice ($p<0.0001$). Saline-treated Nrf2 knockout mice performed significantly better than saline-treated wild type mice ($p=0.0151$), while valproic acid-treated Nrf2 knockout mice performed significantly worse than valproic acid-treated wild type mice ($p<0.0001$). The valproic acid-treated wild type mice ($p=0.0033$) and valproic acid-treated Nrf2 knockout mice ($p<0.0001$) performed significantly worse compared to their saline-treated counterparts. [Figure 12]

Table 1. Summary of results from Experiment One

Behavioral Outcome Measures	Genotype Difference		Significant Treatment Effect
	Saline	VPA	
Weight (P5-P24)	**Yes[1]**	nd[2]	**p<0.05[3]**
Surface Righting	nd[2]	nd[2]	ns[4]
Mid-Air Righting	nd[2]	nd[2]	**p<0.05**
Hanging Wire	nd[2]	nd[2]	**p<0.05**
Negative Geotaxis	nd[2]	nd[2]	ns
Open Field Activity	nd[2]	**Yes[1]**	**p<0.05**
Rotorod	nd[2]	**Yes[1]**	**p<0.05**
Water Maze (Visible)	nd[2]	**Yes[1]**	**p<0.05**
Water Maze (Hidden)	nd[2]	**Yes[1]**	**p<0.05**

Table 1. 1 denotes "Yes" = genotype difference found within treatment group. 2 denotes "nd"= no difference between wild type and knockout mice. 3 denotes "$p<0.05$" = significant difference between saline-treated and valproic-acid treated mice at $p<0.05$. Not the actual p values; some p values well below 0.05 level. 4 denotes "ns" = no significant difference; p greater than 0.05.

Conclusions for Experiment One:

Table one summarizes the results of Experiment One. Overall, valproic acid was found to induce behavioral changes in both wild type and Nrf2 knockout mice. The Nrf2 knockout mice treated with saline were found to be heavier than the wild type mice treated with saline, however there were no weight differences between the valproic acid-treated wild type mice and the valproic acid-treated Nrf2 knockout mice. The one behavorial test run before exposure to valproic acid, the surface righting test, did not show differences between Nrf2 knockout mice and wild type mice. The Nrf2 knockout mice also appear to be more sensitive to the effects of valproic acid on certain behavioral tests, including the rotorod, the water maze, and the males in open field activity.

Significant differences in body weight were found over the course of the developmental testing period. Prior to valproic acid treatment, the Nrf2 knockout mice weighed more than the wild type mice. This difference held true for the saline-treated animals. Valproic acid treatment was found to cause a drop in body weight in both genotypes, where the average body weight of the knockout mice dropped about 9% between P13 and P16, and the average body weight of the wild type mice dropped about 7% between P13 and P16. The average body weight of the valproic acid-treated knockout mice and the average body weight of the valproic acid-treated wild type mice was not found to be different following P14 exposure to valproic acid. While there was a difference in weight between the saline-treated wild type mice and the saline-treated knockout mice, they tended to perform similarly on the behavioral tests. The valproic acid-treated wild type mice and valproic acid-treated knockout mice did not differ in weight; however, they did tend to perform differently on the behavioral tests. The

valproic acid-treated wild type mice tended to perform better on the behavioral assays compared to the Nrf2 knockout mice treated with valproic acid. Therefore, the difference in weight for the saline-treated animals did not appear to have an effect on behavior.

Prior to valproic acid treatment on postnatal day 14, the wild type mice and Nrf2 knockout mice appear to develop normally. Evidence of this normal and similar development was seen in the results of the surface righting test. The ability to turn over from a supine to prone position is a normal developmental reflex that appears between postnatal day 5 and postnatal day 9 in mice. This reflex has been linked to cerebellum development (Petrosini et al., 1990). No difference between the genotypes in ability to turn over was found. All of the mice improved and were able to turn over faster from P5 through P9, at which point the latency to turn had reached its peak. This suggests that from P5-P9 all of the mice have normal cerebellar development.

The mid-air righting test, the negative geotaxis test, and the hanging wire grip strength test were all evaluated from postnatal day 13-19 and provided evidence of a detrimental effect of valproic acid on these behaviors. Mid-air righting was another test of a skill that normally develops in mice around postnatal day 14 that reflects the development of dynamic postural adjustments (Iezhitsa et al., 2001). It tests the animal's ability to turn over from a supine to prone position in the air and land on all four paws. This righting reflex has been linked to cerebellum development (Petrosini et al., 1990). Prior to treatment with valproic acid, mice of both genotypes were successful in mid-air righting. On postnatal day 15, 24 hours after valproic acid exposure, the valproic acid-treated mice failed to mid-air right, while the saline-treated mice continued to improve. The significant drop in the percent of mice on postnatal day 15 could suggest a

pharmacological effect of valproic acid. However, Wagner et al. (2006) conducted an experiment that suggests this effect is not due to a pharmacological effect. The half life of valproic acid was determined from the literature and a dose of 50 mg/kg was chosen to estimate the concentration of valproic acid that would still be present in a mouse 21 hours after it was given 400 mg/kg of valproic acid (Wagner et al., 2006). Mice were given the 50 mg/kg dose of valproic acid on postnatal day 14 and behavioral testing was done 3 hours later. No differences in the percent of mice able to mid air right were found between the saline and valproic acid treated groups (Wagner et al., 2006). This suggests that the differences in performance between the mice treated with 400 mg/kg on postnatal day 14 and the mice treated with saline are not due to a pharmacological effect of the valproic acid and instead are due to the lesion being created by the valproic acid in the cerebellum. From postnatal day 15 through the end of testing on postnatal day 19, the valproic acid-treated mice performed significantly worse compared with their saline-treated counterparts. In this test, no differences between genotypes were found; the saline-treated mice of both genotypes performed similarly, as did the valproic acid-treated mice of both genotypes. Rats with a hemicerebellectomy show a delay in the development of this righting reflex although recovery was found to be almost complete (Petrosini et al., 1990). While the rats in that experiment show a delay, they were also given the hemicerebellectomy earlier in postnatal life than these mice were treated with valproic acid. Rather than a delay in ability, we found a regression where the mice lost the ability to right that they already could successfully perform. The poor performance of the valproic-acid treated groups suggests that the development in the cerebellum has been disturbed.

The negative geotaxis test was another test of a skill that becomes acquired around postnatal day 14 in mice. It tested the latency of the mice to turn 180 degrees on an inclined plane, such that they were facing upward along the plane. This test is considered to evaluate vestibular or proprioceptive function and it has been linked to cerebellum development (Frankel and Gunn, 1968; Petrosini et al., 1990). An overall effect of genotype and day of testing was found, as was an interaction between day and treatment. However, no overall effect of treatment was found. It is clear from figure four the data were highly variable and affected the statistical values of total latency performance presented in figure five. The valproic acid-treated wild type mice performed similarly to the saline-treated wild type mice and the valproic acid-treated knockout mice performed similarly to the saline-treated knockout mice. The saline-treated wild type and Nrf2 knockout mice were found to perform similarly, however, the valproic acid-treated wild type mice performed significantly worse compared to the valproic acid-treated knockout mice. The results of this test were somewhat variable and difficult to interpret. As the saline-treated mice became comfortable with the incline as days of testing went on, many of the mice began to explore the maze rather than turn 180 degrees, thus increasing their latency to turn around. In figure four we see this steady increase in latency for both the saline-treated wild type mice and the saline-treated knockout mice. In addition, the Nrf2 knockout mice treated with valproic acid consistently turned 180 degrees with approximately the same latency throughout testing. The valproic acid-treated wild type mice did experience a longer latency to turn in the two days immediately following treatment, but then improved from P17-P19. It should also be noted that the latency to turn 180 degrees for all of the groups is very fast. The age range

for the appearance of the reflex in mice is between postnatal day 3 and postnatal day 15 and the average age for mice to respond to this reflex is postnatal day 7 (Crawley, 2007). It is possible that we do not see an improvement on latency to turn 180 degrees or an effect of valporic acid treatment in these mice because they are already performing the reflex successfully skewing the data. In addition, Petrosini et al (1990) found that rats with hemicerebellectomy early in postnatal life had a delay in the appearance of the negative geotaxis response, but that they improved almost to control levels. This further indicates that even with cerebellar damage, the negative geotaxis response can still be acquired in rodents.

The hanging wire grip strength test evaluated the forelimb strength of the animal to hold on to a wire by looking at the latency to fall from that wire. Neuromuscular abnormalities can be evaluated with this test. Normally a mouse can hang for several minutes, but a 60 second maximum can be used test session (Crawley, 2000). No difference between genotypes was found for this test. A sex difference was observed and therefore, the males and females were analyzed separately. For the male mice, there was no effect of valproic acid-treatment. All of the male mice improved over the testing days and could hold on to the wire without falling for a longer latency each day. For the female mice, there was a treatment effect. The valproic acid-treated mice were significantly weaker than the saline-treated mice. However, by the last day of testing there were no differences in grip strength between the valproic acid-treated mice and the saline-treated mice. Deficits in this test are typically attributed to muscle dysfunction, however other factors can affect performance on this test including weight, fatigue, cognition, and volition (Crawley, 2007). The valproic acid-treated mice do weigh less

than the saline-treated mice and this may contribute to the significant reduction in ability to hold on to the wire. However, the valproic acid-treated male mice also weigh less than the saline-treated mice and they do not exhibit a difference in latency to fall from the wire. All of the mice, regardless of treatment or genotype, did improve their latency to hold onto the wire over the days of testing from an average of approximately 5 seconds to an average of approximately 20 seconds. By the end of testing for both males and females there was no difference in latency to fall from the wire. In addition, the female mice in the valproic acid-treated group were found to perform worse on postnatal day 14 prior to exposure to valproic acid .This suggests that the effect of valproic acid seen in the female mice may not be indicative of valproic acid inducing a muscular defect, but perhaps the female mice in the group that would be treated with valproic acid were not as strong as the female mice in the saline-treatment group regardless of treatment.

With regard to the behavioral tests evaluated from postnatal day 13 through postnatal day 19, it appears that the mid-air righting test provided the strongest evidence for a behavioral deficit cause by valproic acid, although there were no differences between the Nrf2 knockout mice and the wild type mice.

The developmental behavioral tests evaluated from postnatal day 20 through postnatal day 25 provide the strongest evidence in support of the hypothesis that valproic acid would cause behavioral deficits and the Nrf2 knockout mice would be more sensitive to these effects. The three tests evaluated from P20 through P25 were the open field activity, the rotorod, and the Morris water maze.

The open field activity chambers measured the horizontal locomotor activity of the animal by counting the number of times the animal broke a photocell beam in the

chamber. Upon initial analysis, sex differences were found and therefore, the males and females were analyzed separately. The valproic acid-treated mice were found to be significantly less active overall compared to the saline-treated mice. Specifically, the Nrf2 knockout mice treated with valproic acid were significantly less active compared to Nrf2 knockout mice treated with saline. However, the wild type mice treated with valproic acid were not found to be significantly less active compared to the wild type mice treated with saline. This indicated that treatment with valproic acid on postnatal day 14, six days before this test was performed, resulted in decreased activity levels and that the Nrf2 knockout mice showed even less activity compared to wild type mice. Because activity was evaluated 6 days after exposure to valproic acid, the difference in activity cannot be attributed to the pharmacological effects of valproic acid. Further insight into the implications of these results will be discussed following the female results.

For the female mice, the saline-treated wild type and the saline-treated Nrf2 knockout mice had similar activity levels. The valproic acid-treated mice were significantly less active compared to the saline-treated mice. While the valproic acid-treated Nrf2 knockout mice were less active compared to the valproic acid-treated wild type mice, this effect was not significant. Again, treatment with valproic acid on postnatal day 14 resulted in behavioral changes in both wild type and Nrf2 knockout mice. The male Nrf2 knockout mice show an increased sensitivity to this effect compared with the wild type mice treated with valproic acid. For the female mice, there was a trend toward an increased sensitivity to valproic acid in the Nrf2 knockout mice, although the results did not reach significant levels.

In this behavioral test, the vertical activity and center distance could also be quantified. The ratio of center distance to total distance could also be used to evaluate anxiety, whereby highly anxious mice would avoid the center of the open field (Crawley and Paylor, 1997). The only variable that was recorded for this test was horizontal beam breaks, therefore an evaluation of the anxiety level of these mice could not be determined. However, another test of anxiety was used in evaluation of the adult mice. The number of beam breaks was recorded in 2 minutes bins for 10 minutes of testing by an experimenter, blind to genotype and treatment, standing silently in the room. The experimenter wrote down the number of beam breaks that were automatically recorded by the apparatus every two minutes. Since the bins were so close together, and the experimenter did not observe any animals standing still, it is not likely that the lower activity level exhibited by the valproic acid-treated mice was due to total inactivity or anxiety preventing the animal from exploring. As we can see in figure 8 and figure 9, the least active mice, the knockout mice treated with valproic acid, are still moving enough to break the beams over 200 times every two minutes. Motor activity is associated with cerebellar function and treatment with valproic acid on postnatal day 14 has been associated with granule cell death and cerebellar hypoplasia as well as damage to the Purkinje cell layer (Yochum et al., 2008; Banji et al., 2011). This decrease in motor activity may be caused by damage to the cerebellum. Valproic acid has also been shown to increase levels of reactive oxygen species in rodents and the deficits caused by valproic acid in rodents have been shown to be ameliorated by the use of antioxidant treatment (Banji et al., 2011; Cheh et al., 2010). Nrf2 knockout mice have also been found to be more sensitive to oxidative stress inducing toxicants and to have higher levels

of oxidative stress markers (Itoh et al., 1997; Ishii et al., 2005; Liu et al., 2010; Innamorato et al., 2010). Given this information, the impaired performance on the rotorod in Nrf2 knockout mice treated with valproic acid could suggest that these mice have increased damage to their cerebellum compared to wild type mice with normal levels of antioxidant defense mechanisms.

The rotorod tests motor function and coordination in addition to testing motor learning. This test required the use of the cerebellum and therefore is related to cerebellar function. An overall effect of genotype, treatment and day of trial was found. In addition, a significant interaction between genotype and treatment and a significant interaction between treatment and day of trial was found. All mice improved over the three days of testing. The saline-treated wild type and saline-treated knockout mice performed similarly and these two groups were able to remain on the rotorod the longest. The valproic acid-treated wild type mice performed significantly worse than the saline-treated wild type mice, as the valproic acid-treated knockout mice performed significantly worse than the saline-treated knockout mice. Interestingly, the valproic acid-treated Nrf2 knockout mice performed significantly worse compared to the valproic acid-treated wild type mice. This indicated that treatment with valproic acid on postnatal day 14 resulted in deficits in motor function and coordination in both wild type and Nrf2 knockout mice, and that the Nrf2 knockout mice were more sensitive to this detrimental effect. These deficits in motor function and coordination could be related to damage to the cerebellum. Mice that have motor coordination deficits, perhaps due to abnormalities in the cerebellum, have difficulty staying on the rotorod even at low speeds (Barlow et al., 1996; Sango et al., 1995). As discussed previously in regard to the deficits in locomotor

activity, the results of the rotorod also indicate that the Nrf2 knockout mice may have more damage to their cerebellum compared to wild type mice following valproic acid exposure (Yochum et al., 2008; Banji et al., 2011; Banji et al., 2011; Cheh et al., 2010; Itoh et al., 1997; Ishii et al., 2005; Liu et al., 2010; Innamorato et al., 2010).

The Morris water maze tests spatial learning and memory and the hidden platform paradigm of the test is correlated with the function of the hippocampus. The basis for the test is that rodents are highly motivated to escape from water by the fastest route possible (Crawley, 1999). In the visible platform paradigm of the test, the latency to find the platform that was above the water level and highly visible was recorded for five trials over one day. This tests the visual ability of the animal to see the room cues and the motor ability of the mice to swim (Crawley, 1999). The wild type mice were found to perform significantly better than the Nrf2 knockout mice. The saline-treated mice were able to find the visible platform significantly faster than their valproic acid-treated counterparts. In this paradigm, the saline-treated wild type mice found the platform significantly faster than the Nrf2 knockout mice. This was not consistent with the other behavioral tests in which the saline-treated mice of both genotypes performed similarly. Poor performance in the visible water maze could indicate dysfunction in vision and visual neurons or the motor ability of the mice to swim (Crawley, 1999). Given the results of the activity chambers, where the saline-treated Nrf2 knockout mice were found to be just as active as the saline-treated wild type mice, it is unlikely that the Nrf2 knockout mice have impairments in motor ability. It is not clear if the Nrf2 knockout mice have impairments in vision because no other vision specific evaluations were done, however no one has reported visual impairment in Nrf2 knockout mice in the literature.

In addition, the Nrf2 knockout mice improve significantly over 5 trials to the level of the saline-treated wild type mice indicating that they can learn and swim as well as the wild type mice and therefore do not have impairments in their motor neurons or hippocampus. In addition to this anomaly, the valproic acid-treated knockout mice performed similarly to the valproic acid-treated wild type mice, although both valproic acid groups performed worse than the saline groups. Treatment with valproic acid on postnatal day 14 appears to result in deficits in the latency to find the visible platform in the Morris water maze six days after treatment. This impairment indicates that there are impairments in either vision or motor neurons in the valproic acid treated mice. There are reports of vision defects in people taking valproic acid, however it is related to long term treatment and to GABAergic mechanism of valproic acid(Hosking and Hilton, 2002; Tilz et al., 2007). Since the water maze test is run six days after the valproic acid exposure, there is no longer an increase in GABA present, nor was the exposure chronic in these mice. No further evaluation of vision was conducted and so it cannot be ruled out entirely, however it is unlikely the reason for the deficits found in the valproic-acid treated mice. In addition to motor and visual neuron damage, impairments to find the visible platform have also been seen with lesions to the striatum (Packard and Teather, 1997). The deficits in visible platform water maze performance for the valproic acid-treated mice could be due to motor impairments. As discussed previously, valproic acid exposure on P14 results in damage to the cerebellum that may lead to deficits in motor behavior (Yochum et al., 2008; Wagner et al., 2006; Banji et al 2011; Cheh et al., 2010).

The hidden platform paradigm of the water maze was associated with the hippocampus. This tests the spatial learning and memory of the mice (Crawley, 1999). In

this paradigm the latency to find a platform below the water and not visible is evaluated for five trials a day for four days. In hidden platform paradigm of the water maze, there again was an effect of valproic acid treatment and an effect of genotype. The Nrf2 knockout mice treated with saline were significantly faster to find the platform compared to the saline-treated wild type mice, although, by the end of testing, the saline-treated wild type mice performed similarly to the saline-treated Nrf2 knockout mice. The valproic acid-treated mice of both genotypes performed significantly worse compared with their saline-treated counterparts. Interestingly, the Nrf2 knockout mice treated with valproic acid performed significantly worse than the wild type mice treated with valproic acid. In fact, the valproic acid-treated Nrf2 knockout mice were not successful in finding the platform even after 4 days of testing. Diminishing latency to find the platform indicates learning. Learning and memory are associated with hippocampus functioning and hippocampal lesions impair acquisition of this task in rats and mice (Morris et al., 1982; Logue et al., 1997). The poor performance of the valproic acid-treated animals may indicate damage to the hippocampus. This is consistent with previously reported work that indicates treatment with valproic acid results in increased apoptosis in the hippocampus and difficult in learning (Yochum et al., 2008; Banji et al., 2011; Wagner et al., 2006). In addition exposure to valproic acid on postnatal day 14 has also been shown to cause dendritic atrophy of memory processing hippocampal functions (Devi et al., 2003; Jarrard, 1986). The even longer latency to find the hidden platform indicates that the Nrf2 knockout mice treated with valproic acid may have more damage to their hippocampus compared to the wild type mice. The Nrf2 knockout mice have been shown to be more sensitive to oxidative stress inducing toxicants and valproic acid has been

shown to induce oxidative stress (Itoh et al., 1997; Ishii et al., 2005; Liu et al., 2010; Innamorato et al., 2010; Banji et al., 2011; Cheh et al., 2010).

These findings indicate that early postnatal treatment with valproic acid does result in deficits in behavior as seen in the mid –air righting test, the hanging wire grip strength test, the open field activity chambers, the rotorod, and the Morris water maze. In particular, the rotorod, a cerebellum dependent test, and the water maze, a hippocampus dependent test, have given consistent results with previous findings from valproic acid treatment. It has been shown that 12 and 24 hours after P14 treatment with valproic acid, there is increased apoptosis in the cerebellum and hippocampus of mice (Yochum, 2008). The results of the mid-air righting test in this experiment are also consistent with previous work. Postnatal exposure to valproic acid has been shown to result in regression of the previously acquired skills to perform mid-air righting (Wagner et al., 2006; Banji et al., 2011). In addition to replicating previously reported deficits in behavior for valproic acid-treated mice, this experiment provided some evidence that the Nrf2 knockout mice are more sensitive to the damaging effects of valproic acid. This was shown most profoundly in the rotorod test and in the hidden platform paradigm of the Morris water maze, although it can also be seen to a lesser degree in the open field activity chambers.

Figure 1

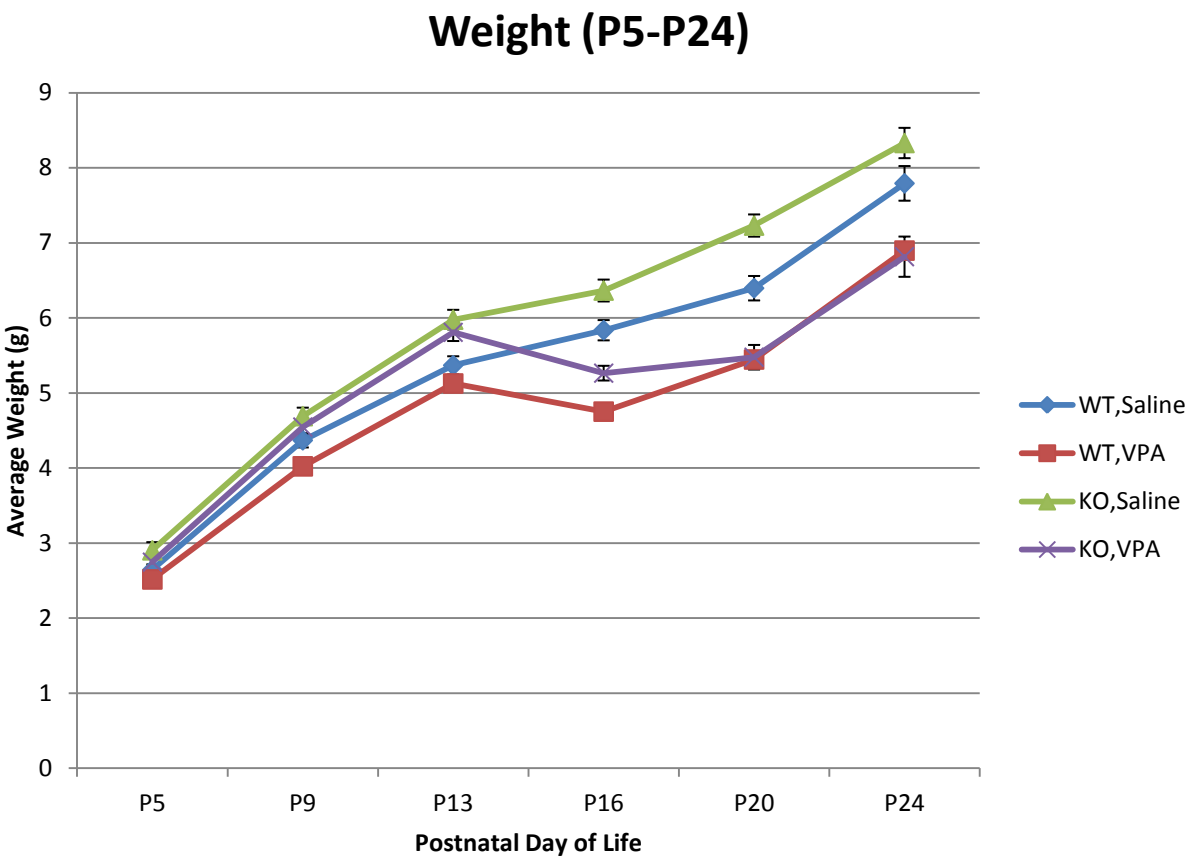

Figure 1. Total body weight change from P5-P24. The WT mice weighed significantly less than the KO mice ($p = 0.0001$). Prior to P14 treatment, there was not a significant difference in weight within genotype. Following P14 treatment, the VPA-treated mice weighed significantly less than the saline treated mice ($p < 0.0001$). WT = wild type mice. KO = Nrf2 knockout mice. VPA= valproic acid. (N = 114 total; 17 male WT saline-treated mice, 16 male WT VPA-treated mice, 11 WT female saline-treated mice, 19 female WT VPA-treated mice, 16 male KO saline-treated mice, 14 male KO VPA-treated mice, 10 female KO saline-treated mice, and 11 female KO VPA-treated mice.)

Figure 2

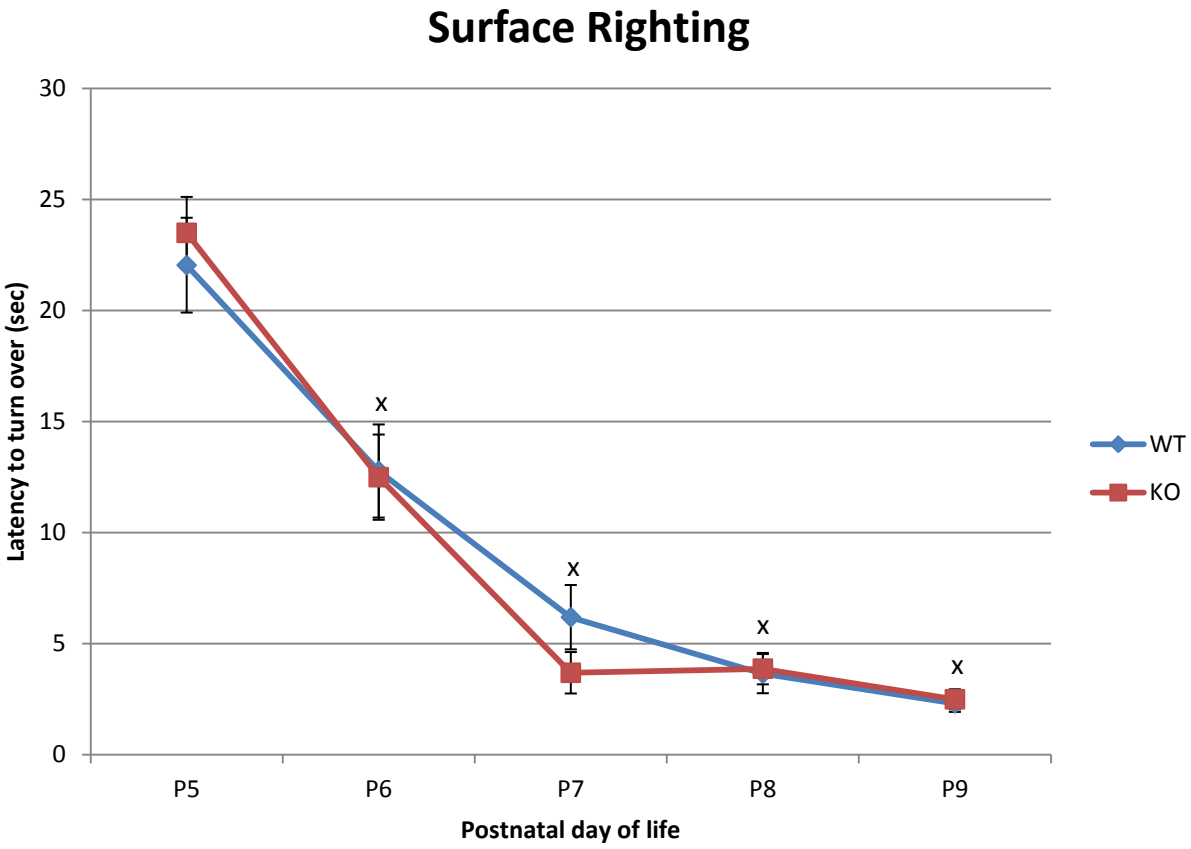

Figure 2. Surface righting. The latency to turn over was found to significantly improve from P5 through P7 for both genotypes ($p<0.0001$). x denotes $p < 0.05$ compared to P5. (N = 61 total; 15 WT males; 15 WTfemales; 15 Nrf2 KO males and 16 Nrf2 KO females)

Figure 3

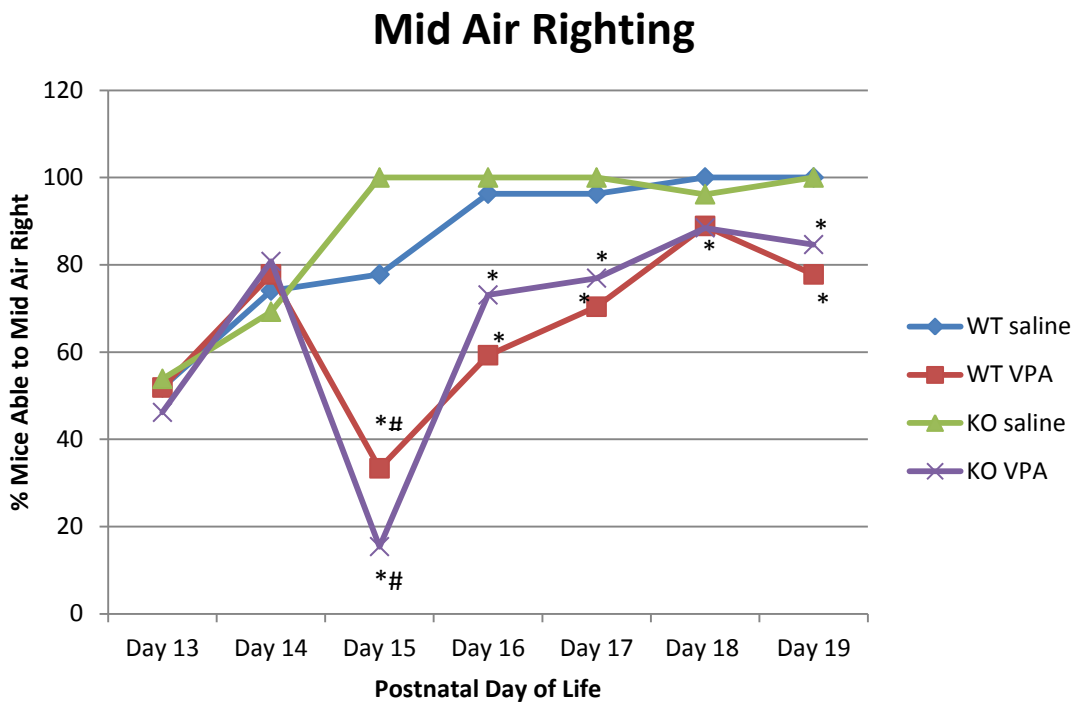

Figure 3. Percentage of mice able to successfully mid-air-right. There was no significant difference between saline-treated WT and saline-treated KO mice, nor was there a significant difference between VPA-treated WT and VPA-treated KO mice. The VPA-treated mice were found to be significantly impaired on P15 ($p=0.0032$). The WT mice treated with VPA were found to be significantly impaired compared to the saline-treated WT mice ($p = 0.009$) and the VPA-treated KO mice were also found to be significantly impaired compared to the saline-treated KO mice ($p < 0.0001$). * denotes $p < 0.05$ compared to within genotype saline-treated counterparts. #denotes $p < 0.05$ compared to other days of testing. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 106 total; 15 male WT saline-treated; 13 male WT VPA-treated; 12 female WT saline-treated; 14 WT VPA-treated; 16 male KO saline-treated; 14 male KO VPA-treated; 10 female KO saline-treated; and 12 female KO VPA-treated)

Figure 4

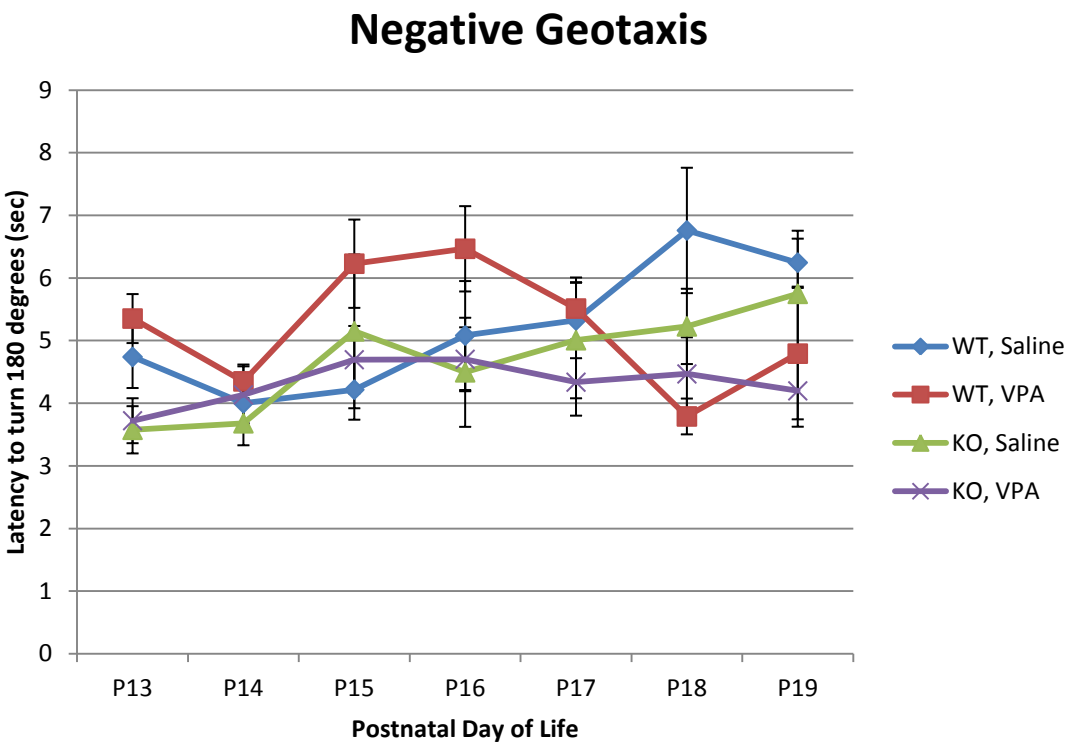

Figure 4. Latency to turn 180 degrees. An overall effect of genotype [F(1,102)=5.389, p=0.0223] was found as was an overall effect of day of testing [F(6,612)=2.195), p=0.0419] and a day by treatment interaction [F(6, 612)=2.982, p=0.0070]. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 106 total; 15 male WT saline-treated; 13 male WT VPA-treated; 12 female WT saline-treated; 14 WT VPA-treated; 16 male KO saline-treated; 14 male KO VPA-treated; 10 female KO saline-treated; and 12 female KO VPA-treated)

Figure 5

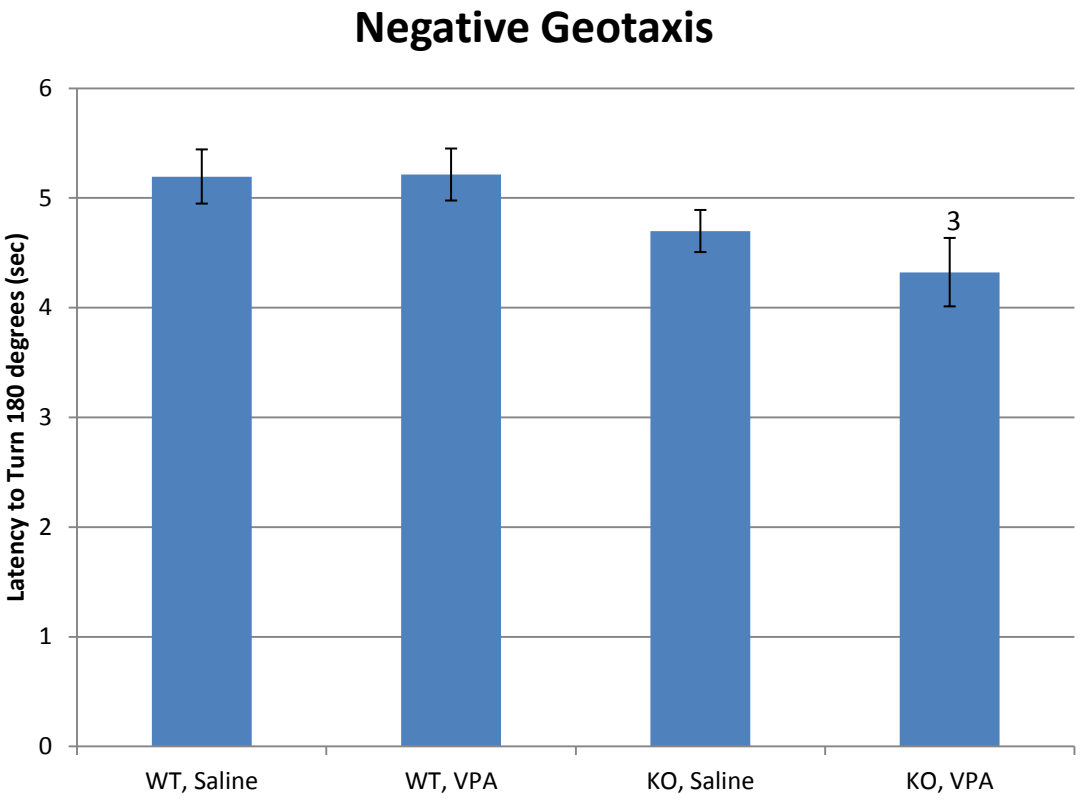

Figure 5. Average total latency to turn 180 degrees. VPA-treated WT mice were significantly slower to turn 180 degrees compared with the VPA-treated KO mice ($p = 0.0107$). 3 denotes $p < 0.05$ compared to WT,VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 106 total; 15 male WT saline-treated; 13 male WT VPA-treated; 12 female WT saline-treated; 14 WT VPA-treated; 16 male KO saline-treated; 14 male KO VPA-treated; 10 female KO saline-treated; and 12 female KO VPA-treated)

Figure 6

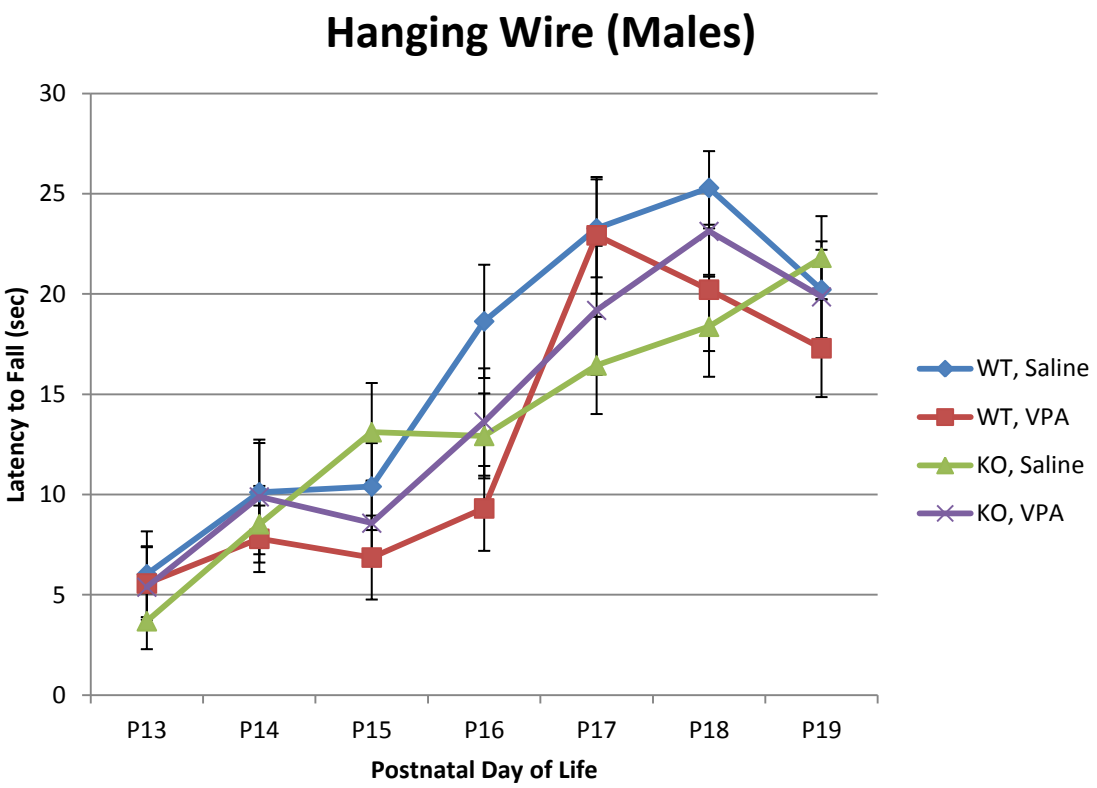

Figure 6. Male latency to fall from wire. The only significant effect found was a significant effect of day of testing [$F(6,324)=38.941$, $p<0.0001$] whereby the mice improved each day and were able to hang onto the wire longer each consecutive day. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 58 total; 15 male WT saline-treated; 13 male WT VPA-treated; 16 male KO saline-treated; 14 male KO VPA-treated)

Figure 7

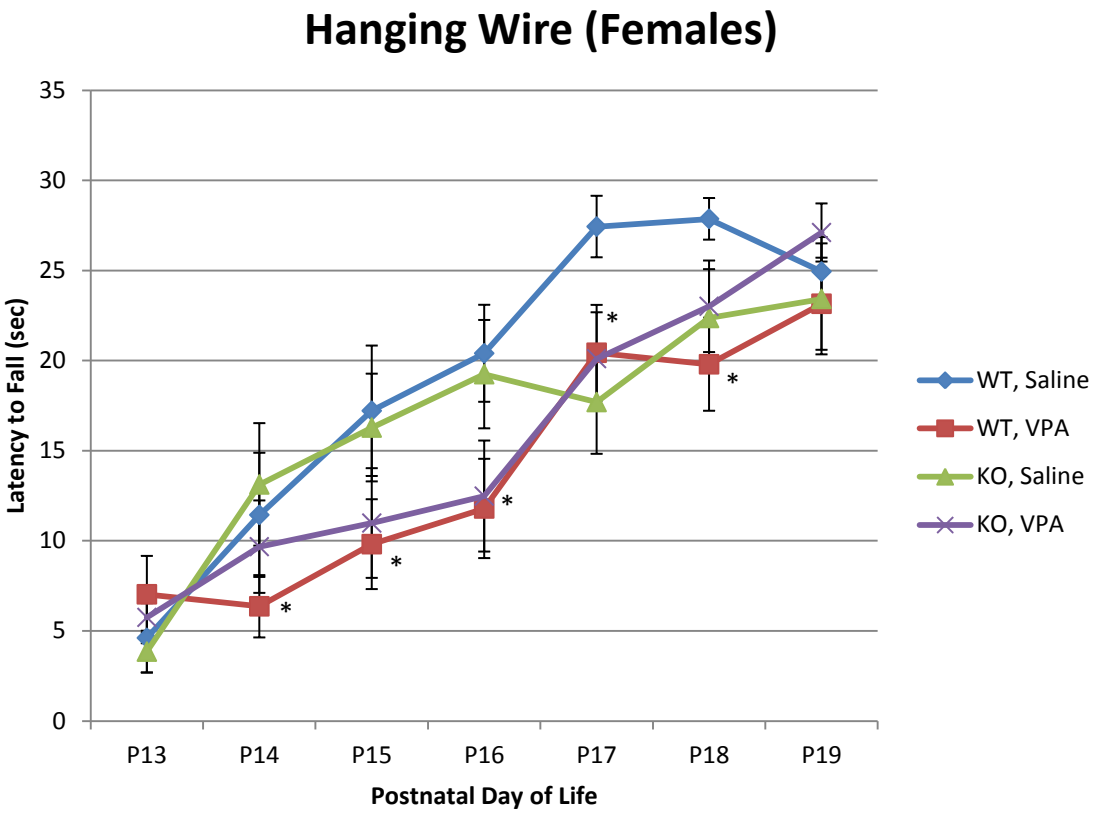

Figure 7. Female latency to fall from wire. VPA-treated mice performed significantly worse compared with the saline-treated mice (p=0.0395). * denotes p< 0.05 compared to saline-treated mice. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 48 total; 12 female WT saline-treated; 14 WT VPA-treated; 10 female KO saline-treated; and 12 female KO VPA-treated)

Figure 8

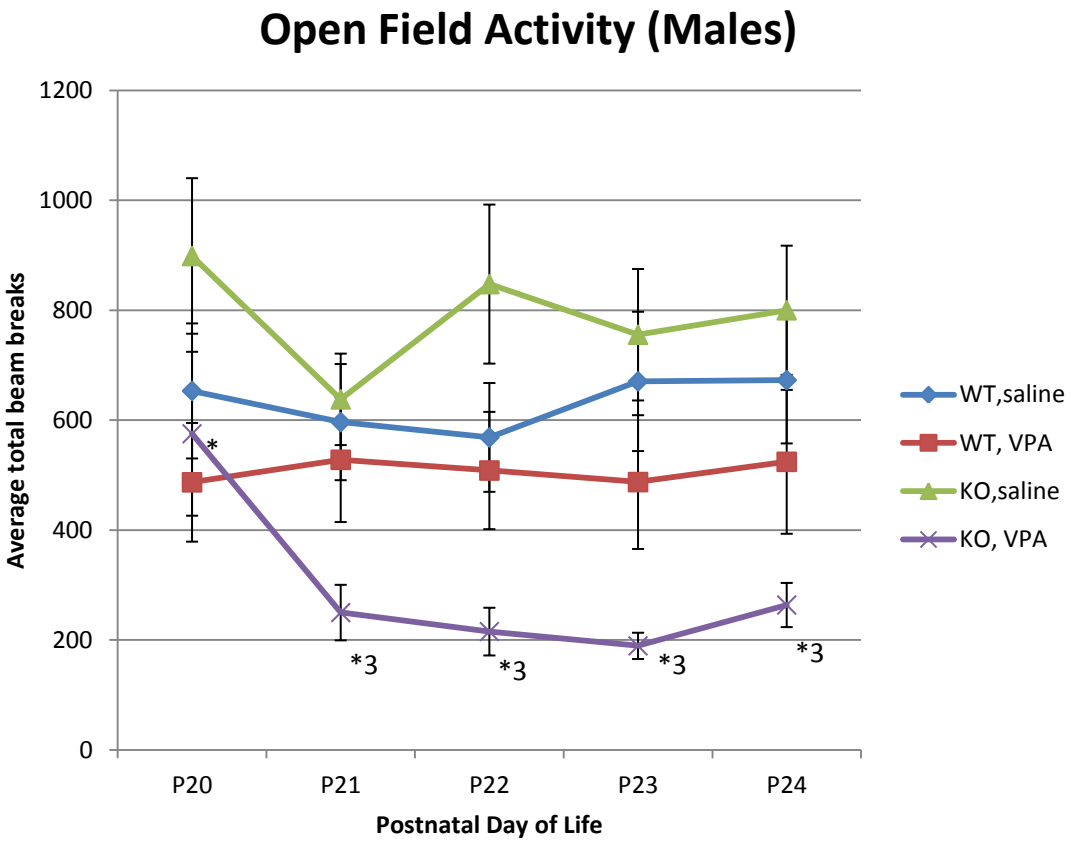

Figure 8. Male average total beam breaks. VPA-treated mice were less active compared to the saline-treated mice (p=0.0018). WT mice treated with VPA were not significantly different compared to the WT mice treated with saline (p=0.4203), while the KO mice treated with VPA were significantly less active compared to the KO mice treated with saline (p=0.0004). * denotes p<0.05 compared to within genotype saline-treated mice. 3 denotes < 0.05 compared to WT, VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 57 total; 15 male WT saline-treated; 12 male WT VPA-treated; 16 male KO saline-treated; 14 male KO VPA-treated)

Figure 9

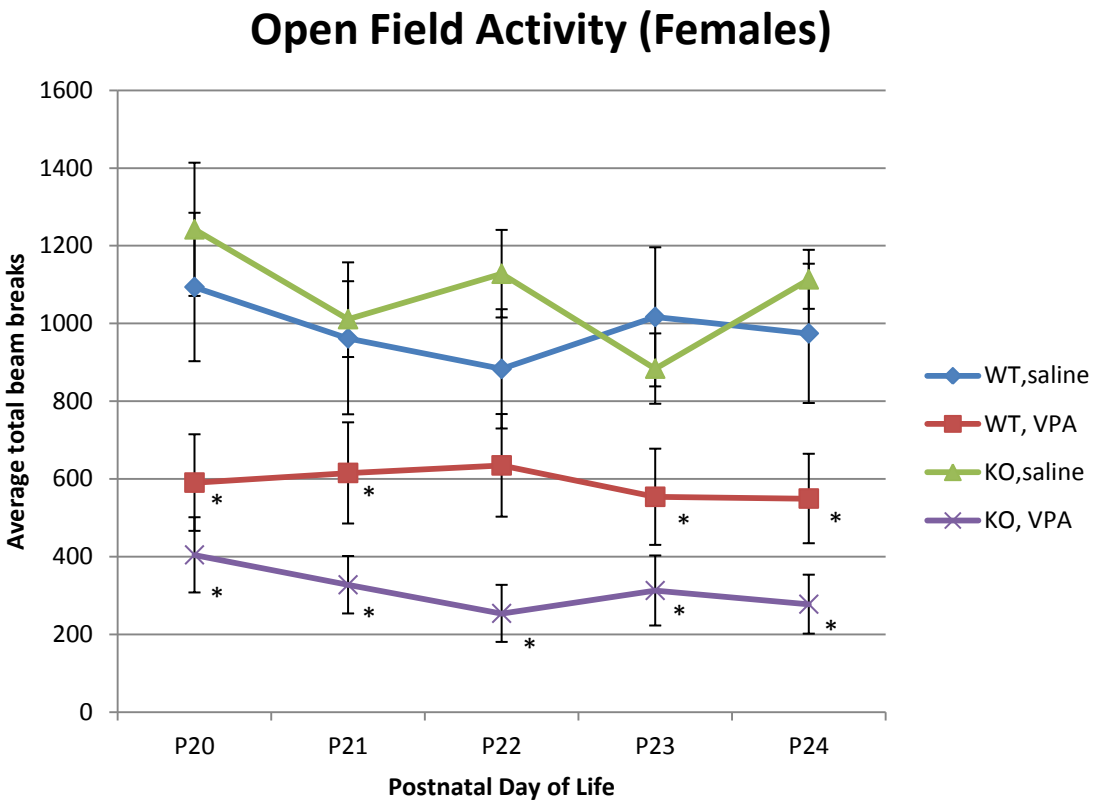

Figure 9. Female average total beam breaks. VPA-treated mice were significantly less active compared with the saline-treated mice ($p<0.0001$). * denotes $p<0.05$ compared to within genotype saline-treated mice. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 45 total; 11 female WT saline-treated; 14 female WT VPA-treated; 10 female KO saline-treated; and 10 female KO VPA-acid treated)

Figure 10

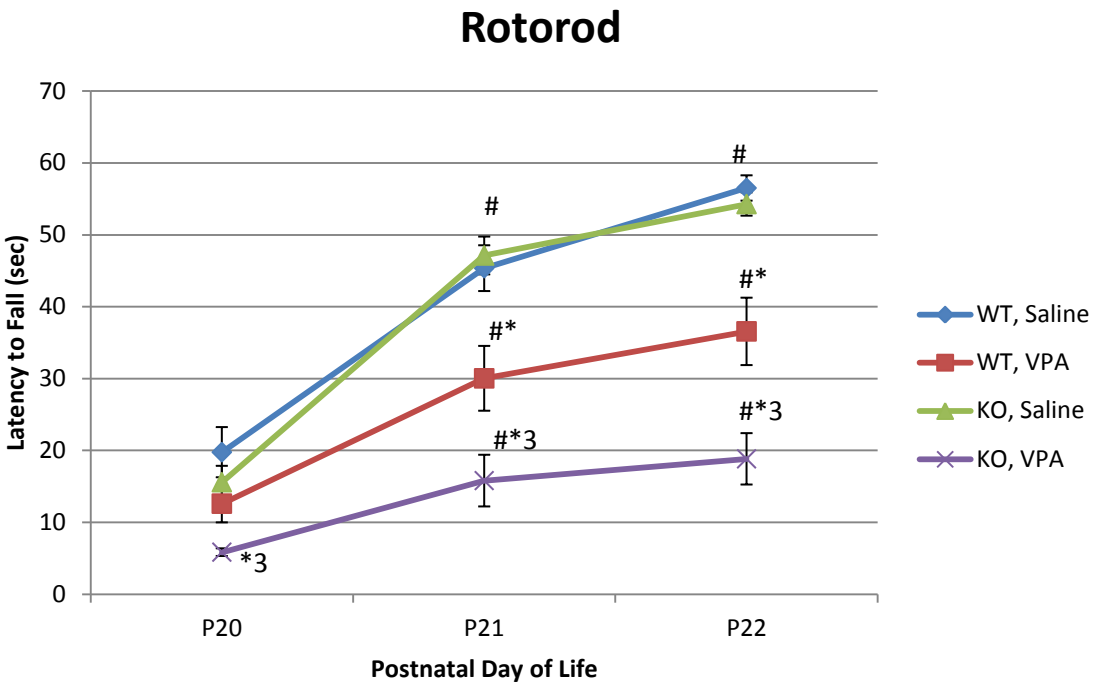

Figure 10. Average latency to fall from the rotorod. The WT mice performed significantly better than the KO mice ($p=0.0101$), the saline-treated mice performed significantly better than the VPA-treated mice ($p<0.0001$), and the latency to fall was significantly higher each day compared to the day before ($p<0.0001$). The saline-treated WT mice and the saline-treated KO mice were found to perform similarly, while the VPA-treated KO mice performed significantly worse than the VPA-treated WT mice ($p=0.0043$). # denotes $p<0.05$ compared to P20. * denotes $p<0.05$ compared to within genotype saline-treated counterparts. 3 denotes $p<0.05$ compared to WT, VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 102 total; 15 male WT saline-treated; 12 male WT VPA-treated; 11 female WT saline-treated; 14 female WT VPA-treated; 16 male KO saline-treated; 14 male KO VPA-treated; 10 female KO saline-treated; and 10 female KO VPA-acid treated)

Figure 11

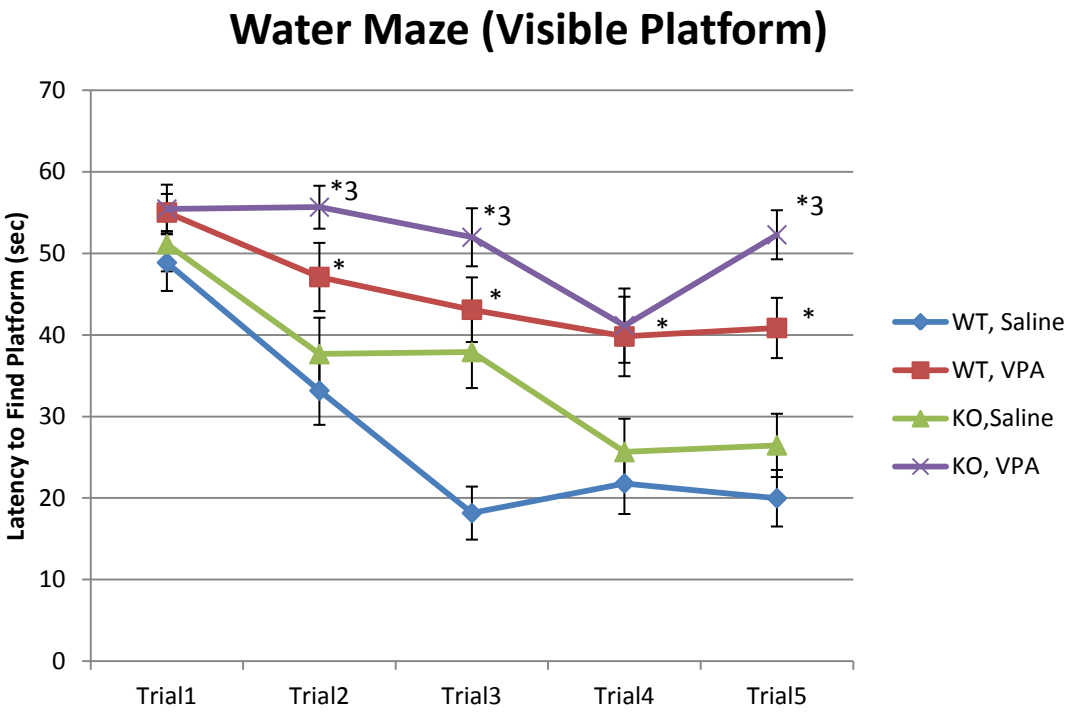

Figure 11. Latency to find the visible platform in the water maze. The WT mice performed significantly better than the KO mice overall (p=0.0063) and the saline-treated mice performed significantly better compared to the VPA-treated mice overall (p<0.0001). The saline-treated WT mice performed significantly better than the saline-treated KO mice (p=0.0353). Although the WT VPA-treated mice performed better than the KO VPA-treated mice, the difference only approached significance (p=0.0532). * denotes p<0.05 compared to within genotype saline-treated. 3 denotes p<0.05 compared to WT, VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 102 total; 15 male WT saline-treated; 12 male WT VPA-treated; 11 female WT saline-treated; 14 female WT VPA-treated; 16 male KO saline-treated; 14 male KO VPA-treated; 10 female KO saline-treated; and 10 female KO VPA-acid treated)

Figure 12

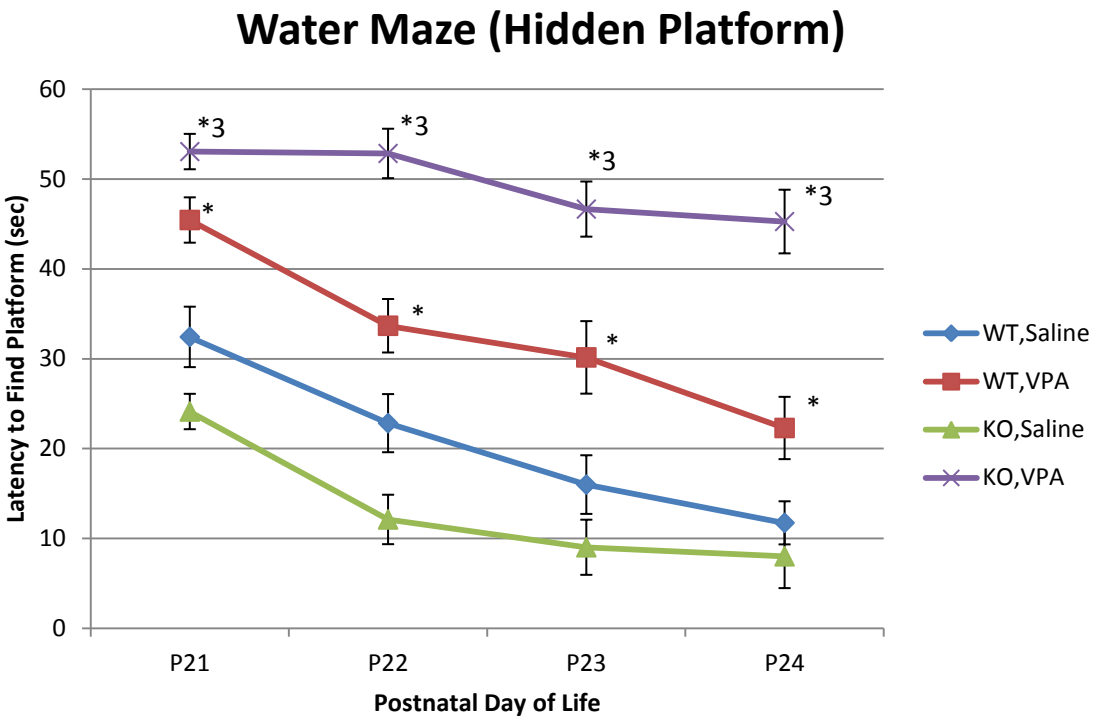

Figure 12. Latency to find the hidden platform in the water maze. VPA-treated mice performed significantly worse than saline-treated mice ($p<0.0001$). Saline-treated KO mice performed significantly better than saline-treated WT mice ($p=0.0151$), while VPA-treated KO mice performed significantly worse than VPA-treated WT mice ($p<0.0001$). The VPA-treated WT mice ($p=0.0033$) and VPA-treated KO mice ($p<0.0001$) performed significantly worse compared to their saline-treated counterparts. * denotes $p<0.05$ compared to within genotype saline-treated counterparts. 3 denotes $p<0.05$ compared to WT, VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 102 total; 15 male WT saline-treated; 12 male WT VPA-treated; 11 female WT saline-treated; 14 female WT VPA-treated; 16 male KO saline-treated; 14 male KO VPA-treated; 10 female KO saline-treated; and 10 female KO VPA-acid treated)

EXPERIMENT TWO

LONG TERM BEHAVIORAL EFFECTS AND ASSESSMENT OF DIFFERENTIAL SENSITIVITY TO AMPHETAMINE FOLLOWING EARLY POSTNATAL EXPOSURE TO VALPROIC ACID

Rationale:

While the behavioral and neurochemical effects of early postnatal and embryonic exposure to valproic acid have been assessed during development and as far as eleven weeks postnatally, mice exposed to valproic acid on postnatal day 14 have not been assessed behaviorally or neurochemically at time points over 90 days. At 11 weeks postnatally, the mice were only evaluated for self-injurious behavior following exposure to another toxicant (Wagner et al., 2006). It would be important to determine if the deficits in behavior that were found during development continued into adulthood to determine if those deficits would still be present, if a compensation may occur, or if the deficits worsen as the mice age. This would allow for further hypothesis testing on the progression of the lesions caused by early postnatal exposure to valproic acid. Mice treated with valproic acid on P14 have been assessed for sociability in the play behavior test at postnatal day 30 and were found to be less social compared with the saline-treated controls (Yochum et al., 2008). At 11 weeks (77 days), mice treated with valproic acid have been evaluated for differences in self-injurious behavior following amphetamine treatment, although no differences were found between valproic acid-pretreated mice and saline-pretreated mice (Wagner et al., 2006). Rats treated with valproic acid on embryonic day 12.5 have been tested behaviorally from postnatal day 90 through

postnatal day 150 (Schneider et al., 2008). These experiments revealed that the valproic acid-treated rats had a lower sensitivity to pain, increased repetitive/stereotypic-like activity, higher anxiety, and decreased level of social interaction (Schneider et al., 2008). Therefore, the following experiment evaluated the behavioral effects of early postnatal exposure to valproic acid in adult mice aged 90 days. The behavioral assays included the rotorod and water maze to determine if the deficits found in those skills earlier in development were long lasting. In addition to repeating those tests, other behaviors were assessed in this experiment. The elevated plus maze was used to evaluate anxiety, the social chambers were used to evaluate social approach, and the target biting chambers were used to evaluate defensive aggression.

In addition to investigating the long term behavioral effects of early postnatal valproic acid exposure, this experiment also investigated a potential differential sensitivity to amphetamine. It has been shown previously that pretreatment with one toxicant can cause a sensitization to a second toxicant later in life. Pretreatment with methylmercury, for instance, has been shown to increase serotonin concentrations after treatment with amphetamine. Pretreatment with methylmercury and then exposure to amphetamine has also been shown to alter the amphetamine-induced changes in DOPAC/DA ratio and HVA/DA turnover rates (Wagner et al., 2007). Exposure to the Gram (-) bacterial toxin, lipopolysaccharaide (LPS) at embryonic day 10.5 has also been shown to increase sensitivity to the dopaminergic toxicity caused by amphetamine. Animals exposed to LPS were found to have 33% fewer dopamine neurons compared to controls (Ling et al., 2004). Given that pretreatment with one toxicant may induce greater effects after exposure to another toxicant, this experiment investigated if pretreatment

with valproic acid causes an increased sensitivity to the dopaminergic toxicity caused by amphetamine. In addition, it also investigated if there were any neurochemical differences between the wild type and Nrf2 knockout mice.

Hypothesis: Early exposure to valproic acid will cause deficits in behaviors well into adulthood (over 90 days) and the Nrf2 knockout mice will continue to exhibit greater deficits. In addition, early postnatal exposure to valproic acid will make the mice more sensitive to the dopaminergic toxicity caused by amphetamine and the Nrf2 knockout mice may show enhanced effects.

Specific Aims:

- **Assess behavioral differences in adult C57BL/6 wild type mice and adult Nrf2 knockout mice**
- **Assess long term effects of early postnatal treatment with valproic acid on behaviors in adult C57BL/6 wild type mice and Nrf2 knockout mice**
- **Assess neurochemical differences between wild type mice and Nrf2 knockout mice in the striatum, hippocampus, and cerebellum using HPLC to determine concentrations of dopamine, serotonin and their metabolites DOPAC, 5-HIAA, and HVA**
- **Assess the potential increased sensitivity of mice pretreated with valproic acid to the neurochemical toxicity caused by amphetamine**

Methods:

Animals

The same mice from the early developmental experiment were used for this experiment. Once the mice completed the developmental tests and were weaned on postnatal day 25, they were group housed by litter and sex in shoebox cages with woodchip bedding and with free access to food and water in a temperature and humidity regulated room with a 12 hr light/dark cycle. The mice began adult behavioral testing after they were aged to 90 days. All behavioral testing was done in the morning approximately 2 hours following the beginning of the light cycle in the colony room. All weights were taken prior to behavioral testing.

A minimum of 10 mice per genotype per treatment group per sex were evaluated in the behavioral experiments. An N of 10 is sufficient for meaningful statistical interpretations of behavioral experiments (Crawley, 2000).

Rotorod

Each mouse was placed on the rotorod with a circumference of 6 inches, rotating at 12 revolutions per minute. The rotorod was 60 inches above a padded receptacle. The latency to fall from the rotorod was recorded for each mouse for three trials, with each trial lasting no more than 60 seconds. For this test 113 mice were evaluated: 17 male wild type saline-treated mice, 12 male wild type valproic acid-treated mice, 22 female wild type saline-treated mice, 13 female wild type valproic acid-treated mice, 16 male Nrf2 knockout saline-treated mice, 12 male Nrf2 knockout mice valproic-acid treated mice, 10

female Nrf2 knockout saline-treated mice, and 11 female Nrf2 knockout valproic acid-treated mice.

Morris Water Maze

The maze was a circular tub measuring 71 cm in diameter and 29 cm in height. The tub was painted white on the interior, filled ¾ full with water maintained at 23-26^{o}C and made opaque with white non-toxic latex paint. A starting point was determined randomly from one of four equally spaced quadrants. In the visible platform paradigm of the water maze, the water was only allowed to fill enough so that the platform sat 1.5 cm above the surface. Each mouse was given one 60 second trial to find the visible platform in each of 5 different positions in the water maze. In the hidden platform paradigm, a platform that was painted white and sat below the surface of the water was placed in one quadrant and the mouse was given 5 trials a day starting at different locations in the maze to find the hidden platform. The hidden platform paradigm was repeated for 4 days. A maximum of 60 seconds was given for each trial in both the hidden or visible platform versions. For the water maze 5 male saline-treated wild type mice, 5 male valproic acid-treated wild type mice, 5 male saline-treated knockout mice, and 5 male valproic acid-treated knockout mice were evaluated.

Elevated Plus Maze

The elevated plus maze was a plus shaped apparatus that stands 60 cm above the floor and has two long closed arms (65 cm long and 8 cm wide), two short open arms (30 cm long and 9 cm wide), and a central neutral 5 cm by 5 cm square. Each mouse was

placed in the center square and observed for 10 minutes. The number of times the animal crossed into a closed arm, open arm, or jumped off the maze was recorded as was the number of fecal boli. The length of time spent in the closed arms and open arms was also recorded. 90 mice were evaluated in the elevated plus maze: 15 male wild type saline-treated mice, 12 male wild type valproic-acid treated mice, 11 female wild type saline-treated mice, 13 females wild type valproic acid-treated mice, 16 male Nrf2 knockout saline-treated mice, 12 male Nrf2 knockout valproic acid-treated mice, 10 female knockout saline-treated mice, and 11 female knockout valproic acid treated mice.

Social Chambers

The social chamber was a 40 cm x 40 cm x 36.6 cm Plexiglas chamber with a stainless steel grid floor. Within the chamber were two cylinders, 11 cm in diameter and 13 cm tall, made of the same stainless steel grid as the floor, located in opposite corners of the chamber. An adult C57BL/6 mouse was placed in one of the cylinders, the target cylinder. The other cylinder was left empty, the control cylinder. The mouse that was being evaluated for social level was placed in the center of the chamber between the cylinders. The mouse that was being evaluated and the mouse in the target cylinder were matched for sex. Each time the subject placed one paw on a cylinder and had at least one paw on the ground, a contact was recorded automatically by a computer program attached to the chambers. The number of contacts with either the target cylinder, containing the C57BL/6 mouse, or the control cylinder, containing nothing, were recorded. The length of time spent touching either cylinder was also automatically recorded by the computer program. The mice were evaluated for thirty minutes a day for three days. For the social

chambers, 89 mice were evaluated: 13 male wild type saline-treated mice, 12 male wild type valproic acid-treated mice, 10 female wild type saline-treated mice, 13 female wild type valproic acid-treated mice, 11 male knockout saline-treated mice, 10 male knockout valproic acid-treated mice, 10 female knockout saline-treated mice, and 10 female knockout valproic acid-treated mice.

Target Biting

The mouse was placed in a plastic tube (2.8 cm diameter, 10 cm long) that had very small hole in one end and a latch door that only the tail could reach out of at the other end. Once the mouse was in the plastic tube with its tail free, the tail was taped to 2 brass bar electrodes. The tube was then placed in the chamber. Within the chamber was a cable tie mounted on an omnidirectional switch. The cable tie was just long enough to reach through the small hole in the end of the plastic tube, such that the mouse could reach it enough to tug on it if the mouse extended its body. Once this was set up, the computer program began. There were 10, two minute trials per day. Each two minute trial was divided into 8 bins that were 15 seconds each. During bin 8 the mouse had a 15 second buzzer and light conditioned stimulus. At the end of bin 8, the mouse received a 150 msec, 2 mA tail shock. The number of times the mouse bit the target cable tie was automatically recorded by the program. A total of 94 mice were evaluated in the target biting chambers: 15 male wild type saline-treated mice, 12 male wild type valproic acid-treated mice, 11 female wild type saline-treated mice, 13 female wild type valproic acid-treated mice, 11 male knockout saline-treated mice, 11 male knockout valproic acid-

treated mice, 10 female knockout saline-treated mice, and 11 female knockout valproic acid-treated mice.

Amphetamine Challenge

Forty male mice from the behavioral experiments were evaluated in an amphetamine challenge following the completion of their adult behavioral testing. The mice were 10 C57BL/6 wild type mice treated with saline on P14, 10 C57BL/6 wild type mice treated with valproic acid on P14, 10 Nrf2 knockout mice treated with saline on P14, and 10 Nrf2 knockout mice treated with valproic acid on P14. After the mice aged 90 days and completed the adult behavioral tests, 5 mice of each group were treated with saline and 5 mice of each group were treated with amphetamine. HPLC analysis after amphetamine challenge has been published using only 6 mice per group (Halladay et al., 2003).

Mice received four subcutaneous injections, each separated by two hours, of either 12.5 mg/kg amphetamine (50 mg/kg total dose) or saline. The amphetamine (obtained from Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% saline to a concentration of 1.25 mg/ml. The mice were then killed by rapid decapitation 72 hours following the first injection.

HPLC

Following the amphetamine challenge and sacrifice, brains were removed and bilateral striata, bilateral hippocampus, and whole cerebellum were dissected out, frozen in liquid nitrogen, and stored until homogenization in 0.3 ml of 0.4 N perchloric acid with

0.1 mM ethylenediaminetetraacetic acid (EDTA). Homogenized samples were centrifuged at 20,000 x g for 20 min at 4°C and the supernatant was frozen in liquid nitrogen until analyzed. Supernatant was assayed for dopamine, serotonin, and their metabolites using HPLC-electrochemical detection (Bioanalytical System, West Lafayette, Indiana). Samples were delivered through a high-pressure (Rheodyne) valve fitted with a 20 µl sample loop onto a Biophase ODS C-18 reverse-phase column (5 mm, 250 x 4.6 mm i.d.), and oxidized with a +.72 V potential between the glassy carbon electrode and the Ag/AgCl reference electrode. The mobile phase consisted of 0.1375 M sodium phosphate (dibasic), 0.0625 M citric acid, 5.0 mg EDTA, and 14% methanol with a flow rate of 0.7 ml/min. Quantification was measured against external standards injected between every six samples. Neurotransmitter and metabolite levels were reported in micrograms per gram of tissue.

Statistical Analysis

The behavioral tests, weights, and neurotransmitter concentrations were analyzed using an ANOVA or repeated measures ANOVA, where appropriate. When significant main effects were found, differences between groups were determined using Fisher's PLSD post-hoc tests.

Results:

Weight (5 weeks of age – 20 weeks of age)

Separate analysis was done for body weight of adult males and females. Less females were available for weight analysis than males because only non-breeding females were included in the analysis. A repeated measures ANOVA was used to analyze adult male weights and a repeated measures ANOVA was used to analyze adult female weights. The mice were weighed weekly in the morning prior to behavioral testing.

Males

A repeated measures ANOVA revealed an overall significant effect of genotype on weight [$F(1,64)=7.947$, $p=0.0064$], as well as an overall significant effect of week [$F(15,960)=1053.599$, $p<0.0001$], a significant interaction of week by genotype [$F(15,960)=7.106$, $p<0.0001$], and a significant interaction of week by genotype by treatment [$F(15,960)=10.622$, $p<0.0001$]. A total of 68 male mice were weighed and analyzed: 27 wild type saline-treated mice, 16 wild type valproic acid-treated mice, 15 knockout saline-treated mice, and 10 knockout valproic acid-treated mice.

Post hoc tests revealed that the Nrf2 knockout mice weighed significantly more overall compared with the wild type mice ($p=0.0085$) and that there was no overall significant effect of valproic acid treatment on adult weight ($p=.4121$). The average total weight for the knockout mice was 24.112 g while the average total weight for the wild type mice was 22.95 g. The average total weight for the saline-treated mice was 23.516 g, while the average total weight for the valproic acid-treated mice was 23.168 g. The

weights of each week were found to be significantly different compared to every other week ($p<0.0001$), except week 14 and 15 ($p=0.0655$) and week 17 and 18 ($p=0.3132$). The average weight of the mice on week 5 was 14.428 g, and that increased each week to the average weight of the mice on week 20 was 27.137 g. The valproic acid-treated knockout mice (13.180 g) weighed significantly less than the saline-treated knockout mice (15.700 g) at week 5 ($p=0.0385$), however, after that point, there was no significant differences between valproic acid-treated and saline-treated knockout mice. The valproic acid-treated wild type mice weighed significantly less than the saline-treated wild type mice at week 5 ($p=0.0241$) (saline-treated weighed 15.102 g; valporic acid-treated weighed 12.878 g). The valproic acid-treated mice (16.403 g) weighed significantly less than the saline-treated wild type mice (18.374 g) on week 6 ($p=0.0112$), however, after that point, there was no significant differences between valproic acid-treated wild type mice and saline-treated wild type mice. The saline-treated wild type mice and saline-treated knockout mice had no significant weight differences until week 14. At week 14, the saline-treated knockout mice weighed significantly more than the saline-treated wild type mice ($p=0.0406$). The saline-treated knockout mice continued to weigh significantly more compared with the saline-treated wild type mice at week 15 ($p=0.0136$), week 16 ($p=0.0072$), week 17 ($p=0.0009$), week 18 ($p=0.0146$), week 19 ($p=0.0073$), and week 20 ($p=0.0298$). The valproic acid-treated wild type and valproic acid-treated knockout mice did not have any differences in body weight until week 11. At week 11, the valproic acid-treated Nrf2 knockout mice weighed significantly more than the valproic acid-treated wild type mice ($p=0.0389$). The valproic acid-treated knockout mice continued to weigh significantly more than the valproic acid-treated wild type mice at week 12

(p=0.0104), week 13 (p=0.0025), week 14 (p=0.0033), week 15 (p=0.0003), week 16 (p=0.0005), week 17 (p<0.0001), week 18 (p=0.0023), week 19 (p=0.0006), and week 20 (p=0.0094). [Figure 13]

Females

The female weights were analyzed from week 5 of life through week 14 to exclude any skewed weights from breeding. Only mice that were not used in breeding were included in this analysis. In order to include more mice, the weights were only analyzed out to week 14 for the females rather than week 20 as in the male weight analysis. A repeated measures ANOVA was run to analyze these weights. A significant effect of genotype was found [F(1,52)=8.689, p=0.0048], as was a significant effect of week [F(9, 468)=555.222, p<0.0001], a significant interaction of week by genotype [F(9,468)=3.779, p=0.0001], and a significant interaction of week by genotype by treatment [F(9,460)=9.727, p<0.0001]. A total of 56 female mice were analyzed for body weight: 17 wild type saline-treated mice, 18 wild type valproic acid-treated mice, 10 knockout saline-treated mice, and 11 knockout valproic acid-treated mice.

Post hoc tests revealed that the saline-treated mice and valproic acid-treated mice had similar body weights (p=0.8975). *Post hoc* tests also revealed that the knockout mice weighed significantly more overall compared to the wild type mice (p=0.0041), where the overall average weight of the knockout mice was 18.794 g and the average total weight of the wild type mice was 17.472 g. The saline-treated knockout mice weighed significantly more than the saline-treated wild type mice at week 5 (p=.0415). On week 5, the saline-treated knockout mice weighed an average of 14.65 g, while the saline-treated wild type

mice weighed an average of 13.065 g. After week 5, the saline-treated mice of both genotypes were not found to differences in body weight. The valproic acid-treated knockout mice weighed significantly more compared to the valproic acid-treated wild type mice at week 6 ($p=0.0280$), and then again at week 10 ($p=0.0262$), week 11 ($p=0.0010$), week 12 ($p<0.0001$), week 13 ($p<0.0001$), and week 14 ($p<0.0001$). The valproic acid-treated knockout mice weighed an average of 16.005g at week6, 20.255 g at week 10, 21.077g at week 11, 21.514g at week 12, 21.682 g at week 13, an 21.982g at week 14. The valproic acid- treated wild type mice weighed an average of 14.411 g at week 6, 18.592 g at week 10, 18.992 g at week 11, 18.944 g at week 12, 19.444g at week 13, and 19.750 g at week 14.

Post hoc test further showed that there was a significant difference between weights of most weeks, except between the weights of week 11 and week 12. [Figure 14]

Rotorod

A repeated measures ANOVA was used to analyze the average latency to fall from the rotorod for adult mice. No significant effect of genotype or treatment was found, however there was a significant interaction of genotype by treatment [$F(1,109)=4.076$, $p=0.0460$]. A significant effect of day of testing [$F(2,218)=38.823$, $p<0.0001$] and a significant interaction of day by genotype by treatment was found [$F(2,218)=3.448$, $p=0.0336$]. *Post hoc* tests revealed that performance on day 1 of testing was significantly different compared to day 2 ($p<0.0001$) and day 3 ($p<0.0001$), and that performance on day 2 was significantly different compared to performance on day 3 ($p=0.0034$). The average latency to fall from the rotorod on day 1 was 35.895 seconds, on

day 2 it was 46.65 seconds, and on day 3 the average latency to fall was 52.330 seconds. The Nrf2 knockout mice treated with valproic acid were found to perform better than the wild type mice treated with valproic acid ($p=0.0137$), while Nrf2 knockout mice treated with saline did not perform differently than wild type mice treated with saline. The saline-treated wild type mice had an average latency to fall from the rotorod of 45.067 seconds, while the Nrf2 knockout mice treated with saline had an average latency to fall of 44.647 seconds. The wild type mice treated with valproic acid had an average latency to fall of 40.368 seconds, while the knockout mice treated with valproic acid had an average latency to fall of 50.114 seconds. Within each genotype, the valproic acid-treated mice and the saline-treated mice did not perform differently. [Figure 15]

Morris Water Maze

Visible Platform

A repeated measures ANOVA was run to analyze the latency to find the visible platform in the Morris water maze. No significant effect of genotype and no interaction between genotype and treatment were found. A significant effect of treatment was found [$F(1,16)=26.397$, $p<0.0001$] as was a significant effect of trial [$F(4,64)=9.061$, $p<0.0001$]. *Post hoc* tests revealed that the valproic acid-treated wild type mice performed significantly worse compared to the saline-treated wild type mice ($p=0.0217$) and that the valproic acid-treated Nrf2 knockout mice performed significantly worse compared to their saline-treated counterparts ($p=0.0012$). The saline-treated wild type mice had an average latency to reach the platform of 9.208 seconds, while the valproic acid –treated wild type mice had an average latency to reach the platform of 18.394

seconds. The saline-treated knockout mice had an average latency to reach the platform of 7.881 seconds, while the valproic acid-treated knockout mice had an average latency to find the platform of 18.921 seconds. The saline-treated wild type mice and Nrf2 knockout mice performed similarly, as did the valproic acid-treated mice of both genotypes. The average total latency to reach the platform was 27.966 seconds on trial 1, 19.043 seconds in trial 2, 7.342 seconds in trial 3, 5.939 seconds in trial 4, and 7.716 seconds in trial 5.Latency to find the platform in trial 3, 4, and 5 was found to be significantly shorter compared to trial 1 ($p<0.0001$). Latency to find the platform was found to be significantly shorter in trial 3 ($p=0.0117$), trial 4 ($p=0.0050$), and trial 5 ($p=0.0145$) compared to performance in trial 2. In Trial 2, the valproic acid-treated mice (latency = 28.942 seconds) performed significantly worse compared to their saline-treated (latency = 9.144 seconds) counter parts ($p=0.0272$). In trial 5, the valproic acid-treated mice (latency = 10.249 seconds) performed significantly worse compared to the saline-treated (latency =5.183 seconds) mice ($p=0.0167$) and the Nrf2 knockout mice (latency = 9.802 seconds) performed significantly worse than the wild type mice (latency = 5.630 seconds) ($p=0.0429$). [Figure 16]

Hidden Platform

A repeated measures ANOVA was used to analyze the latency to find the hidden platform in the Morris water maze. It should be noted that all of the latency values were fast enough to consider that the mice already learned to find the platform. An overall effect of genotype [$F(1, 16)=17.037$, $p=0.0008$], an overall effect of treatment

[F(1,16)=6.075, p=0.0254], and an overall effect of day of testing [F(3, 48)=7.639, p=0.0003] were found to be significant. *Post hoc* tests revealed that the saline-treated Nrf2 knockout mice were significantly faster than the saline-treated wild type mice (p=0.0020) as were the valproic acid-treated knockout mice compared to the valproic acid-treated wild type mice (p=0.0499). The saline-treated knockout mice had an average latency to find the hidden platform of 6.029 seconds, while the saline-treated wild type mice had an average latency of 10.07 seconds. The valproic acid-treated wild type mice had an average latency to find the platform of 12.448 seconds, while the valproic acid-treated knockout mice had an average latency of 8.459 seconds. On day 2 (p=0.0070) and day 3 (0.0221), the knockout mice performed better than the wild type mice overall. On day 2 the knockout average latency was 5.281 seconds, while the wild type was 9.386 seconds. On day 3, the knockout average latency was 6.925 seconds, while the wild type average latency was 14.326 seconds. Latency to find the platform on day 2 (7.333 seconds) (p=0.0008) and day 4 (6.303 seconds) (p=0.0001) was found to be significantly shorter compared to day 1 (12.751 seconds). Latency to find the platform on day 3 (10.625 seconds) (p= 0.0354) was found to be significantly longer compared to day 2 and the latency to find the platform on day 4 was found to be significantly shorter compared to day 3 (p=0.0066). [Figure 17]

Elevated Plus Maze

A one way ANOVA was used to analyze the average number of crosses into a closed or open arm of the elevated plus maze. There was an overall significant effect of genotype [F(1,184)=15.567, p=0.0001], an overall significant effect of sex

[F(1,184)=10.505, p=0.0014], an overall significant effect of type of cross [F(1,184)=476.669m p<0.0001], and an overall significant interaction of genotype by type of cross [F(1,184)=21.117, p<0.0001). No significant effect of valproic acid treatment was found.

Post hoc tests revealed the Nrf2 knockout mice made significantly more crosses in total compared to the wild type mice (p<0.0001). The Nrf2 knockout mice made an average of 11.9 crosses, while the wild type mice made an average of 10.03 crosses. The male mice made significantly more crosses in total compared to the female mice (p=0.0011). The males made an average of 11.658 crosses, while the females made an average of 10.126 crosses. Significantly more crosses were made into the closed arms compared to the open arms (p<0.0001). An average of 16.017 crosses were made into the closed arms, while an average of 5.92 crosses were made into the open arm. [Figure 18]

A one-way ANOVA was used to analyze the average time spent in each arm of the elevated plus maze. No effect of genotype, treatment or sex was found. There was an overall significant effect of the arm of the maze [F(1,184)=11561.044, p<0.0001]. The mice spent overwhelmingly more time in the closed arm (an average of 509.7 seconds) of the maze compared to the open arm of the maze (an average of 56.85 seconds). [Figure 19]

The number of fecal boli was analyzed using a one-way ANOVA. No significant differences were found between genotype, sex, or treatment groups.

The number of times mice jumped off the maze was also analyzed using a one-way ANOVA. There were no significant differences between genotype, sex or treatment groups.

Social Chambers

A one way ANOVA was used to analyze the average total number of touches made to either cylinder in the social chamber over the 30 minutes of testing. A significant effect of genotype [$F(1,164)=28.960$, $p<0.0001$] and a significant effect of target verses control cylinder [$F(1,164)=22.365$, $p<0.0001$] was found, as was a significant interaction of genotype by type of cylinder [$F(1,164)=6.285$, $p=0.0131$]. The Nrf2 knockout mice made an average of 226.207 touches compared to the wild type mice that made an average of 152.637 touches to both cylinders. The Nrf2 knockout mice touched both cylinders significantly more times than the wild type mice ($p<0.0001$). Overall, the mice made more contact with the target cylinders (an average of 219.860) compared to the control cylinders (an average of 155.562) ($p<0.0001$). *Post hoc* tests revealed that the saline-treated knockout mice ($p=0.0156$) touched the target cylinder significantly more times compared to the saline-treated wild type mice, but the knockout mice also touched the control cylinder significantly more than the wild type mice ($p=0.0004$). The saline-treated knockout mice touched the target cylinder an average of 273.175 times and made an average of 183.1 contacts with the control cylinder, while the saline-treated wild type mice touched the target cylinder an average of 209.833 times and made an average of 103.217 contacts with the control cylinder. The valproic acid-treated knockout mice and the valproic acid-treated wild type mice did not have a significant difference in the number of times they touched the target cylinder, however the valproic acid-treated knockout mice ($p=0.0012$) touched the control cylinder (223.783 average contacts)

significantly more times than the valproic acid-treated wild type mice (119.720 average contacts). [Figure 20]

An ANOVA used to analyze the percent of touches to the target cylinder indicated that there was a significant effect of genotype [$F(1,82)=5.870$, $p=0.0176$] and treatment [$F(1,82)=7.792$, $p=0.0065$]. 60% of the contacts that the saline-treated wild type mice made with a cylinder were to the target cylinder, while 66% of the contacts made to a cylinder by a valproic acid-treated wild type mice were to the target cylinder. 61% of the saline-treated knockout mice's contacts were with the target cylinder, while 51% of the total contacts made to a cylinder were made to the target cylinder by knockout mice treated with valproic acid. The Nrf2 knockout mice's percentage of touches made to the target chamber was significantly lower than the wild type mice ($p=0.0260$) and the valproic acid-treated mice had a significantly lower percentage of touches made to the target cylinder compared to the saline-treated mice ($p=0.0108$). [Figure 21]

A one way ANOVA was run to analyze the total amount of time the mice spent touching each cylinder over the 30 minutes of testing. No effect of genotype or treatment was found, however an effect of type of cylinder was found [$F(1,164)=38.514$, $p<0.0001$]. All of the mice spent significantly more time touching the target cylinder compared to the empty cylinder. The saline-treated wild type mice spent an average of 61.794 seconds touching the target cylinder and an average of 22.298 seconds touching the control cylinder. The valproic acid-treated wild type mice spent an average of 42.558 seconds touching the target cylinder and an average of 24.027 seconds touching the control cylinder. The saline-treated knockout mice spent an average of 54.148 seconds in contact with the target cylinder and an average of 31.617 seconds in contact with the

control cylinder. The valproic acid-treated knockout mice spent an average of 60.539 seconds in contact with the target cylinder and an average of 30.310 seconds in contact with the control cylinder. *Post hoc* tests revealed that the saline-treated knockout mice spent significantly more time in contact with the control cylinder compared to the saline-treated wild type mice ($p=0.0221$) and that the valproic acid-treated knockout mice also spent significantly more time in contact with the control cylinder compared to the valproic acid-treated wild type mice ($p=0.0288$). [Figure 22]

A one way ANOVA was run to analyze the percentage of time the mice spent touching the target cylinder over the time spent touching either cylinder in the 30 minutes trial averaged over 3 days of testing. No significant effect of genotype and no interaction of genotype by treatment were found. However the effect of treatment approached significance [$F(1,82)=3.850$, $p=0.0531$], whereby the saline-treated mice spent more percentage of time touching the target cylinder compared to the valproic acid-treated mice. The saline-treated mice spent an average 66.086% of their time in contact with a cylinder in contact with the target cylinder, while the valproic acid-treated mice spent an average of 60.857% of the time in contact with a cylinder with the target cylinder. [Figure 23]

Target Biting

A repeated measures ANOVA was used to analyze the average number of target bites over 3 bins. An overall significant effect of genotype [$F(1,90)=8.466$, $p=0.0046$], an overall significant effect of bin [$F(2,180)=63.343$, $p<0.0001$], and an overall interaction of bin by genotype [$F(2,180)=9.727$, $p<0.0001$] was found. There was no significant

effect of valproic acid treatment on target biting. *Post hoc* tests revealed that the wild type mice bit the target significantly more times compared to the Nrf2 knockout mice ($p = 0.0043$). Overall the Nrf2 knockout mice made an average of 2.074 bites, while the wild type mice made an overall average of 4.209 bites. These two averages include the very low number of bites in bin 2 and bin 3, bringing the averages down to 2 and 4 bites. All of the mice bit the target significantly more times in bin 1 compared with bin 2 ($p<0.0001$) and compared with bin 3 ($p<0.0001$). There was an overall average of 8.170 bites in bin 1, 1.005 bites in bin 2, and .521 bites in bin 3. In bin 1 the wild type mice treated with saline bit the target an average of 13 times, the wild type mice treated with valproic acid made an average of 8.64 bites, the knockout mice treated with saline made an average of 4.857 bites and the knockout mice treated with valproic acid made an average of 5.091 bites. The wild type mice treated with saline bit the target significantly more times in bin 1 compared with the knockout mice treated with saline ($p=.0274$), while the wild type mice treated with valproic acid did not bite the target any more or less than the knockout mice treated with valproic acid ($p=.1282$). [Figure 24]

HPLC

Striatum

A factorial ANOVA was used to analyze the neurotransmitter and metabolite levels for dopamine and serotonin and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindolacetic acid (5HIAA). For dopamine, a significant effect of adult amphetamine challenge [$F(1,31)=23.237$, $p<0.0001$] was found, while no significant effect of genotype or P14 treatment was

found. *Post hoc* tests revealed that the mice treated with amphetamine ($p<0.0001$) had significantly lower levels of dopamine compared with their saline-treated counterparts. The saline-treated mice had an average dopamine concentration of 8.256 µg/g tissue, while the amphetamine-treated mice had an average dopamine concentration of 4.082 µg/g tissue. In addition, this effect was true of wild type amphetamine-treated mice (3.662 µg/g tissue) compared to wild type saline-treated mice (7.561 µg/g tissue) ($p=0.0065$) and Nrf2 knockout mice treated with amphetamine (4.502 µg/g tissue) compared to Nrf2 knockout mice treated with saline(9.028 µg/g tissue) ($p=0.0021$). All of the mice treated with amphetamine had approximately 50% decrease in dopamine compared with their saline-treated counterparts. [Figure 25]

For 3,4-dihydroxyphenylacetic acid, a significant effect of adult amphetamine challenge [$F(1,31)= 7.924$, $p=0.0084$] was found, while no overall significant effect of genotype or P14 treatment was found. *Post hoc* tests revealed that the wild type mice treated with amphetamine ($p=0.3652$) did not show a significant decrease in DOPAC compared to the wild type mice treated with saline. Only the Nrf2 knockout mice treated with amphetamine ($p=0.0060$) had significantly lower DOPAC concentrations compared with Nrf2 knockout mice treated with saline. The Nrf2 knockout mice treated with amphetamine had an average concentration of 0.677 µg/g tissue of DOPAC, while the Nrf2 knockout mice treated with saline had an average concentration of 1.158 µg/g tissue. Further, it was found that the Nrf2 knockout mice treated with valproic acid on P14 and amphetamine in adulthood (0.554 µg/g tissue) ($p=0.0191$) had significantly lower concentrations of DOPAC compared to Nrf2 knockout mice treated with valproic acid on P14 and saline in adulthood (1.219 µg/g tissue) , while the Nrf2 knockout mice

treated with saline on P14 and amphetamine in adulthood (p=0.1878) did not show significantly different concentrations of 3,4-dihydroxyphenylacetic acid compared to Nrf2 knockout mice treated with saline on P14 and saline in adulthood. [Figure 26]

The factorial ANOVA used to analyze HVA revealed that there was an overall effect of adult amphetamine challenge [F(1,31)= 16.059, p=0.0004] and an overall interaction of adult amphetamine challenge and genotype [F(1,31)=6.328, p=0.0173]. *Post hoc* tests revealed that Nrf2 knockout mice treated with amphetamine (0.775 µg/g tissue) had significantly lower concentrations of HVA compared with Nrf2 knockout mice treated with saline (1.589 µg/g tissue) (p=0.0003), while there was no significant difference between wild type mice treated with amphetamine and wild type mice treated with saline (p=0.2976). [Figure 27]

For 5-hydroxytryptamine (5-HT or serotonin), an overall significant effect of amphetamine challenge [F(1,31)=7.519, p=0.0100] was found. *Post hoc* tests revealed that while there was no significant difference between wild type mice treated with amphetamine and wild type mice treated with saline (p=0.4719), the Nrf2 knockout mice treated with amphetamine (0.466 µg/g tissue)had significantly lower levels of 5-HT compared with saline-treated Nrf2 knockout mice(0.855 µg/g tissue) (p=0.0106). It was also found that the Nrf2 knockout mice treated with valproic acid on P14 and amphetamine in adulthood had significantly less 5-HT (0.479 µg/g tissue) compared with Nrf2 knockout mice treated with valproic acid on P14 and saline in adulthood (1.034 µg/g tissue) (p= 0.0438), while the Nrf2 knockout mice treated with saline on P14 did not show significant differences in concentration of 5-HT (p=0.1471). [Figure 28]

For 5-hydroxyindolacetic acid, a significant interaction of genotype by amphetamine challenge was found [F(1,31)=6.556, p=0.0155]. *Post hoc* tests revealed that while there was no significant difference in 5-HIAA concentration between the wild type mice treated with amphetamine and the wild type mice treated with saline (p=0.4646), the Nrf2 knockout mice treated with amphetamine (0.286 µg/g tissue)had a significantly lower concentration of 5-HIAA compared with the Nrf2 knockout mice treated with saline(0.471 µg/g tissue)(p=0.0128). In addition, there was a significant difference in 5-HIAA concentration between Nrf2 knockout mice treated with valproic acid on P14 and amphetamine in adulthood (0.260 µg/g tissue) compared with the Nrf2 knockout mice treated with valproic acid on P14 and saline in adulthood (0.560 µg/g tissue) (p=0.0215), while no difference in 5-HIAA concentration was found between Nrf2 knockout mice treated with saline on P14 and amphetamine in adulthood and Nrf2 knockout mice treated with saline on P14 and saline in adulthood (p=.5411). [Figure 28]

Hippocampus

The 5-hydroxytryptamine and 5-hydroxyindolacetic acid concentrations in the hippocampus were analyzed using a factorial ANOVA for each neurotransmitter. No effect of genotype, P14 treatment, or adult amphetamine challenge was found for either 5-HT or 5-hydroxyindolacetic acid. [Figure 29]

Cerebellum

Analysis with a factorial ANOVA revealed no significant difference in serotonin levels of the cerebellum for the effects of genotype, P14 treatment or adult amphetamine

challenge. A one-way ANOVA was used to analyze 5-hydroxyindolacetic acid levels in the cerebellum and no significant effect of genotype, P14 treatment, or adult amphetamine challenge was found. [Figure 30]

Table 2. Summary of results of Experiment 2.

Behavioral Outcome Measures	Significant Genotype Effect	Significant VPA Effect	Gene x Treatment Interaction	Significant Amphetamine Effect
Weight	**p<0.05**[1]	ns[2]	ns	na[3]
Rotorod	ns	ns	**p<0.05**	na
Water Maze Visible	ns	**p<0.05**	ns	na
Water Maze Hidden	**p<0.05**	**p<0.05**	**p<0.05**	na
Elevated Plus Maze	**p<0.05**	ns	ns	na
Social chambers	**p<0.05**	**p<0.05**	ns	na
Target biting	**p<0.05**	ns	ns	na
HPLC	ns	ns	ns	**p<0.05**

Table 2. 1 denotes "p<0.05" = significant effect observed. Not the actual p values. 2 denotes "ns" = no significant effect observered. 3 denotes "na"= not applicable because amphetamine was only a variable in HPLC analysis not in the behavioral tests.

Conclusions for Experiment Two:

The results of experiment two are summarized in Table 2. The adult behavioral experiments revealed some interesting information about differences between Nrf2 knockout mice and wild type mice and about the long term effects of early postnatal valproic acid treatment. The social chambers, elevated plus maze, and target biting tests revealed behavioral differences between the Nrf2 knockout mice and the wild type mice. The water maze and the social chambers provided some evidence of long term effects of early postnatal treatment with valproic acid on behavior. The Nrf2 knockout mice were found to be heavier than the wild type mice through adulthood. This effect was seen in both the males and females. In the adult mice, both male and female, no differences in body weight were found between valproic acid-treated and saline-treated mice.

Because significant effects of valproic acid treatment were found in the rotorod and water maze tests during development, these tests were rerun when the mice aged to 90 days. The rotorod measured motor function and coordination as well as motor learning. This test is associated with cerebellum function and mice that abnormalities in the cerebellum have difficulty staying on the rotorod (Barlow et al., 1996; Sango et al., 1995). The mice were evaluated in three 60 second trials for three days. All of the mice improved over the days of testing indicating there were no deficits in motor function and coordination. There were no differences between genotypes or between treatment groups. This indicates that all of the mice now have normal cerebellum functioning given that they were all able to walk on the rotorod. This is inconsistent with the results of the rotorod test during development on P20 through P22. During development the valproic acid-treated mice had difficulty staying on the rotorod, and Nrf2 knockout mice treated

with valproic acid had the most difficult staying on the rotorod. It was suggested that the developing mice had damage to the cerebellum. However, now it appears that the deficits are no longer there and that perhaps there is compensation for the damage in the cerebellum as the mice age.

The visible and hidden platform paradigms of the water maze were both repeated in the adult mice. For the visible platform paradigm, the latency to find a platform above the water level and clearly visible was recorded in five trials on one day. There were no differences between the Nrf2 knockout mice and the wild type mice within treatment groups. However, the valproic acid-treated mice were found to perform significantly worse than the saline-treated mice within genotype groups. While the valproic acid-treated mice were slower to find the visible platform, they did improve their latency to find the platform over the trials and by trial 3 were successfully finding the platform in less than 20 seconds. This indicated that, while there was an initial difference between the valproic acid-treated and saline-treated mice, the valproic acid-treated mice were still capable of learning to find the visible platform quickly. Poor performance in the visible water maze could indicate dysfunction in vision and visual neurons or the motor ability of the mice to swim (Crawley, 1999). Poor performance in the visible water maze has also been associated with lesions of the striatum (Packard and Teather, 1997). As previously discussed, there is some evidence that chronic valproic acid treatment in humans can cause vision defects, however that damage is related to the long term treatment and valproic acid – induced concentrations of GABA, which is no longer present months after a single dose exposure (Hosking and Hilton, 2002; Tilz et al., 2007). This would indicate that the long lasting effect of valproic acid treatment on visible water maze performance

has to do with motor function. In the hidden platform paradigm, the latency to find a platform below the surface of the water level was recorded in five trials per day for four days. The Nrf2 knockout mice were found to find the platform significantly faster than the wild type mice within treatment groups. Although this significant difference was found, all of the mice found the platform in less than 20 seconds from day one of testing. Normal performance on the visible platform paradigm, but impaired performance on the hidden platform paradigm is interpreted as an indication of learning and memory deficits. Hippocampal lesions impair acquisition of the hidden platform task in rats and mice (Morris et al., 1982; Logue et al., 1997). If swimming speed is slower or if the mouse fails to swim at all, this may indicate dysfunction in visual neurons, motor neurons, spinal cord, and cerebellum, rather than a deficit in learning and memory (Crawley, 1999). In this experiment, there was impaired performance on the visible platform and normal performance on the hidden platform. In addition, all the latencies were very short for all groups to find the hidden platform. Therefore, all of the mice had already successfully learned to find the platform and the differences did not indicate deficits in hippocampal functioning, but perhaps just a difference in locomotion, whereby the Nrf2 knockout mice were hyperactive compared to the wild type mice. Results from the elevated plus maze also indicate a hyperactive state of the Nrf2 knockout mice, although the results from the open field activity chambers in the developing mice did not indicate the Nrf2 knockout mice were more active compared to the wild type mice. The results of the adult water maze tests were inconsistent with the findings from the water maze performed during the developmental experiment. During development, there did appear to be deficits in valproic acid-treated mice. The valproic acid-treated mice had much longer latencies to

find both the visible and hidden platform, suggesting they may have hippocampal and cerebellar damage. The Nrf2 knockout mice treated with valproic acid had the longest latencies to find both platforms and that suggests that the Nrf2 knockout mice had more damage to the hippocampus and the cerebellum compared to the wild type mice. While there may still be some motor dysfunction in the valproic acid-treated mice as seen in the visible platform paradigm of the adult test, there no longer appears to be evidence of hippocampal damage. Perhaps as the mice continue to live with this damage, they have a compensation mechanism to allow them to overcome the behavioral deficits associated with damage to the hippocampus. Deficits in the visible platform have also been associated with lesions of the striatum, although evidence that valproic acid exposure leads to striatal lesions have not been reported (Packard and Teather, 1997).

The elevated plus maze, social chambers, and target biting tests all revealed significant differences between the Nrf2 knockout mice and the wild type mice, while only the social chambers revealed long term effects of early postnatal treatment with valproic acid. The elevated plus maze evaluated anxiety level of the mice. Anxiety and fear related behaviors are associated with several brain regions, including the amygdale. Damage to the amygdala has been shown to result in expression of anxiety-related behavior (Anand and Shekhar, 2003). In addition to detecting anxiogenic or anxiolytic effects, the total number of entries into an arm gives a control measure of general hyperactivity or sedation (Crawley, 1999). Under normal conditions, mice spend more time in the closed arm of the maze and make more crosses into the closed arm than the open arm (Lister, 1987). Recording fecal boli can also be an indication of anxiety, whereby more fecal boli would indicate increased anxiety. The data showed that all of the

mice crossed into the closed arm of the maze more times than they crossed into the open arm. In addition, all the mice spent more time in the closed arm of the maze compared to the open arm. This indicated that both the wild type and Nrf2 knockout mice, both saline-treated and valproic acid-treated, have normal anxiety levels and normal functioning of the amgydala. One interesting difference found in this experiment was that the Nrf2 knockout mice crossed into both the open and closed arms significantly more times than the wild type mice. While they crossed into both arms more than the wild type, they still crossed into the closed arm more than the open arm. Therefore, this difference can be viewed as a difference in activity level, rather than a difference in anxiety level between the Nrf2 knockout mice and the wild type mice. This observation is consistent with the results of the water maze. The Nrf2 knockout mice were found to have shorter latencies to find the hidden platform compared to the wild type mice, although all the mice had short enough latencies to suggest that already learned to find the platform. Therefore, it was suggested that this difference might be a hyperactivity response from the Nrf2 knockout mice as we see a hyperactivity response in the Nrf2 knockout mice in the elevated plus maze.

In the social chambers, the number of times the subject mouse touched either a target cylinder containing another mouse or an empty control cylinder was automatically recorded as was the time spent touching either cylinder. For the total number of touches to a cylinder, a significant difference between genotypes was found. The saline-treated Nrf2 knockout mice made more contact with the target cylinder compared to the saline-treated wild type mice, however, they also made significantly more touches to the control cylinder compared to the saline-treated wild type mice. The valproic acid-treated Nrf2

knockout mice made a similar number of touches to the target cylinder as the valproic acid-treated wild type mice, however, the knockout mice made significantly more touches to the empty control cylinder compared to the valproic acid-treated wild type mice. Considering that the Nrf2 knockout mice made more contact with both cylinders, percentage of touches to the target cylinder was analyzed. In percent target touches, not only was a genotype effect found, but also an effect of treatment was found. While the knockout mice made significantly more touches overall to the target cylinder, the percent of touches they made to the target cylinder was significantly less compared to the wild type mice. Time spent in contact with the target and control cylinders was also analyzed. All the mice spent more time overall in contact with the target cylinder compared to the empty control cylinder. The Nrf2 knockout mice spent more time in contact with the control cylinder compared to the wild type mice within treatment groups. When percent of time spent in contact with the target cylinder was analyzed, no difference between genotypes was found. While the percent of time the valproic acid-treated mice spent in contact with the target cylinder was not significantly less than the saline-treated animals, this difference approached significance. Given these results, the Nrf2 knockout mice appeared to be more active in that they made more total contact with both cylinders. The Nrf2 knockout mice may be less social considering they did not show as great a preference for the target cylinder as the wild type mice, however it was not clear because the percent of time spent touching the target cylinder was not different from the wild type mice. While no significant difference in number of touches to a cylinder was found between saline-treated and valproic acid-treated mice, when percent of target touches was analyzed, the valproic acid-treated mice made significantly less contact with the target

cylinder compared to the saline-treated mice. In addition, while there was no difference in total time spent touching a cylinder between the saline-treated mice and valproic acid-treated mice, the difference in percent of time spent touching the target cylinder approached significance with the valproic acid-treated mice spending less time than the saline-treated mice. This indicated that early postnatal treatment with valproic acid may have some long term effects on social behavior well into adulthood in mice. The results of the social chambers are consistent with previously reported findings. Exposure to valproic acid on embryonic day 12.5 in rats was shown to result in decrease social behavior in adulthood (Schneider et al., 2008). Mice treated with valproic acid on postnatal day 14 were also found to exhibit less social behaviors when evaluated for play behavior between postnatal day 30 and postnatal day 40 (Yochum et al., 2008). Early exposure to valproic acid has been associated with damage to the cerebellum (Yochum et al., 2008; Banji et al., 2011). While the cerebellum has typically been solely associated with motor activity, recent work in humans have shown cerebellum function to play a role in attention tasks that may provide a link to the social and cognitive deficits (Allen et al., 2004). Perhaps this link suggests the deficits in social behavior could be due to abnormal cerebellum functioning in the valproic acid-treated mice.

The target biting chamber assessed defensive aggression. The test consisted of ten two minute trials. The two minute trials were divided into eight 15 second bins during which the mice had access to a target cable tie. Immediately following bin 8, and just prior to bin 1, the mice received a 2mA tail shock. The number of times the animal bit the target cable tie was recorded per bin. The number of bites from bins 2-7 was averaged and the data was analyzed as target bites in 3 bins. All animals bit the target significantly

more times in bin 1, immediately following the tail shock, compared to bin 2 and bin 3. No effect of valproic acid treatment was found in this test of defensive aggression. There was, however, a genotype effect. In bin 1, the Nrf2 knockout mice bit the target significantly fewer times than the wild type mice. This indicated that the Nrf2 knockout mice may be less aggressive compared with the wild type mice. The brain regions associated with aggressive behavior include the hypothalamus and midbrain periaqueductal gray, and the regions of the brain associated with modulating functions include the amygdala, hippocampus, and prefrontal cortex (Siegel et al., 2007). Typical treatments for aggressive behavior in humans are serotonergic compounds and GABAergic compounds because the neurotransmitters GABA and serotonin that act through the 5-HT1 receptor inhibit aggressive behavior (Siegel et al., 2007). Fluprazine, a serotonin agonist has been shown to reduce aggressive behavior in the target biting chambers (Carelli and Wagner, 1988). Aggressive behavior is multifaceted and these brain regions and neurotransmitters are not the only ones involved. While these reports indicate that a lack of aggressive behavior may be due to increased GABA expression, increased serotonin, or dysfunction in one of the brain regions involved in aggression, there have not been any published reports to indicate the Nrf2 knockout mice may be linked to any of these factors. This is also the first report on aggression in Nrf2 knockout mice.

After the adult behavioral tests were completed, forty male mice were evaluated for differential sensitivity to a high dose of amphetamine. Amphetamine causes damage to the dopamine neurons in the striatum and, therefore, the striatum of these animals was analyzed for dopamine concentrations as well as the dopamine metabolites

dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) concentrations. The concentration of 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were also analyzed in the striatum. In addition, the hippocampus and cerebellum were also analyzed for concentrations of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid, but not for dopamine and its metabolites because those brain regions are known to have little if any dopamine. An overall effect of amphetamine treatment was found for dopamine concentration in which all of the groups treated with amphetamine had lower concentrations of dopamine compared with their saline-treated counterparts. There was no effect of genotype or postnatal day 14 treatment on the concentration of dopamine in the striatum. The dopamine lesion that resulted from amphetamine treatment in these mice was very large. All of the mice treated with amphetamine had approximately 50% lesion in striatal dopamine. The 50 mg/kg dose that was chosen had been previously reported to induce amphetamine depletion (Halladay et al., 2000). It is possible that the dose that was used in this experiment was too high and that the lesions that resulted were already maximal. If the lesions were already maximal, it would not be possible to detect a significant difference between the wild type and knockout mice. The lesions were similar in the mice pre-treated with valproic acid as they were in the saline-treated mice. Perhaps a lower dose would provide insight into any differences between the knockout and wild type mice and between the P14 valproic acid-treated mice and the P14 saline-treated mice. The dopamine metabolites DOPAC and HVA were also found to be affected by amphetamine treatment. However, the decreases in DOPAC and HVA concentrations were only found in Nrf2 knockout mice. For DOPAC, only the Nrf2 knockout mice treated with valproic acid on postnatal day 14 had

a significantly lower concentration of DOPAC compared to the Nrf2 knockout mice treated with valproic acid on P14 and saline in adulthood. For HVA, the Nrf2 knockout mice treated with saline or valproic acid on postnatal day 14 and amphetamine in adulthood had significantly lower concentrations of HVA compared within P14 treatment group to their adult-saline-treated counterparts. For serotonin and its metabolite 5-hydroxyindoleacetic acid, the same effect was found where significant differences in concentrations were only found in the Nrf2 knockout mice treated with valproic acid on postnatal day 14. In the hippocampus and cerebellum, no differences in concentrations of 5-HT and 5-HIAA were found between genotype, postnatal day 14 treatment, or adult treatment. The greatest effect found from this experiment is that amphetamine causes a depletion in dopamine concentration in the striatum, which is consistent with previous reports (Halladay et al., 2000; Wagner et al., 2007). Changes in dopamine metabolite concentrations and serotonin and its metabolite concentrations were found in the striatum, but only in the Nrf2 knockout mice treated with valproic acid on postnatal day 14. While this finding is interesting, it requires further investigation with more subjects to determine if this is a real effect.

Figure 13

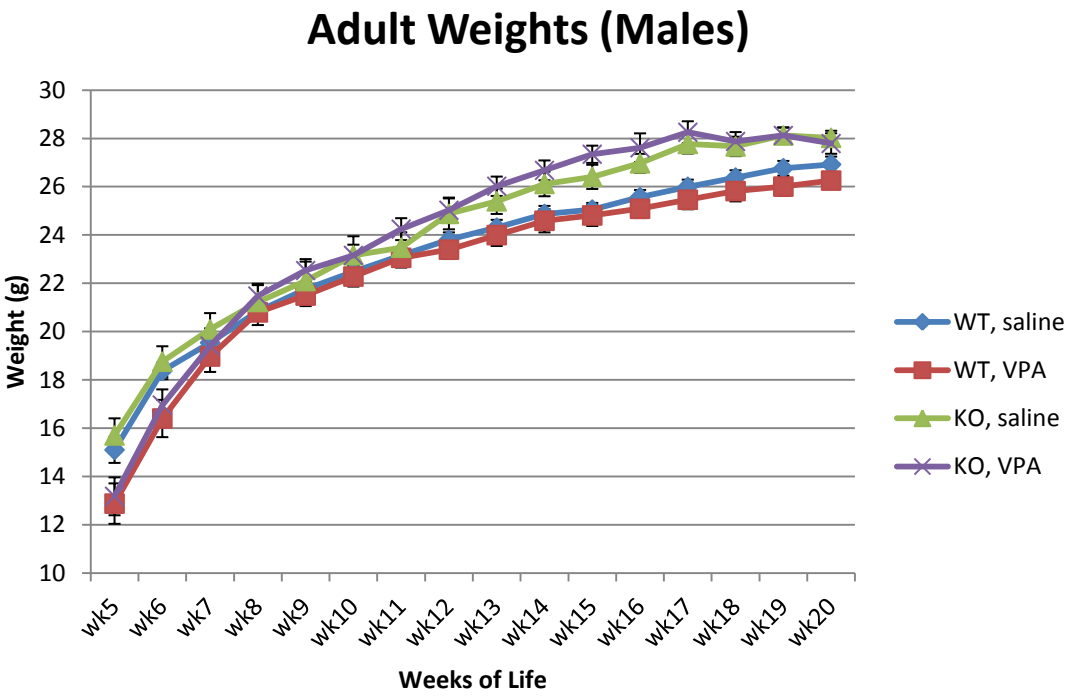

Figure 13. Average male weights over weeks of life. All male mice gained a significant amount of weight over testing ($p < 0.0001$). There was no overall effect of VPA treatment on adult weight. The KO mice continue to weigh significantly more than the WT mice ($p=0.0085$). WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N= 68 total; 27 WT saline-treated mice, 16 WT VPA-treated mice, 15 KO saline-treated mice, and 10 KO VPA-treated mice)

Figure 14

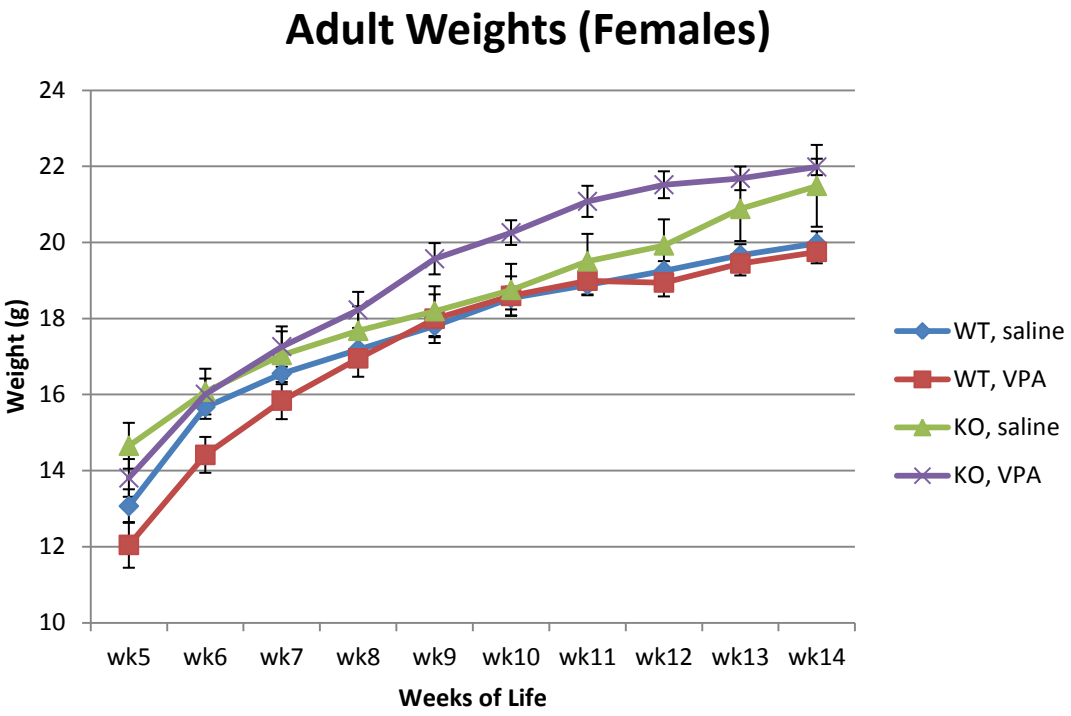

Figure 14. Average female adult weight. All groups of female mice gained a significant amount of weight over testing ($p < 0.0001$). There was no overall effect of VPA treatment on adult weight. The KO mice continue to weigh significantly more than the WT mice ($p=0.0041$). WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N =56 total; 17 WT saline-treated mice, 18 WT VPA-treated mice, 10 KO saline-treated mice, and 11 KO VPA-treated mice)

Figure 15

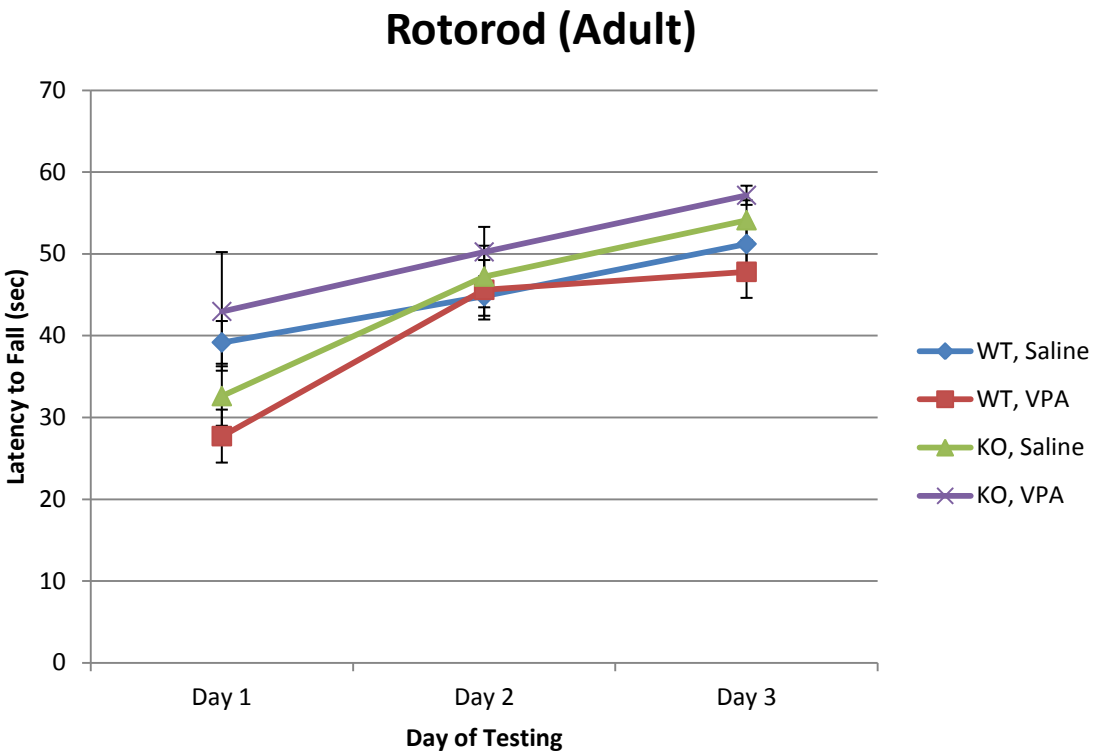

Figure 15. Adult average latency to fall from the rotorod. All groups performed significantly better over days of testing ($p < 0.0001$). WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N= 113 total; 17 male WT saline-treated mice, 12 male WT VPA-treated mice, 22 female WT saline-treated mice, 13 female WT VPA-treated mice, 16 male KO saline-treated mice, 12 male KO VPA-acid treated mice, 10 female KO saline-treated mice, and 11 female KO VPA-treated mice)

Figure 16

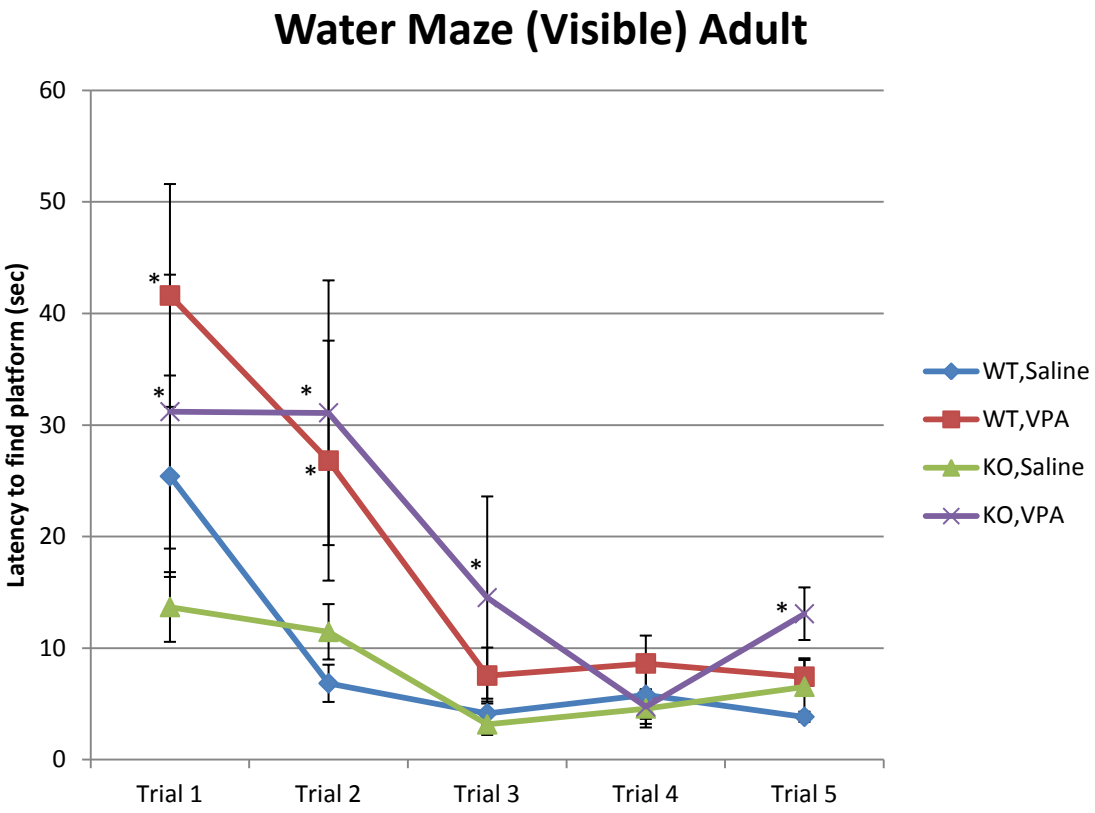

Figure 16. Adult Average Latency to find the visible platform. No difference between genotypes was found. VPA-treated WT mice performed significantly worse compared to the saline-treated WT mice ($p=0.0217$) and that the VPA-treated KO mice performed significantly worse compared to their saline-treated counterparts ($p=0.0012$). The saline-treated WT mice and KO mice performed similarly, as did the VPA-treated mice of both genotypes. * denotes $p<0.05$ compared to within genotype saline-treated counterparts. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 20 total; 5 male WT saline-treated mice, 5 male WT VPA-treated mice, 5 male KO saline-treated mice, and 5 male KO VPA-treated mice)

Figure 17

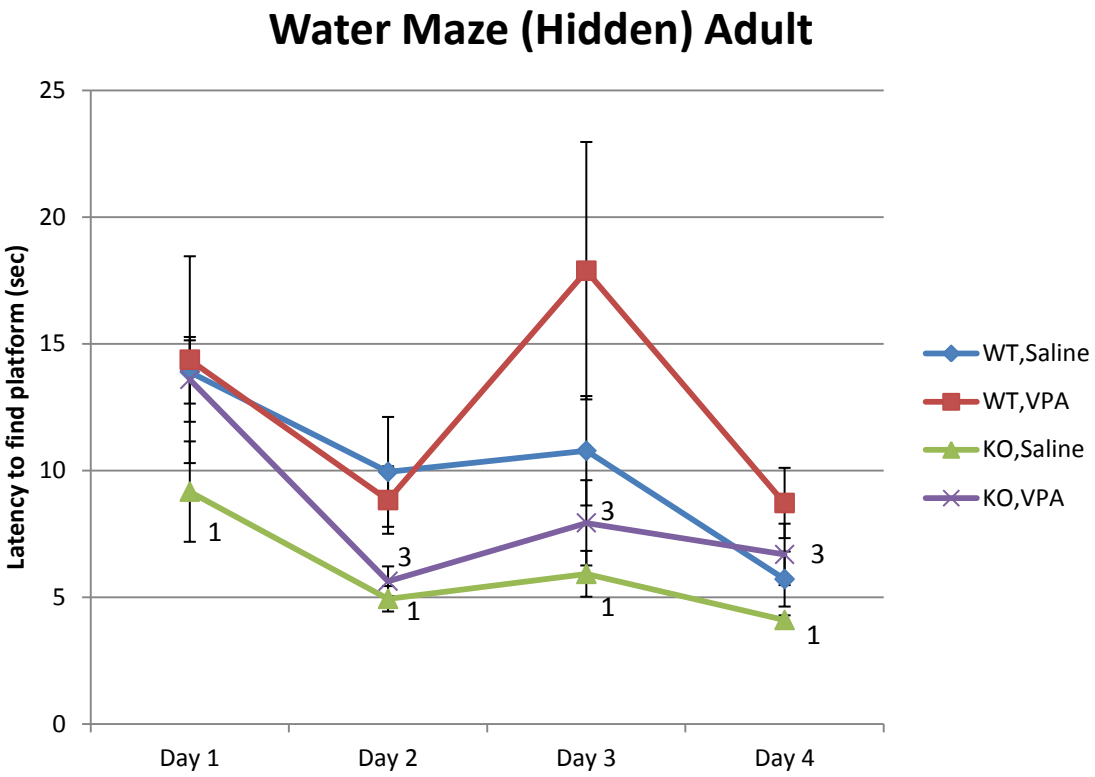

Figure 17. Adult average latency to find the hidden platform. The saline-treated KO mice were significantly faster than the saline-treated WT mice ($p=0.0020$) as were the VPA-treated KO mice compared to the VPA-treated WT mice ($p=0.0499$). 1 denotes $p<0.05$ compared to WT, Saline. 3 denotes $p<0.05$ compared to WT, VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 20 total; 5 male WT saline-treated mice, 5 male WT VPA-treated mice, 5 male KO saline-treated mice, and 5 male KO VPA-treated mice)

Figure 18

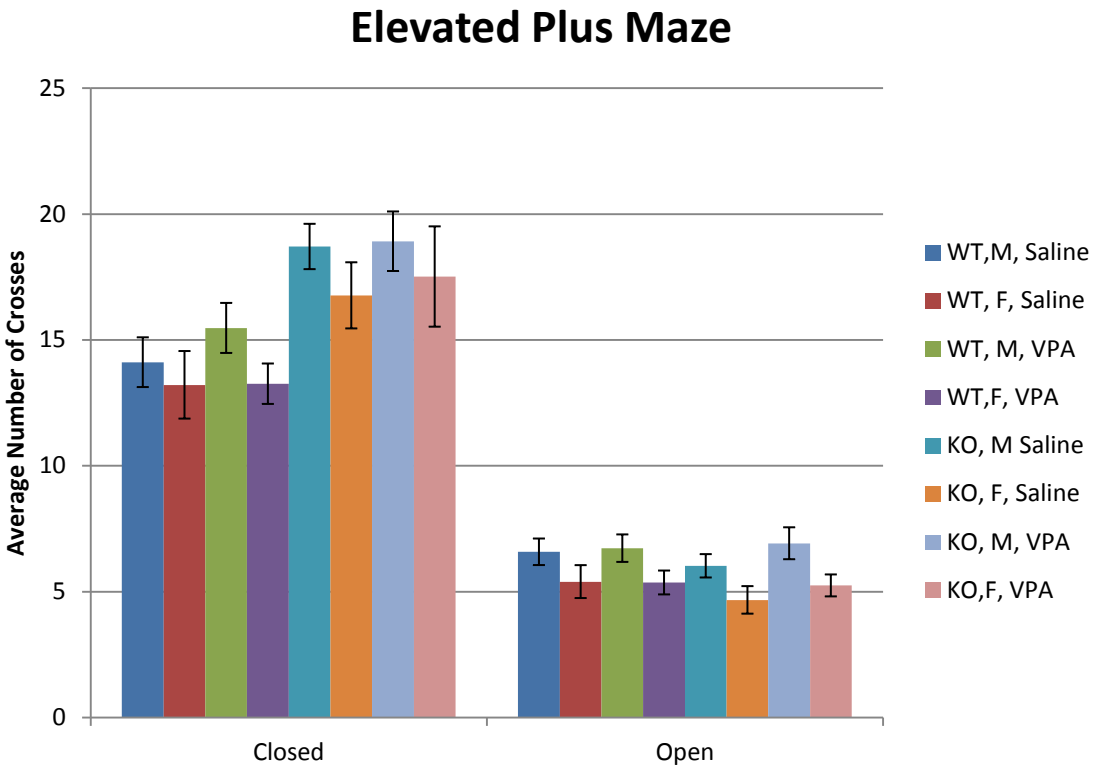

Figure 18. Average number of crosses into closed and open arms of the elevated plus maze. KO mice made significantly more crosses total compared to the WT mice ($p<0.0001$). The male mice made significantly more crosses total compared to the female mice ($p=0.0011$). Significantly more crosses were made into the closed arms compared to the open arms ($p<0.0001$). No effect of VPA treatment was found. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 90 total; 15 male WT saline-treated mice, 12 male WT VPA treated mice, 11 female WT saline-treated mice, 13 females WT VPA-treated mice, 16 male KO saline-treated mice, 12 male KO VPA-treated mice, 10 female KO saline-treated mice, and 11 female KO VPA-treated mice)

Figure 19

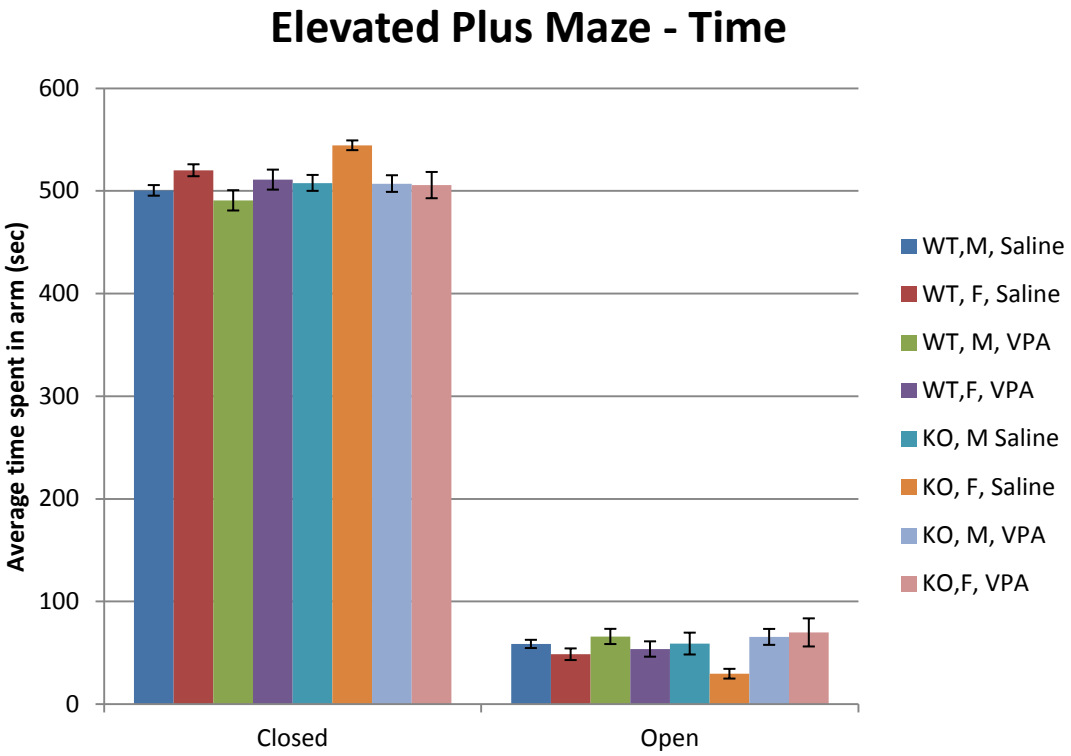

Figure 19. Average time spent in an arm of the elevated plus maze. No effect of genotype, treatment, or sex was found. All mice spent more time in the closed arm compared to the open arm ($p < 0.0001$). WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N = 90 total; 15 male WT saline-treated mice, 12 male WT VPA treated mice, 11 female WT saline-treated mice, 13 females WT VPA-treated mice, 16 male KO saline-treated mice, 12 male KO VPA-treated mice, 10 female KO saline-treated mice, and 11 female KO VPA-treated mice)

Figure 20

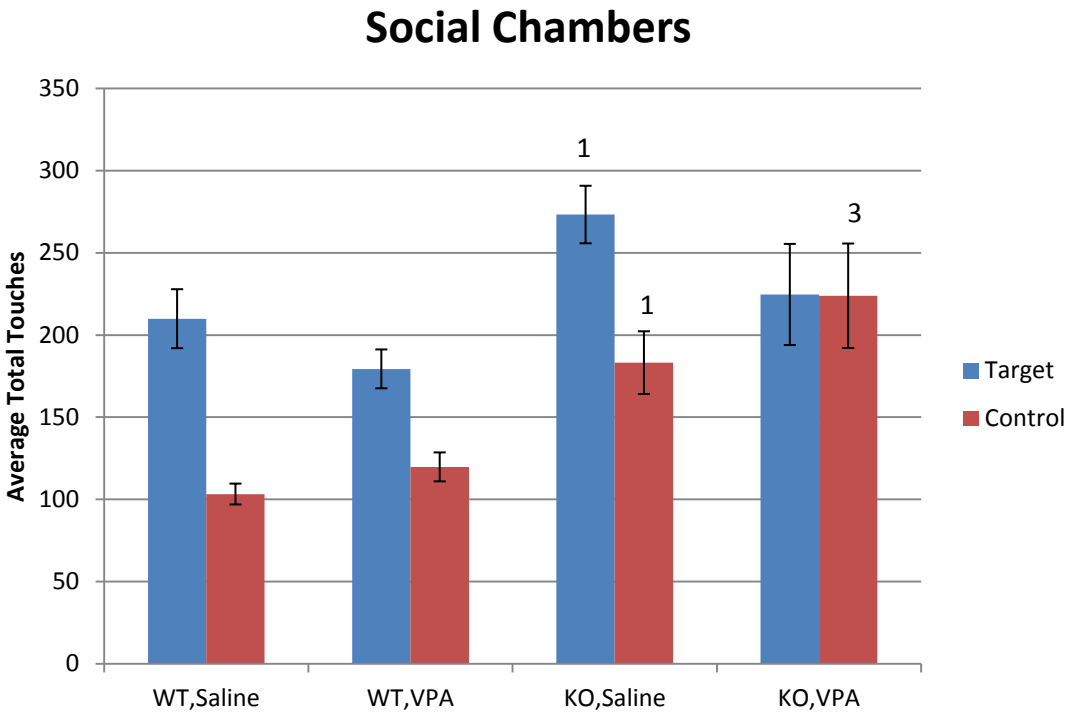

Figure 20. Average total touches to a cylinder in the social chambers. Overall mice made more contact with the target cylinders compared to the control cylinders ($p<0.0001$). Saline-treated KO mice($p=0.0156$) touched the target cylinder significantly more times compared to the saline-treated WT mice, but the KO mice also touched the control cylinder significantly more than the WT mice ($p=0.0004$). The VPA-treated KO mice ($p=0.0012$) touched the control cylinder significantly more times than the VPA-treated WT mice. 1 denotes $p<0.05$ compared to WT, Saline. 3 denotes $p<0.05$ compared to WT, VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N= 89 total; 13 male WT saline-treated mice, 12 male WT VPA-treated mice, 10 female WT saline-treated mice, 13 female WT VPA-treated mice, 11 male KO saline-treated mice, 10 male KO VPA-treated mice, 10 female KO saline-treated mice, and 10 female KO VPA-treated mice)

Figure 21

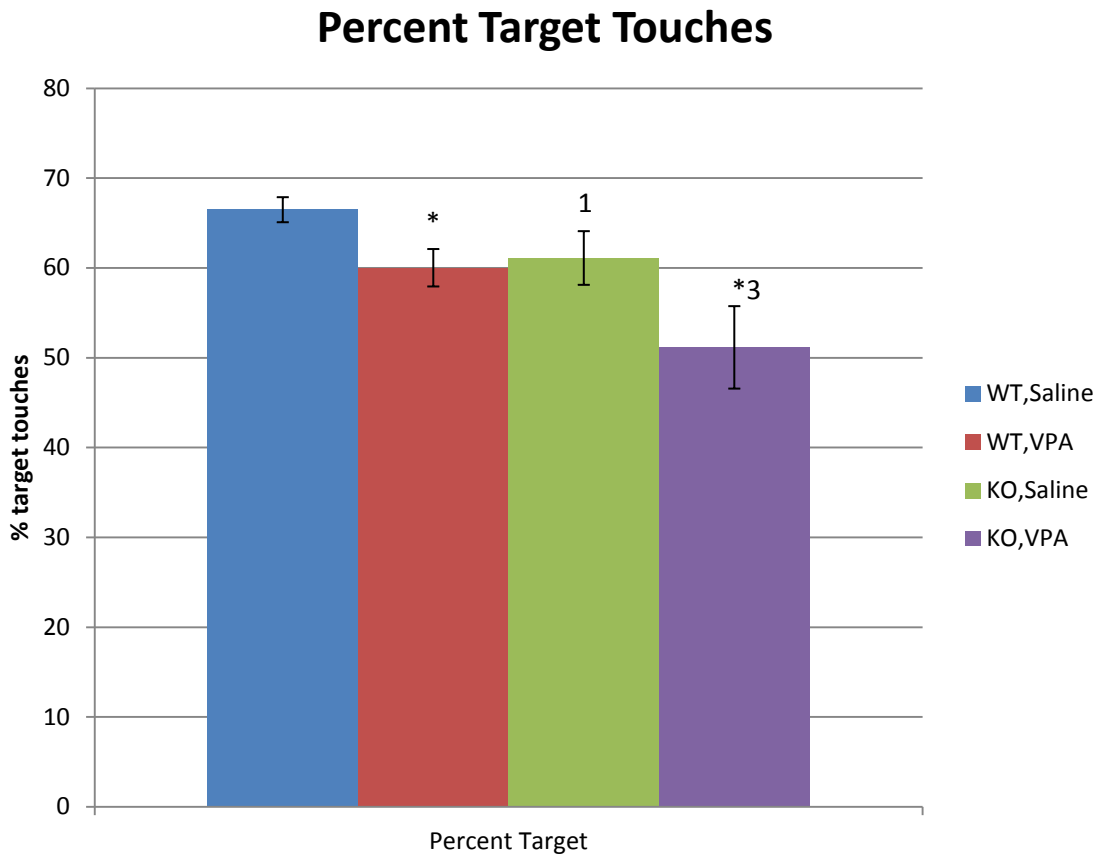

Figure 21. Percent touches to the target cylinder. The KO mice's percentage of touches made to the target chamber was significantly lower than the WT mice (p=0.0260) and the VPA-treated mice had a significantly lower percentage of touches made to the target cylinder compared to the saline-treated mice (p=0.0108). * denotes p<0.05 compared to within genotype saline-treated counterparts. 1 denotes p<0.05 compared to WT, Saline. 3 denotes p<0.05 compared to WT, VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N= 89 total; 13 male WT saline-treated mice, 12 male WT VPA-treated mice, 10 female WT saline-treated mice, 13 female WT VPA-treated mice, 11 male KO saline-treated mice, 10 male KO VPA-treated mice, 10 female KO saline-treated mice, and 10 female KO VPA-treated mice)

Figure 22

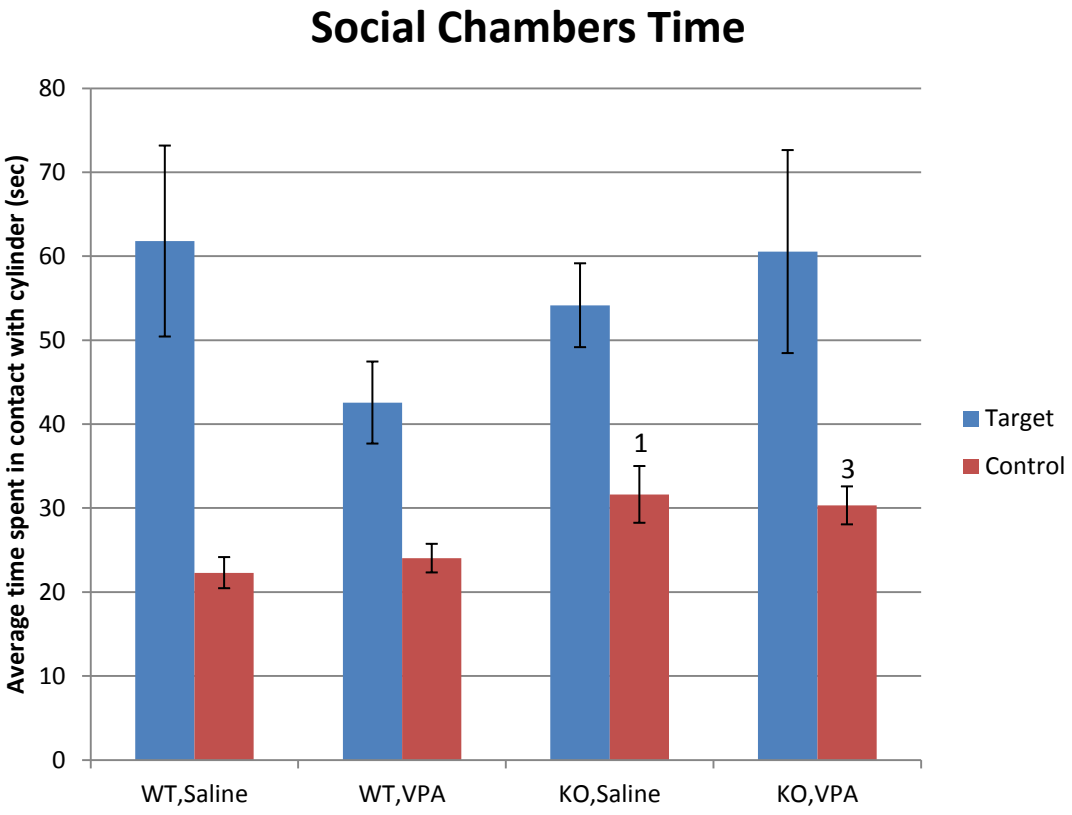

Figure 22. Average time spent in contact with a cylinder. All of the mice spent significantly more time touching the target cylinder compared to the empty cylinder. Saline-treated KO mice spent significantly more time in contact with the control cylinder compared to the saline-treated WT mice ($p=0.0221$) and that the VPA-treated KO mice also spent significantly more time in contact with the control cylinder compared to the VPA-treated WT mice ($p=0.0288$). 1 denotes $p<0.05$ compared to WT, Saline. 3 denotes $p<0.05$ compared to WT, VPA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N= 89 total; 13 male WT saline-treated mice, 12 male WT VPA-treated mice, 10 female WT saline-treated mice, 13 female WT VPA-treated mice, 11 male KO saline-treated mice, 10 male KO VPA-treated mice, 10 female KO saline-treated mice, and 10 female KO VPA-treated mice)

Figure 23

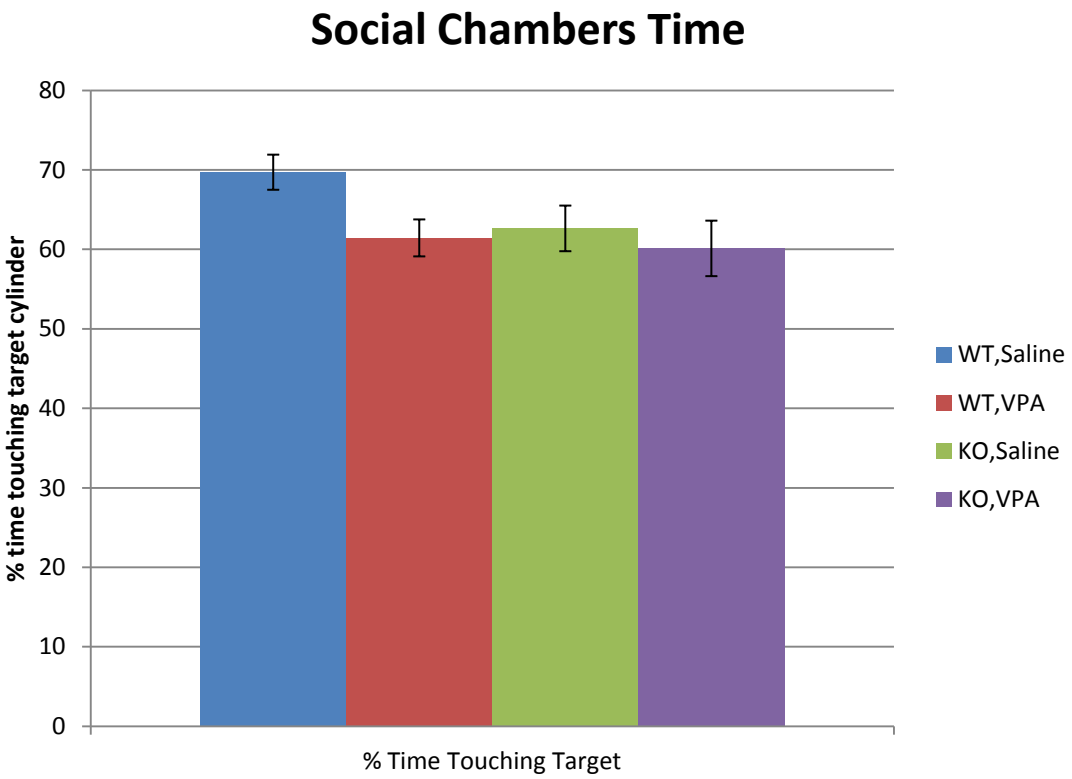

Figure 23. Percent of time spent in contact with the target cylinder. Percent time spent in contact with the target of the total time spent in contact with either cylinder, not of total 30 minute trial. The effect of treatment approached significance [$F(1,82)=3.850$, $p=0.0531$], whereby the saline-treated mice spent more percentage of time touching the target cylinder compared to the VPA-treated mice. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N= 89 total; 13 male WT saline-treated mice, 12 male WT VPA-treated mice, 10 female WT saline-treated mice, 13 female WT VPA-treated mice, 11 male KO saline-treated mice, 10 male KO VPA-treated mice, 10 female KO saline-treated mice, and 10 female KO VPA-treated mice)

Figure 24

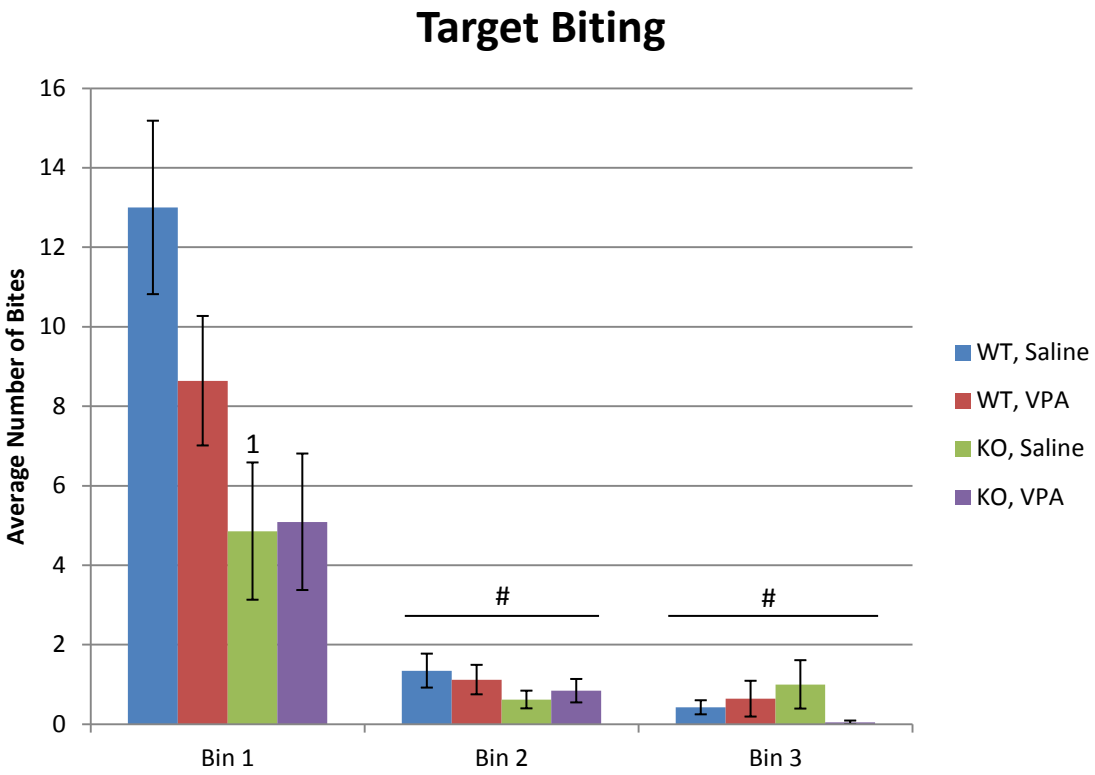

Figure 24. Average number of bites to the target. All of the mice bit the target significantly more times in bin 1 compared with bin 2 ($p<0.0001$) and compared with bin 3 ($p<0.0001$). There was no significant effect of VPA treatment on target biting. The WT mice treated with saline bit the target significantly more times in bin 1 compared with the KO mice treated with saline ($p=.0274$). 1 denotes $p<0.05$ compared to WT, Saline. # denotes $p<0.05$ compared to Bin 1. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. (N= 94 total; 15 male WT saline-treated mice, 12 male WT VPA-treated mice, 11 female WT saline-treated mice, 13 female WT VPA-treated mice, 11 male KO saline-treated mice, 11 male KO VPA-treated mice, 10 female KO saline-treated mice, and 11 female KO VPA-treated mice)

Figure 25

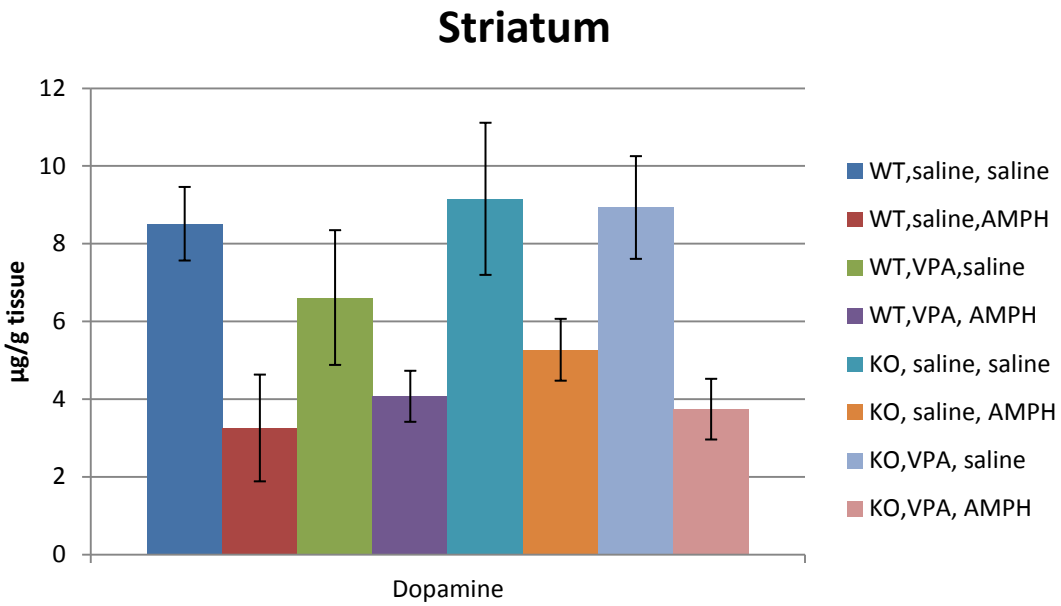

Figure 25. Concentrations of dopamine in the striatum. Mice treated with amphetamine had significantly lower levels of dopamine compared with their saline treated counterparts ($p<0.0001$). WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. AMPH = amphetamine. (N=40; 5 WT P14 saline, adult saline, 5 WT P14 saline, adult AMPH, 5 WT P14 VPA, adult saline, 5 WT P14 VPA, adult AMPH, 5 KO P14 saline, adult saline, 5 KO P14 saline, adult AMPH, 5 KO P14 VPA, adult saline, 5 KO P14 VPA, adult AMPH)

Figure 26

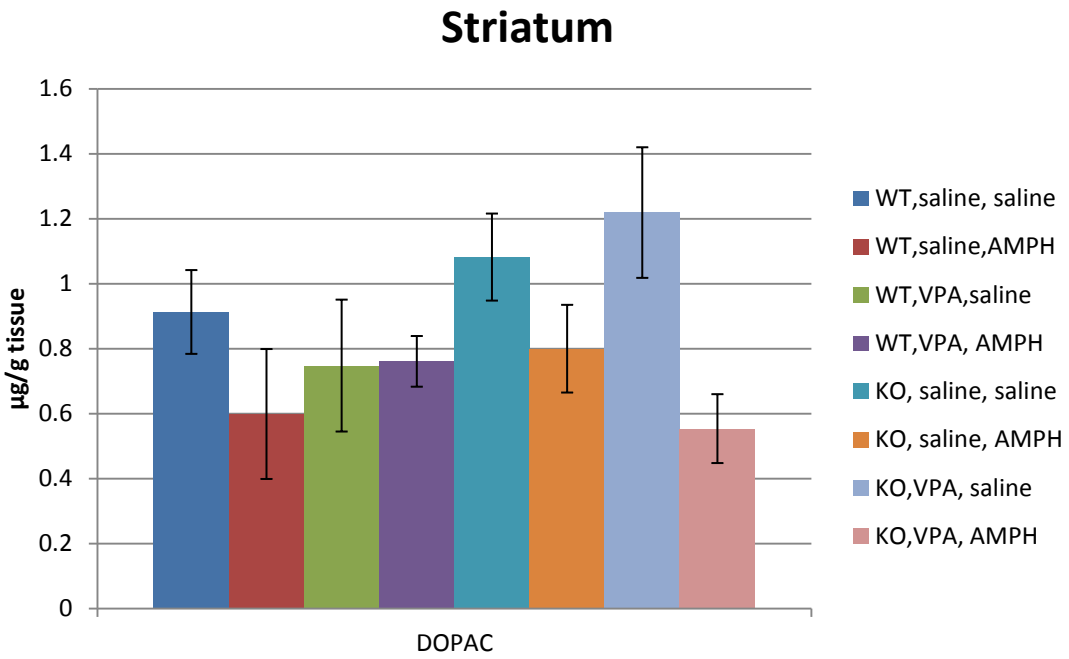

Figure 26. Concentration of DOPAC in the striatum. The KO mice treated with amphetamine (p=0.0060) had lower DOPAC concentrations compared with KO mice treated with saline. KO mice treated with VPA and amphetamine (p=0.0191) had significantly lower concentrations of DOPAC compared to KO mice treated with VPA and saline. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. AMPH = amphetamine. (N=40; 5 WT P14 saline, adult saline, 5 WT P14 saline, adult AMPH, 5 WT P14 VPA, adult saline, 5 WT P14 VPA, adult AMPH, 5 KO P14 saline, adult saline, 5 KO P14 saline, adult AMPH, 5 KO P14 VPA, adult saline, 5 KO P14 VPA, adult AMPH)

Figure 27

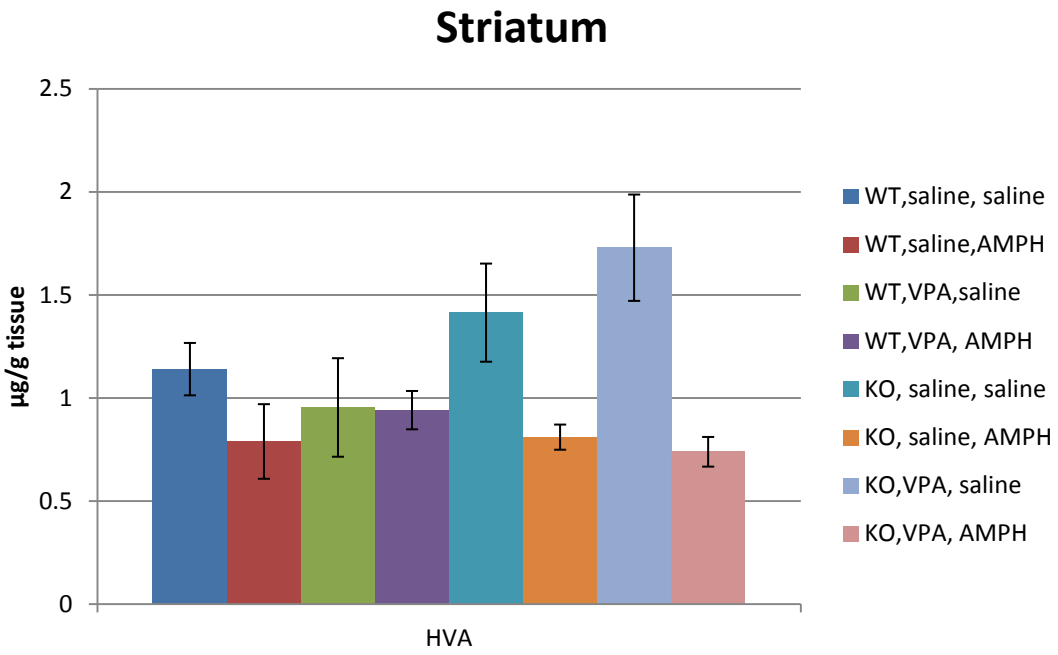

Figure 27. Concentration of HVA in the striatum. The KO mice treated with amphetamine had significantly lower concentrations of HVA compared with KO mice treated with saline (p=0.0003). WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. AMPH = amphetamine. (N=40; 5 WT P14 saline, adult saline, 5 WT P14 saline, adult AMPH, 5 WT P14 VPA, adult saline, 5 WT P14 VPA, adult AMPH, 5 KO P14 saline, adult saline, 5 KO P14 saline, adult AMPH, 5 KO P14 VPA, adult saline, 5 KO P14 VPA, adult AMPH)

Figure 28.

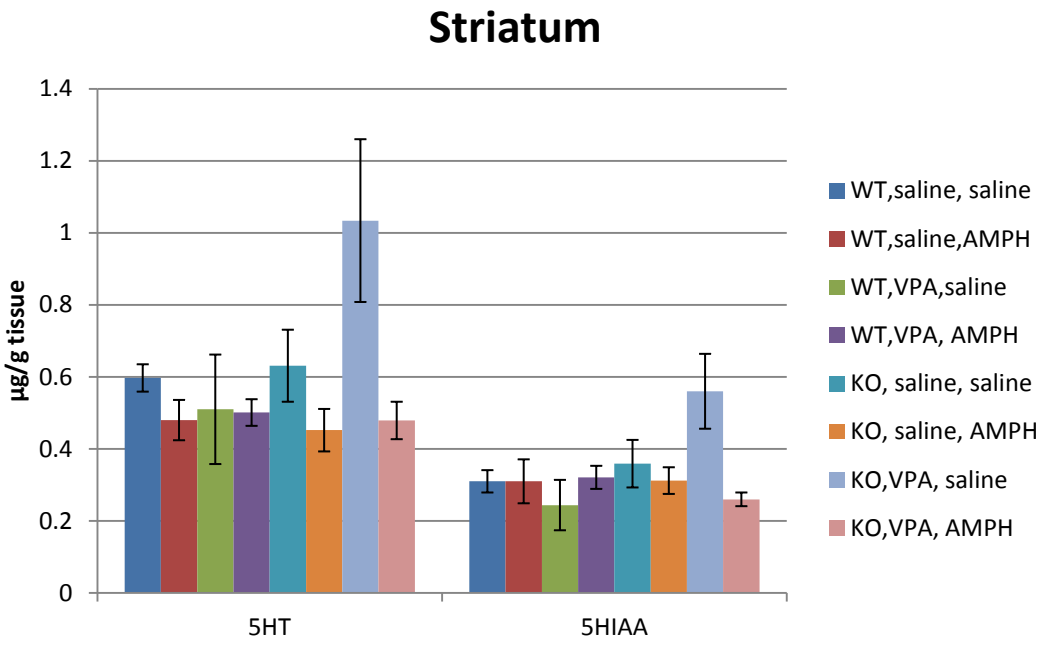

Figure 28. Concentration of 5HT and 5HIAA in the striatum. For 5HT, KO mice treated with amphetamine had significantly lower levels of serotonin compared with saline treated KO mice (p=0.0106). KO mice treated with VPA and amphetamine had significantly less 5-HT compared with KO mice treated with VPA and saline (p= 0.0438). For 5-HIAA, KO mice treated with VPA and amphetamine had lower concentrations compared with the KO mice treated with VPA and saline (p=0.0215). WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. AMPH = amphetamine. (N=40; 5 WT P14 saline, adult saline, 5 WT P14 saline, adult AMPH, 5 WT P14 VPA, adult saline, 5 WT P14 VPA, adult AMPH, 5 KO P14 saline, adult saline, 5 KO P14 saline, adult AMPH, 5 KO P14 VPA, adult saline, 5 KO P14 VPA, adult AMPH)

Figure 29

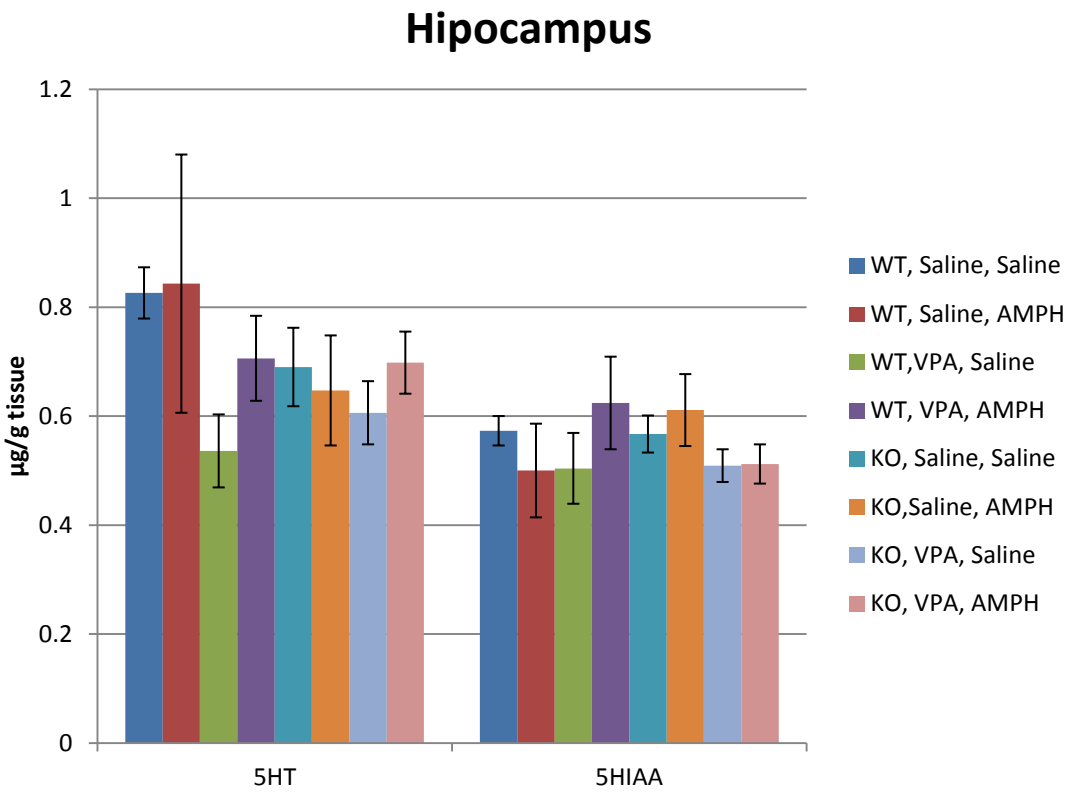

Figure 29. Concentrations of 5HT and 5HIAA in the hippocampus. No effect of genotype, P14 treatment, or adult amphetamine challenge was found for either 5-HT or 5HIAA. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. AMPH = amphetamine. (N=40; 5 WT P14 saline, adult saline, 5 WT P14 saline, adult AMPH, 5 WT P14 VPA, adult saline, 5 WT P14 VPA, adult AMPH, 5 KO P14 saline, adult saline, 5 KO P14 saline, adult AMPH, 5 KO P14 VPA, adult saline, 5 KO P14 VPA, adult AMPH)

Figure 30

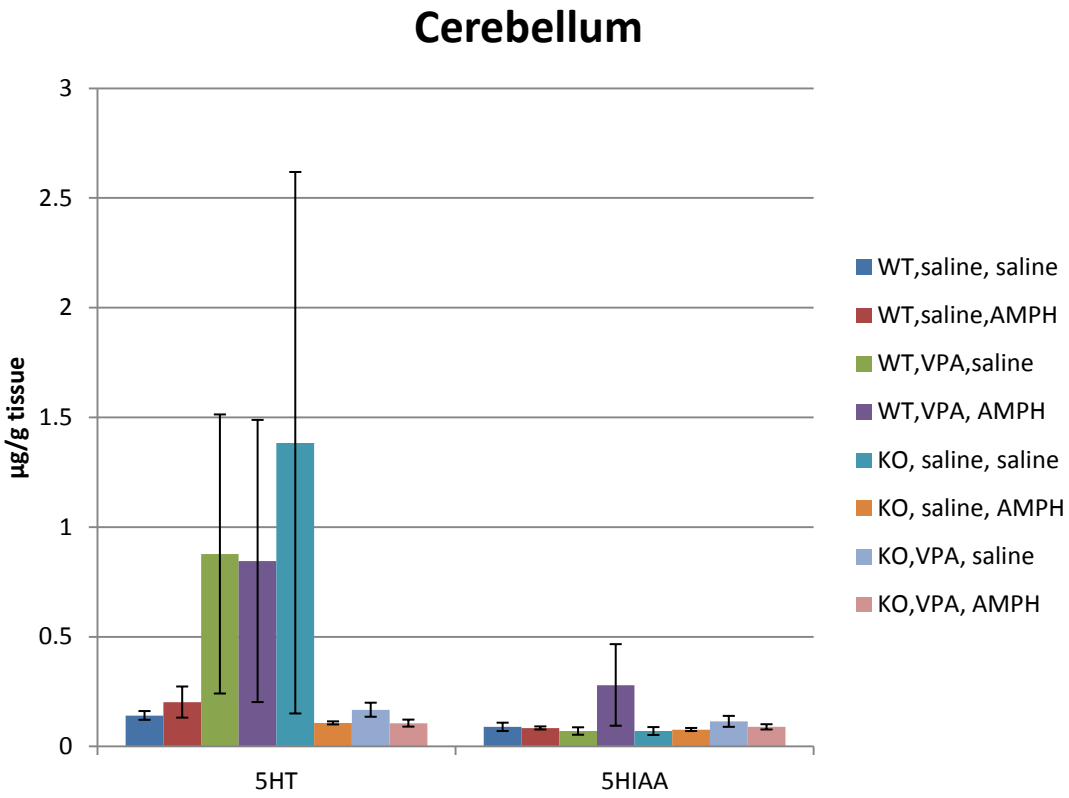

Figure 30. Concentrations of 5HT and 5HIAA in the cerebellum. No significant difference in 5HT or 5HIAA concentrations in the cerebellum for the effects of genotype, P14 treatment or adult amphetamine challenge was found. WT = wild type mice. KO = Nrf2 knockout mice. VPA = valproic acid. AMPH = amphetamine. (N=40; 5 WT P14 saline, adult saline, 5 WT P14 saline, adult AMPH, 5 WT P14 VPA, adult saline, 5 WT P14 VPA, adult AMPH, 5 KO P14 saline, adult saline, 5 KO P14 saline, adult AMPH, 5 KO P14 VPA, adult saline, 5 KO P14 VPA, adult AMPH)

DISCUSSION AND FUTURE DIRECTIONS

Overall early postnatal treatment with valproic acid did appear to result in behavioral deficits both during development and in adulthood, and there appeared to be some differences between the Nrf2 knockout mice and C57BL/6 wild type mice. The developmental behavioral tests that revealed effects of postnatal day 14 exposure to valproic acid included the mid-air righting test, the open field activity chambers, the rotorod, and the Morris water maze. These behavioral tests are associated with cerebellar function (Petrosini et al., 1990;Barlow et al., 1996; Sango et al., 1995; Crawley, 1999; Packard and Teather, 1997). Animals with abnormalities and damage to the cerebellum have been shown to have poor performance on these tests. Treatment with valproic acid on postnatal day 14 has been associated with granule cell death and cerebellar hypoplasia as well as damage to the Purkinje cell layer (Yochum et al., 2008; Banji et al., 2011). Learning and memory are associated with hippocampus functioning and hippocampal lesions impair acquisition of the hidden water maze task in rats and mice (Morris et al., 1982; Logue et al., 1997). Previously reported work indicates treatment with valproic acid results in increased apoptosis in the hippocampus and difficulty in learning (Yochum et al., 2008; Banji et al., 2011; Wagner et al., 2006). In addition exposure to valproic acid on postnatal day 14 has also been shown to cause dendritic atrophy of memory processing hippocampal functions (Devi et al., 2003; Jarrard, 1986). Given this information, it appears that the valproic acid-treated mice likely have damage in their cerebellum and hippocampus.

Valproic acid has been linked to oxidative stress in that it has been shown to induce oxidative stress biomarkers and treatment with antioxidants has been shown to

ameliorate many of the behavioral deficits as well as the Purkinje cell damage (Tabatabaei and Abbott, 1999; Levav-Rabkin et al., 2010; Lombardo et al., 2005;Al Deeb et al., 2000). Nrf2 knockout mice have also been found to be more sensitive to oxidative stress inducing toxicants and to have higher levels of oxidative stress markers (Itoh et al., 1997; Ishii et al., 2005; Liu et al., 2010; Innamorato et al., 2010). The Nrf2 knockout mice treated with valproic acid exhibited even greater deficits than the wild type mice treated with valproic acid in the open field activity chambers, the rotorod, and the water maze. As stated above, these tests are associated with cerebellar and hippocampal functioning and damage to these regions results in poor performance on these tests. Treatment with valproic acid has been shown to cause damage to the cerebellum and the hippocampus. Given this information, the Nrf2 knockout mice treated with valproic acid could have increased damage to their cerebellum compared to wild type mice with normal levels of antioxidant defense mechanisms.

In the future, more experiments should be done to further elucidate the link between oxidative stress and valproic acid, as well as the effect of valproic acid in Nrf2 knockout mice. Valproic acid has been reported to induce a 30-fold increase in apoptosis in the external granule cell layer of the cerebellum and a 10-fold increase in apoptosis in the dentate gyrus of the hippocampus (Yochum et al., 2008). It would be interesting to evaluate apoptosis in the Nrf2 knockout mice in comparison to the wild type mice following valproic acid treatment. While Nrf2 knockout mice have been reported to be more sensitive to oxidative stress inducing toxicants and show increase oxidative stress markers following exposure to these toxicants, no tests for oxidative stress markers were done in these experiments. In addition, exposure to valproic acid on postnatal day 14 has

been shown to increase oxidative stress markers in rodents (Banji et al., 2010). These mice should also be evaluated for oxidative stress biomarkers to confirm the presence of oxidative stress following valproic acid exposure and to evaluate if differential levels of these markers are seen in Nrf2 knockout mice. Others have reported that treatment with antioxidants ameliorates the behavioral impairments as well as the Purkinje cell damage in mice exposed to valproic acid on postnatal day 14 (Cheh et al., 2008; Banji et al., 2010). It may be interesting to evaluate the effect of antioxidants in valproic acid-treated Nrf2 knockout mice both on behavior and histopathological markers of the damage caused by valproic acid.

In adult behavioral testing, some long lasting effects of postnatal day 14 treatment with valproic acid were found. The adult behavioral tests that indicate a lasting effect of valproic acid on behavior included the social chambers and the Morris water maze. The valproic acid-treated mice were found to be less social. This is consistent with the literature that reports treatment with valproic acid has been shown to impair social development and decrease social behaviors in rats (Chapman and Cutler, 1989; Wu and Wang, 2002; Schneider et al., 2008). Social behavior is a complex behavior that is associated with several brain regions including the mesolimbic areas, the parietal cortex, and the cerebellum (Critchley et al., 2000). The mesolimbic areas include the ventromedial prefrontal cortex, the medial temporal lobes, the striatum and the limbic thalamus (Critchley et al., 2000). Rodent studies have found that when two hypothalamic neuropeptides, oxytocin and vasopressin were decreased by knockout mice social behavior was decreased (Winslow et al., 2000; Wersinger et al., 2004; Wersinger et al., 2006).While the cerebellum has typically been solely associated with motor activity,

recent work in humans have shown cerebellum function to play a role in attention tasks that may provide a link to the social and cognitive deficits (Allen et al., 2004). Perhaps this link suggests the deficits in social behavior could be due to abnormal cerebellum functioning in the valproic acid-treated mice. In the future, it may also be interesting to determine if the social behavioral deficits in these mice could be due to damage in one of these other brain regions and if these mice have lower levels of those hypothalamic neuropeptides. In addition, deficits in social behavior in mice treated with valproic acid have been ameliorated by treatment with antioxidants (Banji et al., 2010). Therefore, social behavior should be reevaluated with the use of antioxidants. The valproic acid-treated mice had longer latencies to reach the visible platform in the water maze task. Poor performance in the visible water maze could indicate dysfunction in vision and visual neurons or the motor ability of the mice to swim (Crawley, 1999). In addition to motor neuron damage, impairments to find the visible platform have also been seen with lesions to the striatum (Packard and Teather, 1997). It is possible that cerebellar damage caused the impairments in the visible platform of the water maze and therefore, it would also be interesting to investigate cerebellar damage in adult mice as well as in the young mice.

In adult behavioral testing, some differences between wild type and Nrf2 knockout mice not dependent on valproic acid treatment. Differences between wild type mice and Nrf2 knockout mice were found in body weight, both during development and throughout adult hood. During developmental testing, Nrf2 knockout mice treated with valproic acid were found to show greater impairments compared to wild type mice treated with valproic acid however, no differences were found between saline-treated

wild type and knockout mice. In the adult behavioral tests differences between knockout mice and wild type mice, regardless of treatment were found in the water maze, elevated plus maze, and target biting. The Nrf2 knockout mice were found to be less aggressive in the target biting chambers. The brain regions associated with aggressive behavior include the hypothalamus and midbrain periaqueductal gray, and the regions of the brain associated with modulating functions include the amygdala, hippocampus, and prefrontal cortex (Siegel et al., 2007). No other tests of aggression were done in this experiment; therefore in the future another test of aggression, such as the resident intruder paradigm, may give further insight into the lack of aggression seen in the Nrf2 knockout mice. The Nrf2 knockout mice were found to be more active in the elevated plus maze, and had shorter latencies to find the hidden platform of the water maze compared to the wild type mice that indicated hyperactivity rather than a difference in anxiety level or hippocampal functioning. Adult mice were not evaluated in the open field activity chambers, and perhaps a test of baseline motor activity could indicate if these mice are hyperactive compared to the wild type mice or if they are hyperactive in a novel environment. The water maze and the elevated plus maze both serve as novel environments with for the mice to explore in short testing periods. The open field activity chambers can be run for longer trials of up to 30 minutes, taking into account early hyperactivity and habituation and then revealing a baseline activity level. Hyper activity is associated with abnormal functioning of the basal ganglia and therefore an investigation into the histopathology of the basal ganglia in the Nrf2 knockout mice may be interesting (Wichmann and Delong, 1996).

One limitation of this experiment was that the mice used in the developmental behavioral experiment were the same mice used for the adult studies. These mice have already gone through extensive behavioral testing which could result in improved performance on later behavioral tests. Although this is true, all of the mice were in the same conditions throughout both experiments, therefore any alteration in performance on behavioral tests would be parallel for all the groups and differences between groups could still be evaluated. In the future, it may be interesting to test naïve adult mice that were exposed to valproic acid on postnatal day 14.

Neurochemical analysis after amphetamine challenge showed little difference between genotypes and P14 treatment groups. No differences between wild type and Nrf2 knockout mice or differences between postnatal day 14 treatment groups in dopamine concentration in the striatum were found. All groups treated with amphetamine had a significant decrease in dopamine concentration compared to their saline-treated counterparts in the striatum. The concentrations of the two dopamine metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid, as well as the concentrations 5-hydroxytryptamine and 5-hydroxyindoleacetic acid, were not affected by amphetamine treatment in the wild type mice or the P14 saline-treated Nrf2 knockout mice in the striatum. The P14 valproic acid-treated Nrf2 knockout mice treated with amphetamine in adulthood had lower concentrations of 3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxytryptamine, and 5-hydroxyindoleacetic acid compared to the P14 valproic acid-treated Nrf2 knockout mice treated with saline as adults. No differences in 5-hydroxytryptamine or 5-hydroxyindoleacetic acid were found in the cerebellum or the hippocampus between any groups. In the cerebellum, the neurotransmitters GABA and

glutamate mediate fast excitiation and inhibition (Ottersen 1993). This experiment only evaluated 5HT and 5-HIAA in the cerebellum, but in the future the levels of GABA and glutamate should also be evaluated. The greatest effect found from the amphetamine challenge was that amphetamine caused a depletion in dopamine concentration in the striatum, which is consistent with previous reports (Halladay et al., 2000; Wagner et al.,2007). Changes in dopamine metabolite concentrations and serotonin and its metabolite concentrations were found in the striatum, but only in the Nrf2 knockout mice treated with valproic acid on postnatal day 14. While this finding is interesting, it requires further investigation with more subjects to determine if this is a real effect. The dopamine depletion that was found in these groups was approximately 50% of the saline-treated mice. The fact that no differences were found between the lesion in the wild type mice and the lesion in the knockout mice could indicate that the lesion itself was already maximal. If this is the case, then the knockout mice can't show any greater lesion because the lesion is already at its worst. Perhaps a further investigation with lower doses of amphetamine would reveal differences in sensitivity between the wild type and knockout mice. No differences were found between the valproic acid-pretreated group and the saline-pretreated group. Again this might be due to the already very large lesion.

Another important future experiment in valproic acid-treated and amphetamine-treated Nrf2 knockout mice would look into microglia activation. Microglia are the resident macrophages in the central nervous system. These cells function similarly to the immune cells outside of the central nervous system, responding to changes in homeostasis by producing and releasing cytokines and chemokines. Between 10 and 20 percent of the cells in the central nervous system are microglia (Chew et al., 2006; Imai et

al, 1997). Microglia serve many roles in the central nervous system including scavenging, phagocytosis, cytotoxicity, antigen presentation, promotion of repair, and extracellular signaling. In the early postnatal developing brain, the microglia have been implicated in synaptic pruning and phagocytosis of the unneeded synapses (Trembley et al., 2011). The microglia are also responsible for phagocytosis of apoptotic debris in the adult brain (Trembley et al., 2011). In addition to clearing of debris, these cells may also play a role in protecting and regulating the survival of neuronal cells in that they can release brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) among other neurotrophins (Ousman and Kubes, 2012). In their resting state, the morphology of these cells resembles a spider's web, with a small cell body and many long branched processes (Jonas et al., 2012). Under normal conditions, in their resting state, the microglia act as immune surveillance in the CNS using their processes to communicate with the environment (Ransohoff and Perry 2009). The cell bodies of the microglia remain in place while their processes extend and retract rapidly, avoiding contact with other microglia processes but making contact with surrounding cellular elements (Nimmerjahn et al., 2005). Microglia can become activated in response to a variety of stimuli including abnormal endogenous proteins, exogenous pathogens and injured neurons (Jonas et al., 2012). Once activated a change in morphology occurs where the branches thicken and retract and the cell body enlarges (Jonas et al., 2012). In addition to this change in morphology, the activated microglia cells produce cytokines and chemokines. In response to lipopolysaccharaide, microglia cells have been shown to release the cytokines tumor necrosis factor (TNF), interleukin 1 (IL-1), and interferons (IFNs) (Ousman and Kubes, 2012). The activated microglia cells can also present

antigens to and activate T cells. In the coronavirus-induced mouse hepatitis model, microglia upregulated the antigen presenting molecule major histocompatibility (MHC) I, which then activated $CD8^+$ T cells (Malone et al., 2008). Although the microglia can mount a strong immune defense, they are normally kept in a repressed state. The microglia are kept repressed through inihibitory inputs by the CX3CL1 receptor, CX3R1. CX3CL1 is a chemokine released by neurons in the CNS (Cardona et al., 2006). In addition to this receptor, the communication between neuronal CD200 receptor and the CD200 ligand on microglia has also been shown to repress the activation of microglia (Ousman and Kubes, 2012).

Although microglial activation has not been investigated in early exposure to valproic acid, there is evidence to suggest that valproic acid induces and immune response in rodents. Schneider et al. (2008) found that rats exposed to valproic acid on embryonic day 12.5 exhibited increased basal levels of corticosterone, decreased weight of the thymus, and decreased splenocyte proliferate response to concanavalin A. In addition, they found that the valproic acid-exposed mice had a lower interferon (IFN)-γ/IL-10 ratio and increased production of nitric oxide by macrophages both basally and in response to LPS (Schneider et al., 2008). While it is not known if these mice had increase nitric oxide in their brain, it has been suggested that peripheral nitric oxide measures could reflect central nervous system nitric oxide levels and increased nitric oxide in the brain neurodevelopment and synaptic connectivity (Schneider et al., 2008; Calabrese et al., 2007; Giovannoni et al., 1998).

Methamphetamine and MPTP have been shown to increase microglial activation in the striatum of rodents. Methamphetamine administered to Sprague-Dawley rats

induced microglial activation in the striatum, while saline-treated controls did not show morphological changes in microglia (LaVoie et al., 2004). This increase in microglia activation was found to be maximal at two days post treatment, which is prior to the appearance of dopaminerigic axonal pathology, leading the researchers to suggest microglial activation may contribute to the methamphetamine induced neurotoxicity (La Voie et al., 2004). In a study by Thomas et al. (2004), methamphetamine was found to induce a significant increase in microglial activation at the 24 hour and 48 hour time point after treatment in C57BL/6 mice. In addition, they also found that MPTP treatment resulted in increased microglial activation in the striatum of mice, consistent with other previous studies on microglial activation and MPTP.

Nrf2 knockout mice have been shown to have increased microglia activation following MPTP exposure compared to wild type mice. Inamorato et al. (2010) found Nrf2 knockout mice to have increased basal levels of microglia compared to wild type controls. In addition, they found that MPTP-treated Nrf2 knockout mice had an increase in microglia both over their baseline level and compared to the MPTP-treated wild type controls. Rojo et al. (2009) found that Nrf2 knockout mice also exhibited more microgliosis in the striatum compared to wild type controls and had a stronger response to MPTP treatment. Using microglial primary cells cultures of wild type and Nrf2 knockout mice, they found that MPP^+ induced a strong Phase II activation in wild type mice derived microglia, but not in Nrf2 knockout mice derived microglia and that the MPP^+ induced a slight increase in the expression of IL-6 and TNF-α in the wild type derived microglia and the Nrf2 knockout derived microglia had an enhanced increase in these proinflammatory markers. This lead to their conclusion that oxidative stress may

prime microglia and make them more sensitive to further inflammatory reactions (Rojo et al., 2009).

Given this information, a proposed future study would investigate microglia activation following P14 exposure to valproic acid as well as investigate microglia activation after pre-treatment with valproic acid and later adult treatment with amphetamine. There is evidence to suggest that valproic acid can cause an immune response, however microglia activation in response to valproic acid exposure has not been assessed. It has also been suggested that a possible mechanism of the neurotoxicity caused by valproic acid could be oxidative stress. Specifically, our lab has shown that at 12 and 24 hours following valproic acid exposure, mice have an increase in apoptosis in the hippocampus (Yochum et al., 2008). Therefore, it is possible that exposure to valproic acid on postnatal day 14 could result in the activation of microglia cells in the hippocampus. This experiment would investigate the activation of microglia cells 24 hours after exposure to valproic acid. In addition to investigating the effect of valproic acid on microglia activation, it would also investigate the effect of amphetamine on microglia activation. Methamphetamine and MPTP have been shown to increase activated microglia cells in the striatum of rats and mice 48 hours after exposure (LaVoie et al., 2004; Thomas et al., 2004). Not only have Nrf2 knockout mice been found to have increased basal levels of microglia activation compared to wild type controls, they also have been shown to have an increased sensitivity and increased microglia activation following MPTP exposure compared to wild type controls (Inamorato et al., 2010; Rojo et al.,2009). Given these findings, this experiment would investigate the potential activation of microglia following exposure to amphetamine in both wild type and Nrf2

knockout mice. In addition, this experiment would investigate the sensitization of pretreatment with valproic acid to microglia activation following amphetamine exposure. CD11b immunihistochemistry could be utilized to look at microglia activation. For the study looking into microglia activation following postnatal day 14 exposure to valproic acid, the mice would be sacrificed by rapid decapitation 12 or 24 hours following valproic acid exposure to be consistent with prior work in apoptosis studies. The whole brain could then be fixed in 4% paraformaldehyde and then placed in 30% sucrose until frozen microtome sectioning could be completed. The cerebellum, hippocampus, and striatum would be collected during frozen microtome sectioning. These sections could then be stained for CD11b and counterstained with methylgreen. Cell counting could then be done in sections matched according to the mouse atlas of Franklin and Paxinos (1997). Three sections per brain region should be chosen and matched sections for each mouse should be used in the cell counting. The same procedure for cell staining and counting could be used in the adult study. In the adult study, a time course should be evaluated to determine the day of sacrifice following amphetamine exposure. In our HPLC investigations, the mice are sacrificed 72 hours following exposure to amphetamine, however the literature suggests that microglia activation is maximal 48 hours after exposure to methamphetamine. This study could provide further insight into how the damage in certain brain regions is caused by valproic acid and by amphetamine. This experiment could implicate a role for microglia activation in the oxidative damage caused by valproic acid or amphetamine. If there is increased activation of microglia cells following valproic acid exposure, this might indicate that the oxidative stress induced by valproic acid could be related to microglia activation. In addition, it may provide insight

into whether pretreatment with valproic acid could result in an increased sensitivity to amphetamine. While we did not see increased sensitivity in HPLC analysis, perhaps that damage was already too great to evaluate differences and this study could investigate more subtle changes in toxicity.

Summary

Overall, valproic acid was found to induce behavioral changes in both wild type and Nrf2 knockout mice. The Nrf2 knockout mice also appear to be more sensitive to the effects of valproic acid on certain behavioral tests, including the rotorod, the water maze, and the males in open field activity. These tests are associated with cerebellar and hippocampal functioning and damage to these regions results in poor performance on these tests. Treatment with valproic acid has been shown to cause damage to the cerebellum and the hippocampus. Given this information, the Nrf2 knockout mice treated with valproic acid could have increased damage to their cerebellum compared to wild type mice with normal levels of antioxidant defense mechanisms. The Nrf2 knockout mice appear to be an excellent model to assess the effects of oxidative stress inducing neurotoxicants on behavioral development.

REFERENCES

Anand, A. and Shekar, A. Brain imaging studies in mood and anxiety disorders: special emphasis on the amygdala. *Ann. N.Y. Acad. Sci*. **985** (2003) 370-388.

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A.M., and Cook, J.L. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J. Biol. Chem.* **274** (1999) 26071–26078.

Alan, G., Muller, R.A. and Courchesne, E. Cerebellar function in autism: functional magnetic resonance image activation during a simple motor task. *Biol. Psychiatry.* **56** (2004)269-278.

Al Deeb, S., Moutaery, K.A., Arshaduddin, M. and Tariq, M. Vitamin E decreases valproic acid induced neural tube defects in mice. *Neurosci. Lett.* **292(3)** (2000) 179-182.

Altman, J. and Bayer, S.A. (1997) *Development of the Cerebellar System in Relation to its Evolution, Structure, and Functions*. CPC Press. Boca Raton.

American Psychiatric Association. (2000). *Diagnostic and statistical manual of mental disorders* (4th ed., text rev.). doi:10.1176/appi.books.9780890423349

Aoki, Y., Hashimoto, A.H., Amanuma, K., Matsumoto, M., Hiyoshi, K., Takano, H., Masumura, K., Itoh, K., Nohmi, T. and Yamamoto, M. Enhanced spontaneous and benzo(a)pyrene –induced mutations in the lung of NRf2-deficient gpt delta mice. *Cancer Res.* **67** (2007)5643-5648.

Aoki, Y., Sato, H., Nishimura, N., Takahashi, S., Itoh, K. and Yamamoto, M. Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. *Toxicol. Appl. Pharmacol.* **173** (2001)154-160.

Archer, T. and Frederiksson, A. Functional changes implicating dopaminergic systems following perinatal treatments *Dev. Pharmacol. Ther*. **18** (1992) 201–222.

Atianjoh, F.E., Landenheim, B., Krasnova, I.N., and Lud Cadet, J. Amphetamine causes dopaminevdepletion and cell death in the mouse olfactory bulb. *Eur. J. Pharmacol*. **589(1-3)** (2008) 94-97.

Bailey, A., Luthert, P., Dean, A., Harding, B., Janota, I., Montgomery, M., Rutter, M. and Lantos, P. A clinicopathological study of autism. *Brain.* **121** (1998) 889-905.

Baird, L. and Dinkova-Kostova, A.T. The cytoyprotective role of the Keap1-Nrf2 pathway. *Arch Toxicol.* **85** (2011), 241-272.

Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C.J., Foresti, R., Alam, J. and Motterlini, R. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem. J.* **371** (2003) 887–895.

Banji, D., Banji, O.J., Abbagoni, S., Hayath, M.S., Kambam, S. and Chiluka, V.L. Amelioration of behavioral aberrations and oxidative markers by green tea extract in valproate induced autism in animals. *Brain Res.* **1410** (2011)141-151.

Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Col lins, F., Shiloh, Y., Crawley, J. N., Ried, T., Tagle, D. and Wynshaw- Boris, A. Atm-deficient mice: A paradigmof ataxia telangiectasia. *Cell.* **86** (1996) 159–171.

Behar, T.N., Schaffner, A.E., Scott, C.A., Greene, C.L. and Barker, J.L. GABA receptor antagonists modulate postmitotic cell migration in slice cultures of embryonic rat cortex. *Cereb. Cortex.* **10** (2000) 899-909.

Behar, T.N., Smith, S.V., Kennedy, R.T., McKenzie, J.M., Maric, I. and Barker, J.L. $GABA_B$ receptors mediate motility signals for migrating embryonic cortical cells. *Cereb. Cortex*. **11** (2001) 744-753.

Ben-Ari, Y., Gaiarsa, J.L., Tyzio, R. and Khazipov, R. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol. Rev*. **87** (2007) 1215–1284.

Berne, R.M., Levy, M.N., Koeppen, B.M. and Stanton, B.A. (2008). *Physiology.* Elsevier Inc. Philadelphia.

Biswas, M. and Chan, J.Y. Role of Nrf1 in antioxidant response element-mediated gene expression and beyond. *Toxicol. Appl. Pharmacol.* **244(1)** (2010) 16-20.

Boron, W.F. and Boulpaep, E.L. (2005). *Medical Physiology*. Elsevier Inc. Philadelphia.

Breese, G., Baumeister,A., McCown,T., Emerick,S. Frye,G., Crotty,K. and Mueller, R. Behavioral differences between neonatal and adult 6-hydroxydopamine-treated rats to dopamine agonists: relevance to neurological symptoms in clinical syndromes with reduced brain dopamine *J. Pharmacol. Exper. Ther.* **231** (1984) 343–354.

Bregestovski, P. and Bernard, C. Excitatory GABA: how a correct observation may turn out to be an experimental artifact. *Front. Pharmacol*. **3** (2012) 65.

Burton, N.C., Kensler, T.W. and Guilarte, T.R. In vivo modulation of the Parkinsonian phenotype by Nrf2. *Neurotoxicology*. **27**(2006) 1094–1100.

Cadet, J.L., Sheng, P., Ali, S., Rothman, R., Carlson, E., Epstein, C. Attenuation of methamphetamine-induced neurotoxicity in copper/zinc superoxide dismutase transgenic mice. *J. Neurochem.* **62** (1994) 380–383.

Cagnon, L. and Braissant, O. Hyperammonemia-induced toxicity for the developing central nervous system. *Brain Res. Rev.* **56** (2007) 183–197.

Calabrese, V., Mancuso, C., Calvani, M., Rizzarelli, E., Butterfield, D.A. and Stella, A.M. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nat. Rev. Neurosci*. **8** (2007) 766–775.

Cardona, A.E., Pioro, E.P., Sasse, M.E., Kostenko, V., Cardona, S.M., Dijkstra, I.M., Huang, D., Kidd, G., Dombrowski, S., Dutta, R., Lee, J.C., Cook, D.N., Jung, S., Lira, S.A., Littman, D.R. and Ransohoff, R.M. Control of microglial neurotoxicity by the fractalkine receptor. *Nat. Neurosci*. **9(7)** (2006) 917-924.

Chan, K., Han, X.D. and Kan, Y.W. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proc. Natl. Acad. Sci. USA.* **98** (2001) 4611-4616.

Chan, K., Lu, R., Chang, J.C. and Kan, Y.W. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc. Natl. Acad. Sci. USA.* **93(24)** (1996)13943-13948.

Chapman, J.B. and Cutler, M.G. Effects of sodium valproate on development and social behavior in the Mongolian gerbil. *Neurotoxicol. Teratol.* **11(2)** (1989) 193-198.

Chateauvieux, S., Morceau, F., Dicato, M. and Diederich, M. Molecular and therapeutic potential and toxicity of valproic acid. *J. Biomed. Biotechnol.* **2010** (2010) 479364.

Chen, C.Y., Jang, J.H., Li. M.H. and Surh, Y.J. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2- related factor 2 in PC12 cells. *Biochem. Biophys. Res. Commun*. **331**(2005) 993–1000.

Chen, G., Yuan, P., Jiang, Y., Huang, L. and Manji, H.K. Valproate robustly enhances AP-1 fmediated gene expression. *Brain. Res. Mol. Brain. Res.* **64** (1999) 52-58.

Cherubini, E. , Gaiarsa, J. L. and Ben-Ari, Y. GABA: an excitatory transmitter in early postnatal life.*Trends. Neurosci*. **14** (1991) 515–519.

Chew, L.J., Takanohashi, A. and Bell, M. Microglia and inflammation: impact on developmental brain injuries. *Ment. Retard. Dev. Disabil. Res. Rev.* **12** (2006) 105–112.

Chevillard, G., Nouhi, Z., Anna, D., Paquet, M. and Blank, V. Nrf3-deficient mice are not protected against acute lung and adipose tissue damages induced by butylated hydroxytoluene. *FEBS Lett.* **584(5)** (2010) 923-928.

Chinta, S.J. and Anderson, J.K. Dopaminergic neurons. *Int. J. Biochem. Cell. Biol.* **37(5)** (2005) 942-946.

Cho, H.Y., Jedlicka, A.E., Reddy, S.P., Kensler, T.W., Yamamoto, M., Zhang, L.Y. and Kleeberger, S.R. Role of Nrf2 in protection against hyperoxic lung injury in mice. *Am. J. Respir. Cell Mol. Biol.* **26** (2002)175-182.

Chung, K.M., REavis, S., Mosconi, M., Drewry, J., Matthews, T. and Tasse, M.J. Peer-mediated social skills training program for young children with high-functioning autism. *Res. Dev. Disabil.* **28(4)** (2007) 423-436.

Cohen, I., Navarro, V., Clemenceau, S., Baulac, M. and Miles R. On the origin of interictal activity in human temporal lobe epilepsy in vitro. *Science* **298** (2002) 1418–1421.

Cope, Z.A., Huggins, K.N., Sheppard, A.B., Noel, D.M., Roane, D.S. and Brown, R.W. Neonatal quinpirole treatment enhances locomotor activation and dopamine release in the nucleus accumbens core in response to amphetamine treatment in adulthood. *Synapse*. **64** (2010) 289–300.

Crawley, J.N. and Paylor, R. A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. *Horm. Behav.* **31** (1997) 197–211.

Crawley, J. Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. *Brain Res*. **835(1)** (1999) 18–26.

Crawley, J. (2000) *What's wrong with my mouse? Behavioral phenotyping of transgenic and knockout mice.* Wiley-Liss. New York.

Critchley, H.D., Daly, E.M., Bullmore, E.T., Williams, S.C.R., Van Amelsvoort, T., Robertson, D.M., Rowe, A., Phillips, M., McAlonan, G., Howlin, P. and Murphey, D.G.M. The functional neuroanatomy of social behavior: changes in cerebral blood flow when people with autistic disorder process facial expressions. *Brain*. **123(11)** (2000) 2203-2212.

Cubells, J.F., Rayport, S., Rajendran, G. and Sulzer, D. Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress. *J. Neurosci*. **14** (1994)2260–2271.

Defoort, E.N., Kim, P.M. and Winn, L.M. Valproic acid increases conservative homologous recombination frequency and reactive oxygen species formation: a potential mechanism for valproic acid-induced neural tube defects. *Mol. Pharmacol.* **69(4)** (2006) 1304-1310.

Derjuga, A., Gourley, T.S., Holm, T.M., Heng, H.H., Shivdasani, R.A., Ahmed, R., Andrews, N.C. and Blank, V. Complexity of CNC transcription factors as revealed by gene targeting of the Nrf3 locus. *Mol. Cell. Biol*. **24(8)** (2004) 3286-3294.

Devi, L., Diwakar, L., Raju, T.R. and Kutty, B.M. Selective neurodegeneration of hippocampus and entorhinal cortex correlates with spatial learning impairments in rats with bilateral ibotenate lesions of the ventral subiculum. *Brain. Res.* **960** (2003) 9-15.

Devito, M.J. and Wagner, G.C. Methamphetamine-induced neuronal damage: a possible role for free radicals. *Neuropharmacology*. **28** (1989) 1145-1150.

Dickinson, D. A., Iles, K. E., Zhang, H., Blank, V. and Forman, H. J. Curcumin alters EpRE and AP-1 binding complexes and elevates glutamate-cysteine ligase gene expression. *FASEB J.* **17**, (2003) 473–475.

Dolske, M.C., Spollen, J., Mckay, S., Lancashire, E., and Tolbert, L. A preliminary trial of ascorbic acid as supplementation therapy for autism. *Prog. NeuroPsychopharmacol. Biol. Psychiatry.* **17** (1993) 765-774.

Dröge, W. Free radicals in the physiological control of cell function. *Physiol. Rev.* **82(1)** (2002)47-95.

Dzhala, V., Valeeva, G., Glykys, J., Khazipov, R. and Staley K. Traumatic alterations in GABA signaling disrupt hippocampal network activity in the developing brain. *J. Neurosci*. **32** (2012) 4017–4031.

Fraenkel, G.S. and Gunn, D.L. (1961) *The Orientation of Animals: Kineses, Taxes, and Compass Reactions*. Dover Publications. New York.

Franklin, K.B.J. and Paxinos, G. (1997) *The Mouse Brain in Stereotaxic Coordinates*. Academic, San Diego.

Friedman, S.D., Castañeda, E. and Hodge, G.K. Long-term monoamine depletion, differential recovery, and subtle behavioral impairment following methamphetamine-induced neurotoxicity. *Pharmacol. Biochem. Behav*. **61** (1998) 35–44.

Fridovich, I**.** Oxygen toxicity: a radical explanation. *J Exp Biol*. **201(Pt 8) (**1998)1203-1209.

Fukuchi, M., Nii, T., Ishimaru, N., Minamino, A., Hara, D., Takasaki, I., Tabuchi A. and Tsuda, M. Valproic acid induces up- or down-regulation of gene expression responsible for the neuronal excitation and inhibition in rat cortical neurons through it epigenetic actions. *Neurosci. Res.* **65(1)** (2009)35-43.

Fumagalli, F., Gainetdinov, R.R., Valenzano,K.J., Caron, M.G. Role of dopamine transporter in methamphetamine-induced neurotoxicity: evidence from mice lacking the transporter. *J. Neurosci.* **18** (1998) 4861–4869.

Funatsu, N., Inoue, T. and Nakamura, S. Gene expression analysis of the late embryonic mouse cerebral cortex using DNA microarray: identification of several region- and layer-specific genes. *Cereb. Cortex.* **14(9)** (2004) 1031-44.

Gibb, J.W., Bush, L., and Hanson, G.R. Exacerbation of methamphetamine-induced neurochemical deficits by melatonin. *J. Pharmacol. Exp. Ther*. **283(2)** (1997) 630-635.

Gimenez-Llort, L., Ahlbom, E., Dare, E., Vahter, M.,nOgren, S.O. and Ceccatelli, S. Prenatal exposure to methylmercury changes dopamine-modulated motor activity during early ontogeny: age and gender-dependent effects. *Environ. Toxicol. Pharmacol*. **9** (2001) 61–70.

Giovannoni, G., Miller, R.F., Heales, S.J., Land, J.M., Harrison, M.J. and Thompson, E.J. Elevated cerebrospinal fluid and serum nitrate and nitrite levels in patients with central nervous system complications of HIV-1 infection: a correlation with blood–brain-barrier dysfunction *J. Neurol. Sci*. **156** (1998) 53–58.

Go, H.S., Seo, J.E., Kim, K.C., Han, S.M., Kim, P., Kang, Y.S., Han, S.H., Shin, C.Y. and Ko, K.H. Valproic acid inhibits neural progenitor cell death by activation of NF-kappaB signaling pathway and up-regulation of Bcl-XL. *J Biomed Sci.* **18** (2011) 48.

Gottlicher, M., Minucci, S., Zhu, P., Kramer, O.H., Schimpf, A., Giavara, S., Sleeman, J.P., Lo Coco, F., Nervi, C., Pelicci, P.G. and Heinzel, T. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* **20(24)** (2001) 6969-6978.

Halladay, A.K., Coyne, T., Sharifi, J., Seto, J., and Wagner, G.C. Avoidance responding following amphetamine-induced dopamine depletion. *Pharmacol. Toxicol*. **87(5)** (2000)211-217.

Halladay, A.K., Kusnecov, A., Michna, L., Kita, T., Hara, C. and Wagner, G.C. Relationship between methamphetamine-induced dopamine release, hyperthermia, self-injurious behaviour and long term dopamine depletion in BALB/c and C57BL/6 mice. *Pharmacol Toxicol*. **93(1)** (2003) 33-41.

Han, D.D. and Gu, H.H. Comparison of the monoamine transporters from human and mouse in their sensitivities to psychostimulant drugs. *BMC Pharmacol*. **3(6)** (2006) 6.

Hashimoto, T., Tayama, M., Murakawa, K., Yoshimoto, T., Miyazaki, M., Harada, M. and Kuroda, Y. Development of the brainstem and cerebellum in autistic patients. *J. Autism Dev. Disord.* **25** (1995)1-18.

Hayes, J.D., Flanagan, J.U. and Jowsey, I.R. Glutathione transferases. *Anuu. Rev. Pharmacol. Toxicol.* **45** (2005)51-88.

He, X., Chen, M.G., Lin, G.X., and Ma, Q. Arsenic induces NAD(P)H-quinone oxidoreductase I by disrupting the Nrf2 x Keap1 x Cul3 complex and recruiting Nrf2 x Maf to the antioxidant response element enhancer. *J. Biol. Chem.* **281** (2006) 23620-23631.

He, X., Chen, M.G., and Ma. Q. Activation of Nrf2 in defense against cadmium-induced oxidative stress. *Chem. Res. Toxicol.* **21** (2008) 1375-1383.

Hosking, S.L. and Hilton, E.J. Neurotoxic effects of GABA-transaminase inhibitors in the treatment of epilepsy: ocular perfusion and visual performance. *Ophthalmic. Physiol. Opt*. **22(5)** (2002) 440-447.

Hong, Y., Yan, W., Chen, S., Sun, C.R. and Zhang, J.M.The role of Nrf2 signaling in the regulation of antioxidants and detoxifying enzymes after traumatic brain injury in rats and mice. *Acta Pharmacologica Sinica*. **31** (2010)1421–1430.

Hong, J.T., Ryu, S.R., Kim, H.J., Lee, J.K., Lee, S.H., Kim, D.B., Yun, Y.P., Ryu, J.H., Lee, B.M. and Kim, P.Y. Neuroprotective effect of green tea extract in experimental ischemia–reperfusion brain injury. *Brain Res. Bull.* **53** (2000) 743–749.

Hung, C.C., Li, T.M., Wei, I.H. and Huang, C.C.The real mechanism of VPA-induced hyperammonemia remains unknown. *Gen. Hosp. Psychiatry*. **33(1):84** (2011) e3-4.

Iezhitsa, I.N., Spasov, A.A. and Bugaeva, L.I. Effects of bromantan on offspring maturation and development of reflexes. *Neurotoxicol Teratol*. **23(2)** (2001) 213-222.

Imai, F., Sawada, M., Suzuki, H., Kiya, N., Hayakawa, M., Nagatsu, T., Marunouchi, T. and Kanno, T. Migration activity of microglia and macrophages into rat brain. *Neurosci. Lett.* **237** (1997) 49–52.

Ingram, J.L., Peckham, S.M., Tisdale, B. and Rodier, P.M. Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. *Neurotoxicol. Teratol.* **22(3)** (2000)319-324.

Innamorato, N.G., Jazwa, A., Rojo, A.I., García, C., Fernández-Ruiz, J., Grochot-Przeczek, A., Stachurska, A., Jozkowicz, A., Dulak, J., and Cuadrado, A. Different susceptibility to the Parkinson's toxin MPTP in mice lacking the redox master regulator Nrf2 or its target gene heme oxygenase-1. *PLoS One*. **5(7)** (2010) e11838.

Inouye, M. and Murakami, U. Temporal and spatial patterns of Purkinje cell formation in the mouse cerebellum. *J. Comp. Neurol.* **194(3)** (1980) 499-503.

Ishii, Y., Itoh, K., Morishima, Y., Kimura, T., Kiwamoto, T., Iizuka, T., Hegab, A.E., Hosoya, T., Nomura, A., Sakamoto, T., Yamamoto, M., and Sekizawa, K. Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema. *J. Immunol*. **175(10)** (2005) 6968-6975.

Itoh, K. Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Naveshima, Y. An Nrf2/small maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236** (1997) 313-322.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii,T., Igarashi, K. Engel, J.D., and Yamamoto, M. Keap1 represses nuclear activation of antioxidant response elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes. Dev.* **13** (1999) 76-86.

Jakel, R.J., Townsend, J.A., Kraft, A.D. and Johnson, J.A. Nrf2-mediated protection against 6-hydroxydopamine. *Brain Res.* **1144** (2007)192-201.

Jarrard, L.E. Selective hippocampal lesions and behavior: implications for current research and theorizing. *Hippocampus*. (1986) 93-126.

Jin, W., Wang, H., Yan, W., Zhu, L., Hu, Z., Ding, Y. and Tang, K. Role of Nrf2 in protection against traumatic brain injury in mice. *J Neurotrama.* **26(1)** (2009)131-139.

Julien, R.M., Advokat, C.D. and Comaty, J.E. (2005) *A Primer of Drug Action.* Worth publishers, New York.

Kaspar, J.W., Suryakant, K.N. and Jasiwal, A.K. Nrf2:INrf2(Keap1) signaling in oxidative stress. *Free Radical Bio. Med.* **47** (2009) 1304-1309.

Kawai, Y. and Arinze, I.J. Valproic acid-induced gene expression through production of reactive oxygen species. *Cancer Res.* **66(13)** (2006) 6563-6569.

Kim, G.Y., Kim, K.H., Lee, S.H., Yoon, M.S., Lee, H.J., Moon, D.O., Lee, C.M., Ahn, S.C., Park, Y.C. and Park, Y.M. Curcumin inhibits immunostimulatory function of dendritic cells: MAPKs and translocation of NF-kappa B as potential targets. *J. Immunol*. **174(12)** (2005) 8116-8124.

Kita, T., Paku, S., Takahashi, M., Kubo, K., Wagner, G.C. and Nakashima,T. Methamphetamine-induced neurotoxicity in BALB/c, DBA/2N and C57BL/6N mice. *Neuropharmacology*. **37** (1998) 1177–1184.

Kita,T., Matsunari,Y., Shimada,K., O'Hara, K., Wagner,G.C. and Nakashima, T. Methamphetamine-induced striatal dopamine release and behavior changes in BALBc mice. *Int. J. Dev. Neurosci*. **18** (2000) 521–530.

Kita, T., Shimada, K., Mastunari, Y., Wagner, G.C., Kubo, K. and Nakashima, T. Methamphetamine-induced striatal dopamine neurotoxicity and cyclooxygenase-2 protein expression in BALB/c mice. *Neuropharmacology*. **39(3)** (2000) 399-406.

Kobayashi, A., Ito, E., Toki, T., Kogame, K., Takahashi, S., Igarashi, K., Hayashi, N. and Yamamoto, M. Molecular cloning and functional characterization of a new Cap'n' collar family transcription factor Nrf3. *J Biol Chem.* **274(10)** (1999) 6443-6452.

Kobayashi, A., Kang, M.I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., and Yamamoto, M. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol. Cell Biol.* **24** (2004) 7130–7139.

Koch, S., Jager-Roman, E., Losche, G., Nau, H., Rating, D. and Helge, H. Antiepileptic drug treatment in pregnancy: drug side effects in the neonate and neurological outcome. *Acta. Paediatr.* **85(6)** (1996) 739-746.

Kosaka, Y., Kin, H., Tatetsu, M., Uema, I. and Takayama, C. Distinct development of GABA system in the ventral and dorsal horns in the embryonic mouse spinal cord. Brain Res. **1486** (2012) 39-52.

Lagostena, L., Rosato-Siri, M., D'Onofrio, M., Brandi, R., Arisi, I., Capsoni, S., Franzot, J., Cattaneo, A. and Cherubini, E. In the adult hippocampus, chronic nerve growth factor deprivation shifts GABAergic signaling from the hyperpolarizing to the depolarizing direction. *J. Neurosci*. **30** (2010) 885–893.

Langmann, T. Microglia activation in retinal degeneration. *J. Leukoc. Biol*. **81** (2007) 1345–1351.

Laruelle, M., Abi-Dargham, A., van Dyck, C.H., Rosenblatt, W., Zea-Ponce, Y. and Zoghi, S.S. SPECT imaging of striatal dopamine release after amphetamine challenge. *J. Nucl. Med*. **36** (1995)1182-1190.

LaVoie, M.J., Card, J.P. and Hastings, T.G. Microglial activation precedes dopamine terminal pathology in methamphetamine-induced neurotoxicity. *Exp Neurol*. **187(1)** (2004) 47-57.

Lecavalier, L. Behavioral and emotional problems in young people with pervasive developmental disorders: relative prevalence, effects of subject characteristics, and empirical classification. *J. Autism Dev. Disord.* **36(8)** (2006) 1101-1114.

Levav-Rabkin T., Melamed O., Clarke G., Farber M., Cryan J. F., Dinan T. G., Grossman Y. and Golan H. M. A sensitive period of mice inhibitory system to neonatal GABA enhancement by vigabatrin is brain region dependent. *Neuropsychopharmacology*. **35** (2010) 1138–1154.

Li, K. and Xu, E. The role and the mechanism of gamma-aminobutyric acid during central nervous system development. *Neurosci. Bull.* **24 (3)** (2008) 195–200.

Li, W., Yu, S.W. and Kong, A.N. Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain. *J. Biol. Chem.* **281** (2006) 27251-27263.

Ling, Z.D., Chang, Q., Lipton J.W., Tong, C.W., Landers, T.M. and Carvey, P.M. Combined toxicity of prenatal bacterial endotoxin exposure and postnatal 6-hyroxydopamine in the adult rat midbrain. *Neuroscience* **124** (2004) 619–628.

Lister, R.G. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology.* **92** (1987)180-185.

Liu, F., Ichihara, S., Valentine, W.M., Itoh, K., Yamamoto, M., Sheik Mohideen, S., Kitoh, J., and Ichihara, G. Increased susceptibility of Nrf2-null mice to 1-bromopropane-induced hepatotoxicity. *Toxicol. Sci*. **115(2)** (2010) 596-606.

Logue, S.F., Paylor, R. and Wehner, J.M. Hippocampal lesions cause learning deficits in inbred mice in the Morris water maze and conditioned-fear task. *Behav. Neurosci.* **111**(1998) 104-113.

Lombardo S. A., Leanza G., Meli C., Lombardo M. E., Mazzone L., Vincenti I. and Cioni M. Maternal exposure to the antiepileptic drug vigabatrin affects postnatal development in the rat. *Neurol. Sci*. **26** (2005) 89–94.

Luchins, D.J. SIB: introduction *Prog. Neuro-Psychopha.* **14** (1990) S63–64.

Malone, K.E., Stohlman, S.A., Ramakrishna, C., Macklin, W. and Bergmann, C.C. Induction of class I antigen processing components in oligodendroglia and microglia during viral encephalomyelitis. *Glia*. **56** (2008) 426–435.

Manent, J.B., Jorquera, I., Mazzucchelli, I., Depaulis, A., Perucca, E., Ben-Ari, Y. and Represa, A. Fetal exposure to GABA-acting antiepileptic drugs generates hippocampal and cortical dysplasias. *Epilepsia.* **48(4)** (2007) 684-93.

McCann, U.D., Wong,D.F., Yokoi, F., Villemagne, V., Dannals, R.F. and Ricaurte, G.A. Reduced striatal dopamine transporter density in abstinent methamphetamine and methcathinone users: evidence from positron emission tomography studies with [^{11}C]WIN-35,428. *J. Neurosci.* **18(20)** (1998) 8417–8422.

McCord, J.M. and Day, E.D. Superoxide dependent production of hydroxyl radical catalyzed by iron-EDTZ complex. *FEBS Lett.* **86(1)** (1978) 139-142.

McMahon, M., Itoh, K., Yamamoto, M. and Hayes, J.D. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of

antioxidant response element-driven gene expression. *J. Boil. Chem*. **278** (2003) 21592-21600.

McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., and Hayes, J.D. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. *J. Biol. Chem.* **281** (2006) 24756-24768.

Meneghini, R. Iron homeostasis, oxidative stress, and DNA damage. *Free Radic. Biol. Med.* **23** (1997) 783-792.

Mesdjian, E., Ciesielski, L., Valli, M., Bruguerolle, B., Jadot, G., Bouyard, P. and Mandel, P. Sodium valproate: kinetic profile and effects on GABA levels in various brain areas of the rat. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* **6(3)** (1982) 223-233.

Moi, P., Chan, K., Asunis, I., Cao, A. and Kan, YW. Isolation of NF-E2-related factor 2(Nrf2), a NF-E2 like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. USA.* **91(21)** (1994) 9926-9930.

Morimitsu, Y., Nakagawa, Y., Hayashi, K., Fujii, H., Kumagai, T., Nakamura, Y., Osawa, T., Horio, F., Itoh, K., Iida, K., Yamamoto, M. and Uchida, K. A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. *J. Biol. Chem*. **277(5)** (2002) 3456–3463.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P. and O'Keef, J. Place navigation impaired in rats with hippocampal lesions. *Nature.* **297** (1982) 681-683.

Moore, S.J., Turnpenny, P., Quinn A., Glover, S., Lloyd, D.J., Montgomery, T. and Dean, J.C. A clinical study of 57 children with fetal anticonvulsant syndromes. *J. Med. Genet.* **7(7)** (2000) 489-497.

Na, L., Wartenberg, M., Nau, H., Hescheler, J. and Sauer, H. Anticonvulsant valproic acid inhibits cardiomyocyte differentiation of embryonic stem cells by increasing intracellular levels of reactive oxygen species. *Birth Defects Res.* **67** (2003) 174-180.

Nguyen, T., Huang, H.C., and Pickett, C.B. Transcriptional regulation of the antioxidant response element. *J. Biol. Chem.* **275** (2000)15466-15473.

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308** (2005)1314–1318.

Ohtsuji, M., Katsuoka, F., Kobayashi, A., Aburatani, H., Hayes, J.D. and Yamamoto, M. Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes. *J. Biol. Chem.* **283** (2008) 33554–33562.

Ottersen, O.P. Neurotransmitters in the cerebellum. *Rev Neurol (Paris).* **149(11)** (1993) 629-36.

Ousman, S. and Kubes, P. Immune surveillance in the central nervous system. *Nat. Neurosci.* **15** (2012) 1096–1101.

Packard, M.G. and Teather, L.A. Double dissociation of hippocampal and dorsal-striatal memory systems by posttraining intracerebral injections of 2-amino-5-phosphonopentaonic acid. *Behav. Neurosci*. **111**(1997) 543-551.

Pepe, A.E., Xiao, Q., Zampetaki, A., Zhang, Z., Kobayashi, A., Hu, Y. and Xu, Q. Crucial role of nrf3 in smooth muscle cell differentiation from stem cells. *Circ Res.* **106(5)** (2010) 870-879.

Petrosini, L., Molinari, M. and Gremoli, T. Hemicerebellectomy and motor behaviour in rats. I. Development of motor function after neonatal lesion. *Exp. Brain Res*. **82(3)** (1990) 472-482.

Ponka, P. Cell biology of heme. *Am J Med Sci*. **318(4)** (1999) 241-256.

Rahman, I., Biswas, S.K. and Kirkham, P.A.. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem. Pharmacol*. **72 (11)** (2006) 1439–1452.

Ramos-Gomez, M., Dolan, P.M., Itoh, K., Yamamoto, M. and Kensler, T.W. Interactive effects of nrf2 genotype and olitpraz on benzo[a]pyrene-DNA adducts and tumor yield in mice. *Carcinogenesis.* **24** (2003)461-467.

Rasmussen, E.B. and Newland, M.C. Developmental exposure to methylmercury alters behavioral sensitivity to D-amphetamine and pentobarbital in adult rats *Neurotoxicol. Teratol.* **23** (2001) 45–55.

Rice, D. and Barone**,** S. Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ. Health. Perspect*. **108 Suppl 3** (2000) 511-533.

Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M. and Kaila, K.. The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature.* **397** (1999) 251–255.

Rodier, P.M., Ingram, J.L., Tisdale, B. and Croog, V.J. Linking etiologies in human and animal models: studies of autism. *Reprod Toxicol.* **11(2-3)** (1997) 417-422.

Rojo, A.I., Innamorato, N.G., Martín-Moreno, A.M., De Ceballos, M.L., Yamamoto, M. and Cuadrado, A. Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. *Glia*. **58(5)** (2010) 588-98.

Ross, D. Functions and distribution of NQO1 in human bone marrow: potential clues to benzene toxicity. *Chem Biol Interact.* **153-154** (2005)137-136.

Rushmore, T.H., Morton, M.R., and Pickett, C.B. The antioxidant responsive element. *J. Biol. Chem.* **266(18)** (1991) 11632-11639.

Russo, A., Longo, R. and Vanella, A. Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. *Fitoterapia.*. **73 Suppl 1** (2002) S21-9.

Sandyk, R. Self-mutilation in Tourette's syndrome. *J. Child Neurol*. **3** (1988) 147–148.

Sango, K., Yamanaka, S., Hoffmann, A., Okuda, Y., Grinberg, A., Westphal, H., McDonald, M. P., Crawley, J. N., Sandhoff, K., Su- zuki, K., and Proia, R. L. Mouse models of Tay-Sachs and Sandhoff diseases differ in neurological phenotype and ganglioside metabolism. *Nature Genet.* **11** (1995)170–176.

Sankaranarayanan, K. and Jaiswal, A.K. Nrf3 negatively regulates antioxidant-response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *J Biol Chem.* **279(49)** (2004) 50810-50817.

Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K. and Przewlocki, R. Gender-specific behavioral and immunological alterations in animal model of autism induced by prenatal exposure to valproic acid. *Psychoneuroendocrino.* **33(6)** (2008) 728-740.

Shah, Z.A., Li, R.C., Thimmulappa, R.K., Kensler, T.W., Yamamoto, M., Biswal, S. and Dore, S. Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury. *Neuroscience.* **147** (2007) 53–59.

Shen, G., Xu, C., Hu, R., Jain, M.R., Nair, S., Lin, W., Yang, C.S., Chan, J.Y. and Kong, A.N. Comparison of (-)-epigallocatechin-3-gallate elicited liver and small intestine gene expression profiles between C57BL/6J mice and C57BL/6J/Nrf2 (-/-) mice. *Pharm. Res.* **22(11)** (2005) 1805-20.

Siegel, A., Bhatt, S., Bhatt, R. and Zalcman, S.S. The neurobiological bases for development of pharmacological treatments of aggressive disorders. *Curr Neuropharmacol*. **5(2)** (2007) 135-47.

Stahl, S.M. (2000).*Essential Psychopharmacology*. Cambridge University Press, New York.

Stefovska, V.G., Uckermann, O., Czuczwar, M., Smitka, M., Czuczwar, P., Kis, J., Kaindl, A.M., Turski, L., Turski, W.A. and Ikonomidou, C. Sedative and anticonvulsant drugs suppress postnatal neurogenesis. *Ann Neurol.* **64(4)** (2008) 434-445.

Surh, Y.J. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer.* **3(10)** (2003) 768-80.

Tabatabaei, A.R. and Abbott, F.S. Assessing the mechanism of metabolism-dependent valproic acid-induced in vitro cytotoxicity. *Chem Res Toxicol.* **12** (1999) 323-330.

Takayama, C. and Inoue, Y.Developmental localization of potassium chloride co-transporter 2 (KCC2) in the Purkinje cells of embryonic mouse cerebellum. *Neurosci Res.* **57(2)** (2007) 322-325.

Tanaka, N., Ikeda, Y., Ohta, Y., Deguchi, K., Tian, F., Shang, J., Matsuura, T. and Abe, K. Expression of Keap1-Nrf2 system and antioxidative proteins in mouse brain after transient middle cerebral artery occlusion. *Brain Res*. **1370** (2011)246–253.

Thomas, D.M., Walker, P.D., Benjamins, J.A., Geddes, T.J. and Kuhn, D.M. Methamphetamine neurotoxicity in dopamine nerve endings of the striatum is associated with microglial activation. *J Pharmacol Exp Ther.* **311(1)** (2004)1-7.

Tilz, C., Wang-Tilz, Y., Jünemann, A., Stefan, H. and Michelson, G. Visual field defect during therapy with valproic-acid. *Eur. J. Neurol*. **14(8)** (2007) 929-932.

Tremblay, M.È., Stevens, B., Sierra, A., Wake, H., Bessis, A. and Nimmerjahn, A. The role of microglia in the healthy brain. *J. Neurosci.* **31(45)** (2011) 16064-16069.

Tyzio R, Represa A, Jorquera I, Ben-Ari Y, Gozlan H, Aniksztejn L. 1999. The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. J Neurosci19:10372–82.

van den Pol, A. N., Obrietan, K.. and Chen, G. Excitatory actions of GABA after neuronal trauma. *J. Neurosci*. **16** (1996) 4283–4292.

Venugopal, J. and Jaiswal, A.K. Nrf1 and Nrf2 positively and C-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinine oxidoreductase1 gene. *Proc. Natl. Acad. Sci. USA* **93** (1996) 14960-14965.

Verrotti, A., Trotta, D., Morgese, G. and Chiarelli, F. Valproate-induced hyperammonemic encephalopathy. *Metab Brain Dis*. **17(4)** (2002) 367-73.

Wagner, G.C., Carelli, R.M. and Jarvis, M.J. Pretreatment with ascorbic acid attenuates the neurotoxic effects of methamphetamine in rats. *Res. Commun. Chem. Path.* **47** (1985) 221–228.

Wagner, G.C., Avena, N., Kita,T., Nakashima, T., Fisher,H. and Halladay, A.K. Risperidone reduction of amphetamine-induced self-injurious behavior in mice. *Neuropharmacology*. **46** (2004) 700–708.

Wagner, G.C., Reuhl, K.R., Cheh, M., McRae, P., and Halladay, A.K. A new neurobehavioral model of autism in mice: pre- and postnatal exposure to sodium valproate. *J. Autism Dev. Disord.* **36** (2006) 779-793.

Wagner, G.C., Reuhl, K.R., Ming, X. and Halladay, A.K. Behavioral and neurochemical sensitization to amphetamine following early postnatal administration of methylmercury (MeHg). *Neurotoxicology*. **28(1)** (2007)59-66.

Wahlstrom, B. and Blennow, G. A study on the fate of curcumin in the rat. *Act. Pharmacol. Toxicol.* **43** (1978) 86–92.

Wakabayashi, N., Itoh, K, Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., Imakado, S., Kotsuji, T., Otsuka, F., Roop, D.R., Harada, T., Engel, J.D, and Yamamoto, M. Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat. Genet.* **35** (2003) 238-245.

Walle, T., Hsieh, F., DeLegge, M.H., Oatis, J.E. Jr. and Walle, U.K.High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab Dispos*. **32(12)** (2004)1377-82.

Wersinger, S.R., Caldwell, H.K., Christiansen, M. and Young, W.S. 3rd. Disruption of the vasopressin 1b receptor gene impairs the attack component of aggressive behavior in mice.*Genes Brain Behav.* **6(7)** (2007) 653-60.

Wersinger SR, Kelliher KR, Zufall F, Lolait SJ, O'Carroll AM, Young WS 3rd. Social motivation is reduced in vasopressin 1b receptor null mice despite normal performance in an olfactory discrimination task. *Horm. Behav*.**46(5)** (2004) 638-45.

Wichmann, T. and DeLong, M.R. Functional and pathophysiological models of the basal ganglia. *Curr. Opin. Neurobiol.* **6 (6)** (1996)751–8.

Woolverton, W.L., Ricaurte, G.A., Forno, L.S. and Seiden, LS. Long-term effects of chronic methamphetamine administration in rhesus monkeys. *Brain Res*. **486** (1989) 73–78.

Wu, Y. and Wang, L. The effects of antiepileptic drugs on spatial learning and hippocampal protein kinase C gamma in rats. *Brain Dev.* **24(2)** (2002) 82-87.

Yamamoto, B.K., Moszczynska, A., and Gudelsky, G.A. Amphetamine toxicities: classical and emerging mechanisms. *Ann. NY. Acad. Sci.* **1187** (2010) 101-21.

Yan, W., Wang, H.D., Feng, X.M., Ding, Y.S., Jin, W. and Tang, K..The expression of NF-E2-related factor 2 in the rat brain after traumatic brain injury. *J. Traum.* **66** (2009) 1431–1435.

Yochum, C.L., Dowling, P., Reuhl, K., Wagner, G.C. and Ming, X. VPA-induced apoptosis and behavioral deficits in neonatal mice. *Brain Res.* **1203** (2008)126-132.

Yu, R., Jiao, J.J., Duh, J.L., Gudehithlu, K., Tan, T.H. and Kong, A.N. Activation of mitogen-activated protein kinases by green tea polyphenols: potential signaling pathways in the regulation of antioxidant-responsive element-mediated phase II enzyme gene expression. *Carcinogenesis*. **18** (1997) 451–456.

Yuasa, S., Kawamura, K., Kuwano, R. and Ono, K. Neuron-glia interrelations during migration of Purkinje cells in the mouse embryonic cerebellum. *Int. J. Dev. Neurosci*. **14** (1996) 429–438.

Zeise, M.L., Lasparow, S. and Zieglgansberger, W. Valproate suppresses N-methyl-o-aspartate evoked, transient depolarizations in the rat neocortex in vitro. *Brain Res*. **544** (1991) 345-348.

Zhao, X., Sun, G., Zhang, J., Strong, R., Dash, P.K., Kan, Y.W., Grotta, J.C. and Aronowski, J. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. *Stroke*. **38** (2007) 3280–3286.

Zhang, Y., Kobayashi, A., Yamamoto, M. and Hayes, J.D. The Nrf3 transcription factor is a membrane-bound glycoprotein targeted to the endoplasmic reticulum through its N-terminal homology box 1 sequence. *J Biol Chem.* **284(5)** (2009) 3195-3210.

Zhang, Y., Crouch, D.H., Yamamoto, M. and Hayes, J.D. Negative regulation of the Nrf1 transcription factor by its N-terminal domain is independent of Keap1: Nrf1, but not Nrf2, is targeted to the endoplasmic reticulum. *Biochem. J.* **399** (2006) 373–385.

Zilberter, Y., Zilberter, T. and Bregestovski, P. Neuronal activity in vitro and the in vivo reality: the role of energy homeostasis. *Trends Pharmacol. Sci.* **31**(2010) 394–401.

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Certificate in Behavioral Pharmacology **Summer 2009**
Internship at Wyeth Pharmaceuticals, Princeton, NJ
Anxiety/Depression In Vivo Group

PUBLICATIONS

Furnari, M.A., Jobes, M.L., Nekrasova, T., Minden, A. and Wagner, G.C. Functional deficits in *Pak5*, *Pak6*, and *Pak5/Pak6* knockout mice. PLOS ONE 2013; 8(4): e61321.

Furnari, M., Saw, C.L., Kong, A-N. T. and Wagner, G.C. Animal model of autistic regression: Link to toxicant-induced oxidative stress. In: *Oxidative Stress in Basic Research and Clinical Practice: Studies on Psychiatric Disorders*; Dietrich-Maszalska, A., Gagnon, S. and Chauhan, V. Eds. Springer Press, New York, (invited chapter, in press).

Furnari, M.A., Jobes, M.L., Nekrasova, T., Minden, A. and Wagner, G.C. Differential sensitivity of PAK5, PAK6, and PAK5/PAK6 double-knockout mice to the stimulant effects of amphetamine and exercise-induced alterations in body weight. In: Nutritional Neuroscience (under revision).

Furnari, M.A., Saw, C.L.L., Kong, A.N., and Wagner, G.C. Altered behavioral development in Nrf2 knockout mice following early postnatal exposure to valproic acid. In: Behavioral Brain Research (submitted)