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Altered behavioral development in Nrf2 knockout mice following early postnatal exposure to valproic acid

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Abstract

Early exposure to valproic acid results in autism-like neural and behavioral deficits in humans and other animals through oxidative stress-induced neural damage. In the present study, valproic acid was administered to genetically altered mice lacking the Nrf2 (nuclear factor-erythroid 2 related factor 2) gene on postnatal day 14 (P14). Nrf2 is a transcription factor that induces genes that protect against oxidative stress. It was found that valproic acid-treated Nrf2 knockout mice were less active in open field activity chambers, less successful on the rotorod, and had deficits in learning and memory in the Morris water maze compared to the valproic acid-treated wild type mice. Given these results, it appears that Nrf2 knockout mice were more sensitive to the neural damage caused by valproic acid administered during early development.

Keywords

Nrf2; Knockout mice; Valproic acid; Developmental behavior; Rotorod; Water maze

1. Introduction

Oxidative stress can occur when there is an overproduction of reactive oxygen species (ROS) and a lack of antioxidant defense mechanisms in an organism. 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 damage (Kaspar et al., 2009). This damage can eventually lead to a variety of disease states such as rheumatoid arthritis, Parkinson's disease, Alzheimer's disease, cystic fibrosis, and cancer (Dröge, 2002). Most relevant to the present study, oxidative stress has

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Conflicts of interest

The authors declare that there are no conflicts of interest.

been implicated in the etiology of autism (James et al., 2004; Ming et al., 2005; Chauhan and Chauhan, 2006).

Valproic acid is a branched short-chain fatty acid that is used clinically in adults as an anticonvulsant agent (Chateauvieux et al., 2010) acting primarily as a GABA (gamma amino butyric acid) agonist (Fukuchi et al., 2009; Mesdjian et al., 1982). However, valproic acid exposure *in utero* and early postnatally has been shown to cause autism-like neural and behavioral deficits in humans and rodents (Rodier et al., 1997; Ingram et al., 2000; Rasalam et al., 2005; Wagner et al., 2006). The neural damage caused by early exposure to valproic acid appears to be due to oxidative stress (Tabatabaei and Abbott, 1999; Cheh et al., 2010). In the developing mammal, GABA is an excitatory neurotransmitter and, therefore, GABA agonists such as valproic acid induce excitotoxic levels of ROS (Levav-Rabkin et al., 2010; Lombardo et al., 2005). Mice exposed to valproic acid on embryonic day 13 or postnatal day 14 have been shown to exhibit developmental behavioral deficits that are similar to deficits observed in autism as well as enhanced neuronal death in the cerebellum and hippocampus, regions known to be affected in humans with autism (Wagner et al., 2006; Banji et al., 2011; Yochum et al., 2008). Thus, early exposure to valproic acid has been used as an animal model of autism (Rodier et al., 1997; Wagner et al., 2006).

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; Sandberg et al., 2014). Nrf2 induces enzymes and other proteins that protect against oxidative stress through transcriptional activation of the antioxidant response element (ARE) of their genes (Alam et al., 1999; Nguyen et al., 2000; Venugopal and Jaiswal, 1996). 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 is highly expressed in kidney, muscle, lung and heart and is expressed but to a much lesser degree in brain (Moi et al., 1994). Nrf2 knockout mice have been shown to have enhanced susceptibility to various environmental toxicants (Itoh et al., 1997; Liu et al., 2010; Ishii et al., 2005; Innamorato et al., 2010) but their response to early valproic acid has not been assessed.

There is evidence to suggest a link between valproic acid and the redox sensitive transcription factor Nrf2. Valproic acid has been shown to increase levels of reactive oxygen species. This increase in reactive oxygen species induced by valproic acid has been implicated in the toxicity caused by early exposure to valproic acid, including neural tube defects (Defoort et al., 2006) as well as the long-lasting behavioral deficits (Cheh et al., 2010). As noted, Nrf2 induces the expression of genes containing the ARE (including HO-1, NQO1, GST, and Gα₁₂) and valproic acid has been shown to induce gene transcription in an ARE-dependent manner for these same genes (Kawai and Arinze, 2006). This provides evidence of a link between the 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, there should be increased sensitivity of the Nrf2 knockout mice to the toxicity induced by valproic acid.

In the present study, Nrf2 knockout mice and C57BL/6J wild type mice were evaluated for behavioral differences following early valproic acid exposure with the prediction that the developmental maturation of these behavioral skills would be more severely affected in the knockout mice as compared to their wild type controls. Several behavioral tests were used that evaluate normal developmental skills, such as surface righting and hanging wire grip strength. Other behavioral tests were used because they are associated with the specific brain regions known to be affected by valproic acid including the rotorod to evaluate cerebellar function and the water maze to evaluate hippocampal function. It was hypothesized that valproic acid administered to Nrf2 knockout mice should cause deficits in these behaviors to a greater extent than that observed in wild type controls.

2. Experimental procedure

2.1. Animals

Four female and two male C57BL/6J mice were purchased from 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, 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 h 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.

2.2. Postnatal valproic acid administration

The litters from these breeding pairs were randomly assigned to either valproic acid treatment group or saline group. The day of birth was considered postnatal day 0. Based upon prior studies, the pups received either saline or 400 mg/kg of valproic acid on postnatal day 14 by subcutaneous injection (Wagner et al., 2006; Yochum et al., 2008). Behavioral testing began on postnatal day 5 of life and continued until postnatal 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 h following the beginning of the light cycle in the colony room. All weights were taken just prior to behavioral testing.

2.3. Behavioral assays

A minimum of 10 mice per genotype per treatment group per sex were randomly selected and evaluated in the behavioral experiments. An *N* of 10 is sufficient for meaningful

statistical interpretations of behavioral experiments (Crawley, 2000). For each experimental group, mice from at least three separate litters were used to make up the *N* of 10. Each mouse was challenged in all of the behavioral tests according to their postnatal day of life. The tests were run in a counter-balanced sequence each day between postnatal day 13 and postnatal day 19. The mice were given a minimum of 10 min rest between behavioral tests.

2.3.1. 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 s was recorded if the mouse failed to right in that time period.

2.3.2. 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 two out of three successful mid-air righting attempts was recorded as ability to right on each day.

2.3.3. 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 through postnatal day 19. The mouse was placed on a grid wire surface (30 cm × 18 cm) on a 45° incline facing downward. The latency to turn 180° such that the head was facing upward along the incline was recorded with a maximum of 30 s for each trial.

2.3.4. 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 s for each trial.

2.3.5. 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 × 22 cm × 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 min for a total of 10 min 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.

2.3.6. Rotorod—On postnatal day 20 through postnatal day 22, each mouse was placed on the rotorod with a circumference of 6 in., rotating at 12 revolutions per minute. The rotorod was 60 in. 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 s. 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.

2.3.7. Morris water maze—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. 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 3/4 full with water maintained at 23–26 °C and made opaque with white non-toxic latex paint.

2.3.7.1. Visible platform: A starting point was determined randomly from one of the four equally spaced quadrants. In the visible platform paradigm of the water maze performed on postnatal day 20, 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 s trial to find the visible platform in each of five different positions in the water maze.

2.3.7.2. Hidden platform: 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 five trials a day starting at different locations in the maze to find the hidden platform. Again, a maximum of 60 s was given for each trial in both the hidden or visible platform versions.

2.3.8. 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 χ^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. When no overall effect of sex was found, the male and female data were combined.

3. Results

3.1. 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 postnatal day 16 were not found to be significantly different from each other [Fig. 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-treatment 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 6.816 g. 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 postnatal day 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.

3.2. 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. No significant difference between wild type mice and Nrf2 knockout mice was found [Fig. 2].

3.3. Mid-air righting

χ^2 analysis revealed no difference between the saline-treated wild type and saline-treated knockout mice ($\chi^2(6) = 2.74, p = 0.840$) and no difference between the valproic acid-treated wild type mice and the valproic acid-treated knockout mice ($\chi^2(6) = 9.26, p = 0.159$). The χ^2 analysis did reveal an overall significant effect of p14 valproic acid treatment on mid-air righting in the wild type mice ($\chi^2(6) = 17.1, p = 0.009$) and in the Nrf2 knockout mice ($\chi^2(6) = 52.4, p < 0.0001$). Fisher's exact test further revealed that there was a significant impairment of valproic acid-treated mice on P15 ($p = 0.0032$) [Fig. 3].

3.4. Negative geotaxis

A repeated measures ANOVA was used to analyze the average latency to turn 180° on a 45° inclined plane over 7 days of testing. An overall effect of genotype [$F(1,102) = 5.389, p = 0.0223$] was found as an overall effect of day of testing [$F(6,612) = 2.195, p = 0.0419$] and a day by treatment interaction [$F(6,612) = 2.982, p = 0.0070$] [Fig. 4a].

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 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 ($p = 0.3972$) [Fig. 4b].

3.5. Hanging wire grip strength

A repeated measures ANOVA was used to analyze the average latency to fall from a wire over 7 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.

3.5.1. Males—A repeated measures ANOVA was used to analyze the average latency to fall from a wire over 7 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 [Fig. 5a].

3.5.2. 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 ($p = 0.0395$) and that, specifically, the valproic acid-treated wild type mice performed significantly worse than the saline-treated wild type mice ($p = 0.0182$), while the valproic acid-treated knockout mice performed similarly to the saline-treated knockout mice ($p = 0.6727$) [Fig. 5b].

3.6. 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.

3.6.1. 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 ($p = 0.0359$). 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 difference over days of testing was also only found to be significant for the Nrf2 knockout mice [Fig. 6a].

3.6.2. 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 ($p < 0.0001$). 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 ($p = 0.0273$) [Fig. 6b].

3.7. Rotorod

A repeated measures ANOVA was used to analyze the average latency to fall from the rotorod over three trials a day for 3 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 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 ($p = 0.0043$) [Fig. 7].

3.8. Morris water maze

3.8.1. 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 five 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. *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 ($p = 0.0532$) [Fig. 8].

3.8.2. 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. 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 [$F(3,294) = 2.819, p = 0.0393$]. *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 [Fig. 9].

4. Discussion

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 differences in body weight between the valproic acid-treated wild type mice and the valproic acid-treated Nrf2 knockout mice. The one behavioral test run before exposure to valproic acid, the surface righting test, did not reveal differences between Nrf2 knockout mice and wild type mice. However, the Nrf2 knockout mice were more sensitive to the effects of valproic acid on certain behavioral tests, including the rotarod, the water maze, and open field activity.

Significant differences in body weight were found over the course of the developmental testing period. Prior to any treatment, the Nrf2 knockout mice weighed slightly more than the wild type mice but this weight difference did not translate into any differences in their performance of the behavioral tasks. Valproic acid treatment on P14 resulted in an equal drop in body weight in both genotypes as compared to the saline treatment. The valproic acid-treated wild type mice and valproic acid-treated knockout mice did not differ in weight but they performed differently on the behavioral tests with the valproic acid-treated wild type mice generally doing 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, the wild type mice and Nrf2 knockout mice appear to develop similarly as assessed by 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 mice improved and were able to right with shorter latencies from P5 through P9, at which point the latency had stabilized.

The mid-air righting test, the negative geotaxis test, and the hanging wire grip strength test provided evidence of a detrimental effect of valproic acid on these behaviors. Mid-air righting normally develops in mice around postnatal day 14 and reflects the development of dynamic postural adjustments mediated by the cerebellum (Iezhitsa et al., 2001; Petrosini et al., 1990). As with surface righting, mice of both genotypes were successful in mid-air righting prior to treatment with valproic acid. On postnatal day 15, 24 h after valproic acid exposure, the valproic acid-treated mice failed to mid-air right, while the saline-treated mice continued to improve. Rats with a hemicerebellectomy show a delay in the development of this righting reflex (Petrosini et al., 1990). The significant drop in the percent of mice able to mid-air right on postnatal day 15 is not a pharmacological effect of residual valproic acid but, rather, could be the result of cerebellar damage (Wagner et al., 2006; Yochum et al., 2008).

As with mid-air righting, the negative geotaxis response matures around postnatal day 14 and evaluates vestibular or pro-prioceptive function and cerebellar development (Fraenkel and Gunn, 1961; Petrosini et al., 1990). The results of this test were somewhat variable and difficult to interpret. It was observed that the valproic acid-treated wild type mice performed significantly worse than the valproic acid-treated knockout mice, which is opposite our hypothesis. We also observed that as the saline-treated mice became comfortable with the incline, many of the mice began to explore the apparatus rather than turn 180°. It should also be noted that the latency to turn 180° for all of the groups on P14 is very fast and, therefore, testing earlier in life should be considered for future studies.

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. Normally a mouse can hang for several minutes, but a 60 s maximum can be used (Crawley, 2000). A significant effect of sex was found with the valproic acid-treated females performing worse 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, 1999). All of the mice, regardless of treatment or genotype, did improve their latency to hold onto the wire over the days of testing.

The open field activity chambers measured the horizontal loco-motor activity of the animal. 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. This indicated that treatment with valproic acid on postnatal day 14, 6 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. In general, motor activity has been 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). 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 in the activity chambers 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 rotarod tests motor function and coordination in addition to testing motor learning requiring cerebellar function. An overall effect of genotype, treatment and day of trial was found. 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. 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). As discussed previously in regard to the deficits in locomotor activity, the results of the rotarod 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; 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 significantly better in the wild type mice than the Nrf2 knockout mice. Further, the saline-treated mice were able to find the visible platform significantly faster than their valproic acid-treated counterparts. 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). The Nrf2 knockout mice did improve significantly over five 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 it appears that they do not have impairments in their motorabilities. There are reports of vision defects in individuals taking valproic acid, however it is related to long term treatment and to the GABAergic effects of valproic acid (Hosking and Hilton, 2002; Tilz et al., 2007). Since the water maze test was run 6 days after the valproic acid exposure, there is no longer GABA agonist action. Although a full evaluation of the visual acuity of the mice was not undertaken, it seems unlikely that this is the reason for the deficits found in the valproic-acid treated mice. Impairments in performing the visible platform component of the water maze have also been attributed to motor deficits associated with damage to the striatum (Packard and Teather, 1997). Thus, 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). In the Yochum et al. (2008) study, striatal damage was not observed.

The hidden platform component of the water maze has been associated with hippocampal mediated spatial learning and memory (Crawley, 1999). There again was an effect of valproic acid treatment and an effect of genotype. The valproic acid-treated mice of both genotypes performed significantly worse compared with their saline-treated counterparts and 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 associated with hippocampus functioning and hippocampal lesions impair acquisition of this task in rats and mice (Morris et al., 1982; Logue et al., 1998). The poor performance of the valproic acid-treated animals may indicate damage to the hippocampus as previously reported (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). However, as noted, the deficits in performance of the visible platform

component of the water maze indicate that additional tests of cognitive performance are warranted.

5. Conclusions

These findings indicate that early postnatal treatment with valproic acid results 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 h after P14 treatment with valproic acid, there is increased apoptosis in the cerebellum and hippocampus of mice (Yochum et al., 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. Finally, histopathological studies are now underway to determine if there might be neurobiological correlates of the enhanced behavioral deficits observed in the Nrf2 knockout mice following valproic acid.

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Fig. 1.

Total body weight change from P5 to 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 ($p < 0.0001$). Please see Section 3 for full explanation of significant values. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid.

Fig. 2. Surface righting. The latency to turn over was found to significantly improve from P5 through P7 for both genotypes ($p < 0.0001$). α denotes $p < 0.05$ compared to P5.

Fig. 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 ($p < 0.0001$). β denotes $p < 0.05$ compared to within genotype saline-treated counterparts. γ denotes $p < 0.05$ compared to other days of testing. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid.

Fig. 4.

(a) Latency to turn 180°. An overall effect of genotype [$F(1,102) = 5.389$, $p = 0.0223$] was found as 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. (b) Average total latency to turn 180°. VPA-treated WT mice were significantly slower to turn 180° compared with the VPA-treated KO mice ($p = 0.0107$). ε denotes $p < 0.05$ compared to WT, VPA. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid.

Hanging Wire (Males)

Hanging Wire (Females)

Fig. 5.

(a) 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. (b) Female latency to fall from wire. VPA-treated mice performed significantly worse compared with the saline-treated mice ($p = 0.0395$).

Specifically, the VPA-treated WT mice performed significantly worse than the saline-treated WT mice ($p = 0.0182$), while the VPA-treated KO mice performed similarly to the saline-

treated KO mice ($p = 0.6727$). δ denotes $p < 0.05$ compared to WT, Saline. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid.

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Fig. 6.

(a) 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 ($p = 0.0004$). β denotes $p < 0.05$ compared to within genotype saline-treated mice. ϵ denotes $p < 0.05$ compared to WT, VPA. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid. (b) Female average total beam breaks. VPA-treated mice were significantly less active

compared with the saline-treated mice ($p < 0.0001$). β denotes $p < 0.05$ compared to within genotype saline-treated mice. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid.

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Fig. 7.

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 ($p = 0.0043$). γ denotes $p < 0.05$ compared to P20. β denotes $p < 0.05$ compared to within genotype saline-treated counterparts. ϵ denotes $p < 0.05$ compared to WT, VPA. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid.

Fig. 8.

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 ($p = 0.0532$). β denotes $p < 0.05$ compared to within genotype saline-treated. ϵ denotes $p < 0.05$ compared to WT, VPA. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid.

Fig. 9.

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. β denotes $p < 0.05$ compared to within genotype saline-treated counterparts. ϵ denotes $p < 0.05$ compared to WT, VPA. WT, wild type mice; KO, Nrf2 knockout mice; VPA, valproic acid.