Hyperactivity, startle reactivity and cell-proliferation deficits are resistant to chronic lithium treatment in adult Nr2e1\textsuperscript{frc/frc} mice

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Although bipolar disorder (BP) is a multifactorial psychiatric disorder that is highly heritable (60–85%) (Bierut et al. 2008), and the 6q chromosomal region has repeatedly shown evidence for genetic linkage to BP and other neurological disorders (Dick et al. 2003; Hayden & Nurnberger 2006; Kohn & Lerer 2005; McQueen et al. 2005; Middleton et al. 2004; Pato et al. 2004; Schulze et al. 2004), the causative genes for BP are just beginning to be identified (Craddock & Sklar 2009; Martinowich et al. 2009; Ogden et al. 2004). The largest meta-analysis of BP, to date, found the strongest genomewide linkage at 6q21-22 (108.5 Mb), with the highest LOD score (4.19) specifically for bipolar I disorder (BPI) that is accompanied by mania (McQueen et al. 2005). One of the genes in the 6q21-22 region is nuclear receptor 2E1 (NR2E1), which encodes a brain- and eye-specific orphan nuclear receptor. Members of the nuclear receptor superfamily encode transcription factors that have previously been implicated in disorders of human brain and behavior, including NR4A2 (Buervenich et al. 2000) and the estrogen receptor (Westberg et al. 2003). Additionally, genes known or proposed to interact with Nr2e1 (Shi et al. 2004; Stenman et al. 2003a) have been implicated in human psychiatric disorders, including PAX6 (Stober et al. 1999) and NR4A2 (Buervenich et al. 2000). A functional role for NR2E1 in BP has further been supported by a significant association, after multiple testing correction, between NR2E1 and BPI, and enrichment of rare candidate-regulatory variants in NR2E1 in BP patients (Kumar et al. 2008).

Mice lacking Nr2e1, the mammalian homolog of the Drosophila Tlx (tailless) gene, have been developed in several laboratories (aka Tlx\textsuperscript{−/−}, Nr2e1\textsuperscript{frc/frc}) and are generally referred to as Nr2e1-null mice. The Nr2e1\textsuperscript{frc} allele, studied here, is a spontaneous deletion of all nine exons of Nr2e1 as well as its proximal promoter (Kumar et al. 2004), while two different targeted deletions of Tlx exist by removing exons two and three (Monaghan et al. 1997) and...
exons three, four and five (Yu et al. 2000) by homologous recombination. Nr2e1 heterozygous mice have little to no phenotypic effects, but collectively Nr2e1-null mice have shown this gene to be critical in the maintenance and cell fate determination of neural stem/progenitor cells (Shi et al. 2004), and when absent results in extreme aggression in mice (Young et al. 2002). The various strains of Nr2e1-null mice exhibit comparable neuroanatomical abnormalities, and of particular interest are those similar to abnormalities seen in some patients with BP, including: increased lateral ventricular volume; reduced volume of the hippocampus, cerebral cortex, corpus callosum, amygdala and cortical layers II and III; olfactory abnormality and dysfunction; reduced neurogenesis; and impairment in GABAergic interneurons (Anand & Shekhar 2003; Brambilla et al. 2003; Goldberg & Chengappa 2009; Kruger et al. 2006; Land & Monaghan 2003; Mccurdy et al. 2006; Monaghan et al. 1997; Roy et al. 2004; Shi et al. 2004; Stenman et al. 2003b; Swayze et al. 1990; Tian et al. 2007; Young et al. 2002; Zhang et al. 2008). Cognitive functioning has only been examined in mice carrying targeted deletions of Nr2e1, and these mice showed reduced fear conditioning, indicating abnormalities in emotion processing, a trait that has been observed in patients with BP and present in rodent models of BP (Calzavara et al. 2009; Roy et al. 2002). Furthermore, altered cell morphology and plasticity in the hippocampus has been detected in Nr2e1frc mice, as well as other mouse models of BP, but have not been examined in targeted knockout mutants (Christie et al. 2006; Kvajo et al. 2008). Collectively, these neurological phenotypes, as well as linkage, association and functional evidence, provide strong support for NR2E1 as a candidate gene for BP, especially BPI. Although the phenotypes listed above does not validate Nr2e1frc mice as a model of BP, nor is BP diagnosed or defined by the phenotypes listed, the presence of these traits provides support that Nr2e1 could play a role in the development of brain regions that might be involved in BP pathogenesis. Despite the mounting support for NR2E1 as a candidate BP gene, Nr2e1-null mice have not been fully characterized for anomalies similar to those seen in some patients with BP, nor phenotypes commonly exhibited in rodent models of BP. Here, we examine Nr2e1frc mice for abnormalities in activity level, learning, information processing and cell proliferation in neurogenic regions. To evaluate the pharmacological validity of Nr2e1frc mice as a model of BP, we tested the effect of lithium treatment on these parameters. Lithium, a mood-stabilizing drug known for its efficacy in the treatment of mania (Malhi et al. 2009), was classically used, and continues to be prescribed today, along with other medications such as valproate and olanzapine. Lithium has been shown to attenuate psychostimulus-induced hyperactivity in rodent models of mania (O’donnell & Gould 2007) and to promote neurogenesis in the dentate gyrus (DG) (Chen et al. 2000). Considering that Nr2e1-null neural stem/progenitor cells (NSCs) showed reduced proliferation that could be rescued by reintroducing Nr2e1 in vitro (Shi et al. 2004), we tested whether lithium could attenuate the proliferative deficit in Nr2e1frc mice and whether any behavioral amelioration would accompany.

Methods and materials

Mice

The B6129F1-Nr2e1 mice used for experimental analysis were all first generation offspring resulting from mating C57BL/6J:129-Nr2e1frc+/− females (backcross generation N17-22) to 129S1/SvImJ-Cg-Nr2e1frc+/− (129-Nr2e1frc−/−) males (N15-20). The Nr2e1frc allele is a 44-kb spontaneous deletion of all nine exons of Nr2e1 that does not affect transcription of neighboring genes (Kumar et al. 2004). In accordance with Mendelian inheritance, approximately 25% of the offspring were homzygous Nr2e1frc mice and 25% were Nr2e1+/- (wild type, Wt) littermates; the latter were used as controls. All mice were weaned at postnatal day (P) 18–21, housed with same-sex littermates, and then individually housed by 4 weeks to avoid aggressive incidence with Nr2e1frc mice and to be consistent for all mice. Mice were provided with food and water ad libitum and were provided standard care according to University of British Columbia animal care policies. Handling of all mice was minimized.

Genotyping

All mice were analyzed by two separate polymerase chain reaction (PCR) assays. Wild-type allele of Nr2e1 was amplified using oEMS1859 (5′-CTGGGCCCTGCGAGATCTC-3′) and oEMS1860 (5′-GGTGGCAGTGAGCTTAAAG-3′), and the fierce allele of Nr2e1 was detected using oEMS650 (5′-GGGGAGGGAGCTTAAATAG-3′), and oEMS1388 (5′-GATTATCTCTTTCCACAAAGTG-3′). Cycling conditions were as follows: 2 min at 92° C, 30 cycles of 30 seconds at 94° C, 30 seconds at 58° C, and 55 seconds at 72° C, and a final extension of 5 min at 72° C.

Testing procedure

All mice were tested in the pathogen-free behavior suite under reverse-light/dark cycle (light 23:00–11:00 h at 320 lux) at the Centre for Molecular Medicine and Therapeutics, Vancouver, Canada, as previously described (Hossain et al. 2004). The multi-room behavior suite consists of a breeding room and dedicated testing rooms, separated by corridors. The lighting in all areas was synchronized. Care was taken not to expose the mice to any inappropriate light, even during testing. When light was needed by the investigator during experiments in the dark phase, a dim red light (8 lux) was used. All adult mice tested were closely age-matched males between the ages of 2 and 6 months, and the majority of mice used in the study were over 2 months. Nr2e1frc mice were used for each test, unless otherwise indicated. The testing chambers and equipment were thoroughly cleaned before testing each subject, using Cidox (Pharmacal Research Laboratories Inc., Naugatuck, CT, USA) and 70% ethanol.

Pup body weight and milk consumption

The body weights of 15 Wt (9 female and 6 male) and 14 Nr2e1frc (5 female and 9 male) pups were measured at P0, 7, 14 and 21. Pups were individually placed on a clean plastic weigh boat and body weight was measured on a bench-top balance. The amount of milk consumed was similarly measured in a different cohort of 11 Wt and 12 Nr2e1frc pups. Pups were removed from their mother and weighed, then kept separate from their mother for 2 h after which the pups were returned to their mother and given 15 min for feeding and were weighed again.

Pup open field activity

Spontaneous exploratory locomotor activity was measured on 10 Wt (8 female and 2 male) and 12 Nr2e1frc (5 female and 7 male) pups at P9, 14 and 18 using a digiscan photocell-equipped automated open-field apparatus 27.5 cm (L) × 27.5 cm (W) × 20.0 cm (H) with lower and upper beams at 1.5 cm and 5.5 cm from the floor, respectively (Med Associates Inc., St. Albans, VT, USA). Each pup was placed in

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the center of the novel arena and allowed to explore for 3 min while the software tallied spatially identified beam breaks.

**Home cage activity**

Home cage activity was measured on a total of 7 Wt (3 female and 4 male) and 8 Nr2e1frc (4 female and 4 male) mice during a 48-h period using identical Cage Rack Systems (San Diego Instruments, San Diego, CA, USA). Each mouse home cage was placed in the center of a metal cage rack frame that generates a uniformly spaced 8 x 4 photobeam grid. The mice were provided with food and water ad libitum throughout the testing period and spontaneous locomotor activity was measured by counting the total number of beam breaks each hour during the 48-h period (Kopp 2001). The apparatus was calibrated to normalize for body weight before testing of each animal. Signals are amplified by a gain value of 4 and the struggle threshold was set at a signal of 15, meaning that only signals above the value of 15 were indicative of struggle. Percent time struggle was then calculated as time spent struggling during which force exceeded the struggle threshold (set to 15) divided by the total testing time (3 min).

**Open field activity and habituation**

Activity and habituation in the open field of 12 Wt and 9 Nr2e1frc male mice were measured using the open-field apparatus described above (Pup open field activity). Mice were introduced to the open-field apparatus for three consecutive days and tested for 10 min each time. The numbers of beam breaks were recorded for all trials.

**Tail suspension**

Struggling during the 3-min tail suspension test was measured on 8 Wt and 4 Nr2e1frc male mice using a PHM-300TSS mouse tail suspension system (Med Associates, St. Albans, VT, USA), as previously described (Abrahams et al. 2005). The apparatus was calibrated to normalize for body weight before testing of each animal. Signals are amplified by a gain value of 4 and the struggle threshold was set at a signal of 15, meaning that only signals above the value of 15 were indicative of struggle. Percent time struggle was then calculated as time spent struggling during which force exceeded the struggle threshold (set to 15) divided by the total testing time (3 min).

**Hot plate and tail flick**

Thermal nociception and pain sensitivity of 8 Wt and 8 Nr2e1frc male mice was measured for each mouse using the hot plate and tail flick tests, respectively, as previously described (Hossain et al. 2004). Mice were placed on the hot plate apparatus (Columbus Instruments, Columbus, OH, USA) thermostatically set at 55.0 ± 0.5 °C. The latency of first licking or kicking of the fore or hind paw was recorded. A cut-off time of 60 seconds was employed to avoid tissue damage. For the tail flick test, mice were placed in a clear restraining tube (Model 33033, Columbus Instruments, Columbus, OH, USA) and the tail was placed freely on a level surface between two photo detector panels of the automated tail flick analgesia meter (Columbus Instruments, Columbus, OH, USA). Immediately after a 90-second habituation period, radiant heat from a 20-V beam of light was focused on the ventral surface of the tail and the time for the mouse to flick its tail was automatically recorded by the apparatus. A 10-second cut-off time was employed to prevent tissue damage. For both tests, the average of two consecutive trials, separated by a 1-min interval, was calculated for each animal.

**Auditory brainstem response**

Auditory functions of 4 Wt and 5 Nr2e1frc male mice were tested using the auditory brainstem response (ABR) procedure, as previously described (Ikeda et al. 1999; Zheng et al. 1999). Briefly, the test was performed on anesthetized mice where subdermal needle electrodes were inserted at the vertex (active) and ventrolaterally to the right ear (reference) and to the left ear (ground). Specific acoustic stimuli were delivered binaurally through 1-cm plastic tubes channeled from high-frequency transducers. Mice were tested with click stimuli and also with 16-KHz tone pips at varying intensity, from low to high (10–90 dB SPL). An ABR threshold was determined for each stimulus frequency by identifying the lowest intensity that produced a recognizable ABR pattern.

**Passive avoidance**

Learning and memory of 9 Wt and 6 Nr2e1frc male mice were tested in the passive avoidance test using the GEMINI™ Avoidance System (San Diego Instruments, San Diego, CA, USA). The equipment has two chambers separated by a sliding door. Mice were introduced to the first chamber in the presence of an auditory stimulus. After 30 seconds in the first chamber, the door separating the two chambers opened and the mouse was allowed to enter the second chamber without the auditory stimulus. The time the mouse took to enter the second chamber after the door opened was recorded. The maximum time allowed to enter the second chamber was 180 seconds. Once the mouse entered the second chamber it received a mild electrical shock (0.2 mA lasting 2 seconds). The mouse was again tested 24 h later and the latency of entering the second chamber was recorded.

**Acoustic startle reactivity**

Acoustic startle reactivity (ASR) was tested using the SR-LAB system (San Diego Instruments, San Diego, CA, USA). Two separate groups of male mice were used: Group 1 (12 Wt, 9 Nr2e1frc) and Group 2 (7 Wt, 7 Nr2e1frc). After a 5-min acclimatization period, each mouse was subjected to 90 acoustic startle stimuli (10 at each of 9 intensities ranging from 75 to 125 dB) in a semi-randomized sequence. The startles had a fixed duration of 50 milliseconds and were separated by a variable inter-stimulus interval (ISI) ranging from 20 to 30 seconds, while the recording window was set at 100 milliseconds. Startle response was measured at each stimulus as well as at 10 no-stimulus trials.

**Lithium administration and testing procedure**

Three Wt and five Nr2e1frc male mice received lithium chloride (LiCl) diets, while four Wt and four Nr2e1frc male mice received control diets. Mice on the control diet were fed with untreated purified diet with Teklad Vitamin Mix (Harlan Teklad, Madison, WI, USA). Mice on the lithium diet were fed with 1.7 g LiCl/kg added to the untreated purified diet with Teklad Vitamin Mix (Harlan Teklad, Madison, WI, USA) for 4 weeks, and then switched to 2.55 g LiCl/kg added to the untreated purified diet with Teklad Vitamin Mix (Harlan Teklad, Madison, WI, USA) for 2 additional weeks, before behavior testing. These mice remained on the 2.55 g LiCl/kg of chow diet throughout the testing period. All mice were also given water ad libitum and a water bottle of 450 mM sodium chloride solution. Each mouse was subjected to behavior tests in the following order: home cage activity, open field activity and habituation, and startle reactivity. The start of each test was performed 1 week after the end of the previous test. Tests were performed as described in the above sections. At the end of behavior testing all animals were killed and bled for serum analysis of lithium level, and brains were harvested for immunohistochemical analysis.

**Serum analysis**

Mice from the lithium-treatment experiment were given a lethal injection of 2,2,2-tribromoethanol in tertiary amyl alcohol (Sigma–Aldrich, St. Louis, MO, USA) (aka avertin) and blood was collected via cardiac puncture using a 25-gauge needle. Blood samples were allowed to separate for 30 min at room temperature. Samples were then centrifuged for 10 min at room temperature at 0.9 g for separation of serum. The serum was then isolated and kept at −20 °C until lithium level analyses. The Department of Pathology and Laboratory Medicine at Vancouver General Hospital, blinded to the experimental conditions, analyzed serum lithium level. The minimum detection limit of lithium serum assay was 0.2 mmol/L.

**Brain harvesting and immunohistochemistry**

Brains of mice from the lithium-treatment experiment were dissected out intact and placed into 4% paraformaldehyde in 1× phosphate-buffered saline (PBS) at 4°C for 48 h, then transferred first to a 20% sucrose solution at 4°C until saturated. Brains were then sectioned at 25 μm using the Cryo-Star HM 560 cryostat (MICROM International, Germany). The number of beam breaks were recorded for all trials.
Walldorf, Germany) and representative sections (every 24th) starting from the most rostral aspect of the ventricles to the most caudal aspect of the hippocampus were analyzed by immunofluorescence. Sections were blocked with 5% normal goat serum (NGS) +5% bovine serum albumin (BSA) in 0.1% Triton-X100 in PBS, incubated overnight at room temperature with rabbit anti-Ki67 polyclonal antibody (1:1000 dil, Cat. #ab15580; Abcam Inc., Cambridge, MA, USA), and further incubated with Alexa Fluor +594 goat anti-rabbit IgG (H + L) (Cat. #A31631; Invitrogen, Carlsbad, CA, USA). Hoechst 33342 was used for nuclear staining for all sections. Sections were mounted onto Superfrost Plus slides (Cat. #12-550-15; Fisher Scientific, Ottawa, ON, Canada) and coveredslipped using Vectashield Hard Set™ (Cat. #H-1400; Vector Laboratories, Inc., Burlingame, CA, USA). Images were captured on an Olympus BX61 motorized fluorescence microscope (Olympus America Inc., Center Valley, PA, USA), and Ki67 cells were analyzed as proliferating cells in the subventricular zone (SVZ) and DG using the ImageJ 1.43u software. Hoechst cell nuclei were analyzed as proliferating cells in the subventricular zone (SVZ) and DG using the ImageJ 1.43u software. Hoechst+ cells were also used to trace SVZ and DG areas in all sections used for cell counting. The number of Ki67+ cells was divided by area traces (mm²) to correct for area differences between the genotypes.

Statistical analysis
All data were analyzed using STATISTICA® 6 (StatSoft, Inc., Tulsa, OK, USA). All data were initially examined using Shapiro-Wilk test for normal distribution. Data that did not fit a normal distribution underwent non-parametric analysis, while data that were normally distributed were subjected to parametric analysis. Body weight, pup open field and ABR data were not normally distributed and therefore underwent non-parametric analysis (Kruskal-Wallis ANOVA). Correction for multiple comparisons when appropriate.

Results
Young Nr2e1frc/frc mice show early hyperactivity
Previously, we showed that Nr2e1frc/frc pups on a C57BL/6J (B6) background failed to gain weight at the rate of their Wt littermates between postnatal weeks 2 and 3 (Young et al. 2002). For the current study, we retested this phenotype at postnatal day (P) 0, 7, 14 and 21 on the B6129F1 background. When the data were analyzed for sex differences as a whole or separately for each genotype, no significant sex effect was detected at any of the postnatal days tested (P > 0.05). We further showed that regardless of sex, there were significant genotype differences at P21 (P < 0.05), but not at P0, 7 or 14 (P > 0.1) (Fig. 1). These findings indicated that B6129F1-Nr2e1frc/frc mice also failed to gain weight at the rate of their Wt siblings, and were significantly smaller by P21. Therefore, small size at wean is a stable phenotype across two genetic backgrounds.

We measured milk consumption in pre-wean pups to test the hypothesis that the failure of Nr2e1frc/frc mice to gain weight normally may depend on a reduction in milk consumption. This hypothesis was not supported by the milk consumption data, where no significant differences were found between the two genotypes (Wt = 0.059 ± 0.004 g, Nr2e1frc/frc = 0.07 ± 0.01 g, P > 0.1). We then measured activity level in the same group of pre-wean pups at P9, 14 and 18 using the open field apparatus. No significant sex effect was detected at any of the postnatal days tested (P > 0.05) when the data were analyzed as a whole or separately for each genotype. We observed age-dependent increased activity level in Nr2e1frc/frc mice compared to Wt controls, as indicated by significant genotype differences at P18 (Fig. 2; P < 0.05), but not at P9 or P14 (P > 0.1). Therefore, the post-wean size reduction of Nr2e1frc/frc mice was not apparently the result of a feeding abnormality but may be a secondary effect of hyperactivity.

Adult Nr2e1frc/frc mice show hyperactivity in three behavioral tests
To fully characterize the extent of the hyperactivity phenotype in Nr2e1frc/frc mice we used the home cage activity monitor, a powerful and ethological test that assesses movement of mice in their home cage. This test showed that Nr2e1frc/frc mice are extremely hyperactive (Fig. 3; genotype effect F(1,11) = 10.6, P < 0.01), regardless of sex (F(1,11) = 2.22, P > 0.1). The mean number of beam breaks per hour was eightfold higher in Nr2e1frc/frc mice than in Wt controls for both light and dark phases (Beam breaks: Light phase: Wt = 189 ± 19.0, Nr2e1frc/frc = 1304 ± 118.9, P < 0.001; Dark phase: Wt = 313 ± 21.6, Nr2e1frc/frc = 2403 ± 148.6, P < 0.001).

Figure 1: Reduced body weight of Nr2e1frc/frc pups. Nr2e1frc/frc pups weighed significantly less than Wt pups by postnatal day 21. *P < 0.001.

Adult Nr2e1frc/frc mice show hyperactivity in three behavioral tests
To fully characterize the extent of the hyperactivity phenotype in Nr2e1frc/frc mice we used the home cage activity monitor, a powerful and ethological test that assesses movement of mice in their home cage. This test showed that Nr2e1frc/frc mice are extremely hyperactive (Fig. 3; genotype effect F(1,11) = 10.6, P < 0.01), regardless of sex (F(1,11) = 2.22, P > 0.1). The mean number of beam breaks per hour was eightfold higher in Nr2e1frc/frc mice than in Wt controls for both light and dark phases (Beam breaks: Light phase: Wt = 189 ± 19.0, Nr2e1frc/frc = 1304 ± 118.9, P < 0.001; Dark phase: Wt = 313 ± 21.6, Nr2e1frc/frc = 2403 ± 148.6, P < 0.001).
Figure 2: *Nr2e1<sup>frc/frc</sup> mice showed hyperactivity as early as postnatal day (P)18. A 3-min open field test showed that *Nr2e1<sup>frc/frc</sup> mice were significantly more active at P18, but not at younger ages. *P < 0.01.

Hyperactivity in *Nr2e1<sup>frc/frc</sup> mice was also seen in the open field test. Throughout the three days of open field habituation testing there was a significant effect of genotype on distance traveled (Fig. 4a–c; F(18, 513) = 80.0, P < 0.001). More specifically, during day 1 of testing Wt mice already showed habituation by the 4th minute of testing (Fig. 4a; P < 0.05), whereas *Nr2e1<sup>frc/frc</sup> mice did not habituate during the 10 min on day 1 (Fig. 4a; P > 0.7). *Nr2e1<sup>frc/frc</sup> mice did eventually show habituation on test days 2 and 3, at 10 min (Fig. 4b; P < 0.01) and 7 min (Fig. 4c; P < 0.05), respectively.

The passive avoidance test depends on the ability of the mouse to react to pain, and therefore prior to this test, we examined our mice for pain sensitivity using the hot plate and tail flick tests. *Nr2e1<sup>frc/frc</sup> mice began licking their paws in significantly less time compared to Wt mice, indicating increased pain sensitivity in the hot plate test (Fig. 6a; P < 0.05). In the tail flick test there was no difference in the time required to remove the tail between *Nr2e1<sup>frc/frc</sup> and Wt mice (Fig. 6b; P > 0.1). Despite the discordance in the results of these two tests we have reason to favor the finding of increased pain sensitivity when *Nr2e1<sup>frc/frc</sup> mice are not restrained (see Discussion). More importantly, both tests showed the ability of *Nr2e1<sup>frc/frc</sup> mice to respond to pain, thus supporting the use of the passive avoidance test.

Figure 3: *Nr2e1<sup>frc/frc</sup> mice showed hyperactivity in the home cage. *Nr2e1<sup>frc/frc</sup> mice broke more beams than their Wt littermates over 48 h.
Figure 4: *Nr2e1<sup>frc/frc</sup> mice showed hyperactivity and habituation deficiency in the open field. Distance traveled was measured in the open field on 3 consecutive days for 10 min each day. *Nr2e1<sup>frc/frc</sup> mice were significantly more active than Wt mice on all 3 days. Wt mice showed habituation on day 1 (a; solid line). *Nr2e1<sup>frc/frc</sup> mice did not show habituation on day 1 (a; dotted line), but showed habituation on days 2 (b; dotted line) and 3 (c; dotted line). *Wt: *P < 0.05. *Nr2e1<sup>frc/frc</sup>: *P < 0.05.
Nr2e1<sup>frc/frc</sup> phenotypes are lithium resistant

The standard protocol for passive avoidance testing is to use light as an adverse stimulus to encourage the animal to cross into the second chamber. However, because Nr2e1<sup>frc/frc</sup> mice have impaired vision, we decided to use sound as the adverse stimulus. We have previously tested 4-month-old Nr2e1<sup>frc/frc</sup> mice on a B6 background and showed that they have normal hearing as measured by ABR (Young et al. 2002). However, because our current mice are on a B6129F1 hybrid background, we retested them for ABR. Nr2e1<sup>frc/frc</sup> mice did not show any significant differences from Wt controls (Click: Wt = 50.0 ± 2.89 dB, Nr2e1<sup>frc/frc</sup> = 45.0 ± 2.74 dB, P > 0.1, 16 kHz: Wt = 22.5 ± 4.33 dB, Nr2e1<sup>frc/frc</sup> = 17.0 ± 2.00 dB, P > 0.1). Therefore, normal ABR in Nr2e1<sup>frc/frc</sup> mice is a stable phenotype across two genetic backgrounds.

Because we confirmed that B6129F1-Nr2e1<sup>frc/frc</sup> mice are able to respond to pain and that their hearing is normal, we used sound to test these mice for passive avoidance. Wt mice showed the expected learning response, showing an average greater than threefold increase in latency to re-enter the second chamber upon the second exposure to the condition stimulus (Fig. 7; P < 0.001). Although Nr2e1<sup>frc/frc</sup> mice also showed an increase in latency to re-enter, this change was much less than that seen in Wt mice, and did not reach statistical significance (Fig. 7; P > 0.05), demonstrating that they did not perform this learning task as well as Wt mice.

**Nr2e1<sup>frc/frc</sup> mice lack startle reactivity**

Hippocampal lesions in rodent models have been well documented to show impairments in prepulse inhibition (PPI), a measure of sensorimotor gating (Kamath et al. 2008; Pouzet et al. 1999). Prior to evaluating PPI, ASR must be tested to establish a startle threshold, as defined as the lowest startle intensity that produces a startle reaction significantly different than at the no-stimulus condition. Nr2e1<sup>frc/frc</sup> mice showed less ASR than Wt controls, as shown by a significant main effect of genotype (Fig. 8; F(1,19) = 17.5, P < 0.001) and a significant interaction between intensity and genotype (F(9,171) = 29.9, P < 0.001, ε = 0.27). Post hoc analysis indicated that the startle threshold for Wt mice was at 105 dB (P < 0.001); interestingly, there was no startle threshold for Nr2e1<sup>frc/frc</sup> mice (P > 0.05). This surprising result was confirmed with a new group of mice (data not shown). Therefore, we conclude that Nr2e1<sup>frc/frc</sup> mice show a lack of normal startle reaction. When we compared the startle magnitudes of Nr2e1<sup>frc/frc</sup>
and Wt mice at the different startle intensities using post hoc analysis, there were significant genotype differences at 115 and 120 dB (P < 0.05). Furthermore, as PPI tests are based on the startle response, PPI results for these mice would be uninformative.

**Nr2e1^frc/frc** hyperactivity resistant to lithium treatment

Lithium chloride is the most effective drug for treatment of mania in patients with BPI, with human therapeutic plasma lithium level between 0.6 and 1.2 mmol/l, which can attenuate psychostimulus-induced hyperactivity (Gould et al. 2001, 2007) and increase neurogenesis in the DG in rodent models (Kim et al. 2004). Using a dietary source of lithium, Wt and Nr2e1^frc/frc mice showed serum lithium level that was on par with human therapeutic levels (Wt and Nr2e1^frc/frc on control diet = below detection limit; Wt on lithium diet = 0.9 ± 0.1 mmol/L; Nr2e1^frc/frc on lithium diet = 0.8 ± 0.1 mmol/L), no significant difference in plasma level between the two genotypes fed with lithium diet, (P > 0.5).

We showed that lithium treatment was unable to alleviate the hyperactivity seen in Nr2e1^frc/frc mice in the 24-h home cage activity test, as shown by the significant effect of genotype (Table 1), but no significant effect of diet (Table 1), nor a significant interaction between genotype and diet (Table 1). The mean number of beam breaks in both light and dark phases was significantly higher in Nr2e1^frc/frc mice compared to Wt controls, regardless of lithium treatment (Light: Wt, control diet = 78.9 ± 10.8, Wt, lithium diet = 110.2 ± 21.6, Nr2e1^frc/frc control diet = 285.5 ± 46.0, Nr2e1^frc/frc lithium diet = 321.4 ± 55.8; Dark: Wt, control diet = 158.5 ± 16.7, Wt, lithium diet = 197.2 ± 24.4, Nr2e1^frc/frc control diet = 997.4 ± 65.1, Nr2e1^frc/frc lithium diet = 1005.7 ± 79.4; for all comparisons between Wt and Nr2e1^frc/frc regardless of diet (P < 0.05).

**Nr2e1^frc/frc** open field habituation deficit is unaffected by lithium treatment

To evaluate the effect of lithium treatment on the habituation deficit in Nr2e1^frc/frc mice, mice fed with control and lithium diets were assayed in the open field habituation test. As before (Fig. 4), there was a significant effect of minutes, day and genotype interaction (Table 1), indicating that Nr2e1^frc/frc mice showed different activity patterns on the different test days compared to Wt controls. The lack of significant interaction between minute, day, genotype and diet (Table 1) indicated that lithium treatment was unable to improve habituation in Nr2e1^frc/frc mice. The lack of lithium effect on Nr2e1^frc/frc habituation deficit was still apparent even after taking into account for activity differences (data not shown).

**Lithium-treated Nr2e1^frc/frc** mice show no improvement in startle reactivity

The lack of startle reactivity was one of the most striking phenotypes shown in Nr2e1^frc/frc mice. To assess the effect of lithium on this behavioral phenotype, Wt and Nr2e1^frc/frc mice fed with control and lithium diets were assayed in the startle reactivity test. Similar to our previous experiments (Fig. 8), the two genotype groups responded differently to the varying acoustic startle stimuli as evidenced by the significant interaction between intensity and genotype (Table 1). We showed that lithium treatment did not significantly correct
Table 1: Summary of lithium findings

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<th>(a) 24-h home cage activity</th>
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<td>Genotype x Diet</td>
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<td>(b) Open field habituation</td>
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<td>(c) Startle reactivity</td>
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The deficient acoustic startle response in $N_{r2e1}^{frc/frc}$ mice compared to that shown by Wt mice, as there was no significant effect of diet (Table 1), and there were no significant interactions between: genotype and diet (Table 1); intensity and diet (Table 1); genotype, intensity and diet (Table 1). We were unable to perform post hoc analysis for effect of diet as there were no significant effects or interactions involving diet. In the post hoc analysis of intensity and genotype effect, Wt mice showed startle threshold at 110 dB ($P < 0.05$), while $N_{r2e1}^{frc/frc}$ mice lacked a startle threshold at any startle intensity ($P > 0.05$), paralleling results shown in Fig. 8. Our results showed an absence of a significant lithium effect on $N_{r2e1}^{frc/frc}$ startle reactivity deficit.

**Cell proliferation in SVZ and DG is unaffected by lithium treatment**

Reduced neural stem/progenitor cell proliferation has been shown in $N_{r2e1}$-knockout mice when compared to their Wt littermates (Shi et al. 2004). Here we show for the first time, using Ki67 staining of proliferating cells, a significant genotype effect for cell proliferation in the two neurogenic zones of the $N_{r2e1}^{frc/frc}$ adult brain, the SVZ and DG (Fig. 9a,b; $F(2,10) = 92.5$, $P < 0.001$). Significant genotype effects in each region were also identified for cell proliferation (SVZ: Fig. 9a; $F(1,11) = 194.5$, $P < 0.001$; DG: Fig. 9b; $F(1,11) = 18.3$, $P < 0.01$). These results indicated that $N_{r2e1}^{frc/frc}$ mice show the same pattern of reduction in cell proliferation as other $N_{r2e1}$-knockout mice (Shi et al. 2004).

**Figure 9:** Lithium treatment did not increase cell proliferation in $N_{r2e1}^{frc/frc}$ mice. There was no significant effect of diet on cell proliferation. (a) In the SVZ, there were significantly less Ki67+ cells in $N_{r2e1}^{frc/frc}$ mice compared to Wt mice ($*P < 0.001$). (b) In the DG, there were also significantly less Ki67+ cells in $N_{r2e1}^{frc/frc}$ mice compared to Wt mice ($*P < 0.01$).
Because lithium has been shown to act through multiple pathways to increase neurogenesis in vivo (Jope 1999, Kim et al. 2004; Wada et al. 2005), we analyzed its effect on cell proliferation in Nr2e1frc mice. We showed that our lithium treatment was unable to alter cell proliferation in either of the two neurogenic zones (SVZ and DG), as evident by no significant effect of diet (SVZ: Fig. 9a; F(1,11) = 1.41, P > 0.5; DG: Fig. 9b; F(1,11) = 1.24, P > 0.5). We also saw no significant interaction between genotype and diet (SVZ: Fig. 9a; F(1,11) = 1.46, P > 0.5; DG: Fig. 9b; F(1,11) = 0.08, P > 0.5), suggesting that our lithium diet was unable to alter cell proliferation in the SVZ and DG of Wt and Nr2e1frc mice.

Discussion

This study was the first to characterize Nr2e1frc mice for a spectrum of phenotypes that have been used in the literature to model aspects of BP (Arban et al. 2005; Cao & Peng 1993; Decker et al. 2000; Einat 2006, 2007; Einat et al. 2003; El-Mallakh et al. 2003; Gessa et al. 1995; Ralph-Williams et al. 2003). In addition, it is the first to evaluate the effect of any drug treatment on Nr2e1-null mice. Results from this study showed new important behavioral phenotypes in Nr2e1frc mice including extreme hyperactivity and deficits in habituation, passive avoidance, and startle reactivity. The presence of reduced cellular proliferation in the SVZ and DG was a novel finding for Nr2e1frc mice, and the resistance of these behavioral and proliferative phenotypes to lithium treatment is a novel finding amongst all Nr2e1-null mice.

In the present study, the extreme hyperactivity phenotype of the Nr2e1frc animals was documented in three different tests: home cage activity, tail suspension and open field habituation. Of these tests, the tail suspension was originally chosen to evaluate depressive behavior in this study, but because of the overwhelming hyperactivity phenotype, the results were not indicative of depressive behavior. Currently, the most frequently used model of mania is psychostimulant-induced hyperactivity (Einat 2007; Machado-Vieira et al. 2004). Interestingly, hyperactivity seen in Nr2e1frc mice was approximately eightfold higher than the basal activity level found in the home cage, while administration of psychostimulant on Wt mice, and other transgenic mice exhibited increased activity levels by approximately two- to fivefold over non-induced or Wt mice, respectively (Arban et al. 2005; Hiroi et al. 2005; Zhuang et al. 2001). Therefore, Nr2e1frc mice show one of the most extreme hyperactivity phenotype currently documented.

Nr2e1-null mice have previously been shown to have hypoplasia of the hippocampus and decreased adult neurogenesis in the granular layer of the DG, regions important for learning and memory (Mainen & Sejnowski 1996; Shi et al. 2004; Young et al. 2002). Our group also shown that not only is the dendritic branching structure of granule cells in Nr2e1frc mice reminiscent of immature neurons in the DG, the mice also lack synaptic plasticity, as shown by the absence of long-term potentiation (LTP) in their DG (Christie et al. 2006). LTP is thought by some to be an electrophysiological measure of learning and memory (Howland & Wang 2008; Kinney et al. 2009). Collectively, learning and memory deficits are expected based on the neuroanatomical abnormalities observed in Nr2e1frc mice. Furthermore, some patients with BP also show cognitive deficits, such as dysfunctions in executive function and verbal memory; however, these deficits are usually less severe and differ from the typical profile seen in patients with schizophrenia (Altshuler et al. 2004; Green 2006; Krabendam et al. 2005). In an attempt to show any cognitive impairment in Nr2e1frc mice, we used two distinct tests of learning and memory. Because Nr2e1frc mice have reduced vision and may also have abnormal olfaction, many conventional behavioral paradigms of learning and memory were not appropriate. In particular, well-established tests of executive memory in rodents such as the Morris water, Barnes, and Y mazes could not be properly employed. Although the open field habituation and passive avoidance tests used in this study do not specifically evaluate the cognitive domains typically affected in patients with BP (Altshuler et al. 2004), these tests were chosen and designed specifically to assess hippocampal-associated learning with minimal use of visual or olfactory cues. We hypothesized that Nr2e1frc mice will have deficits in hippocampal-associated learning based on (1) their hippocampal abnormalities and (2) that the drugs effective in treatment of BP have shown to improve hippocampal-associated learning (Nocjar et al. 2007; Watase et al. 2007; Yan et al. 2007). The passive avoidance test was chosen as Roy et al. (2002) showed that Nr2e1 knockout mice were hyper-responsive to shock, indicating that shock was an appropriate unconditioned stimulus for inducing learning in these mice that have such extensive sensory deficits. Furthermore, both tests also provide an internal control for activity level because they consider the change in activity between the same groups of mice on different days, thus normalizing for activity levels. Nevertheless, we showed that Nr2e1frc mice perform poorly on these tasks compared to Wt mice, as evident by the increased time required to habituate in the open field test and the lack of significant increase in latency to re-enter in the passive avoidance test. Yet, we cannot exclude the possibility that acquisition of environmental cues could be disrupted because of sensory deficits or the hyperactivity phenotype may interfere with the inhibition of locomotor activity in Nr2e1frc mice, which contributes to their deficits in performance in these tasks. Despite these caveats, our data suggest the importance of Nr2e1 in proper brain development, without which there is a reduced performance in hippocampal-associated learning tasks.

This study was also the first to test for ASR in Nr2e1-null mice. Our novel finding of complete lack of startle was unexpected, because previously there has not been a case of hearing mice not showing ASR. ASR was done in preparation for evaluating PPI; however, we are unable to test PPI because PPI requires startle reactivity greater than movements seen at background noise and Nr2e1frc mice showed no startle threshold. This result, along with normal response for the tail flick test, was surprising because our previous results, and those of others (Roy et al. 2002), led us to anticipate a hyper-responsive phenotype. However,
we note that the lack of hyper-responsiveness in these instances correlates with the use of restraint, an extreme stressor in mice (Bain et al. 2004). Brain regions shown to contribute to stress-related response include the amygdala and hippocampus (Liberzon & Martis 2006; Vermetten & Brenner 2002). Regions suggested to be involved in the modulation of ASR include nucleus accumbens, basolateral amygdala and prefrontal cortex (Stevenson & Gratton 2003; Storozheva et al. 2003). All of these regions are structurally abnormal in the Nr2e1frc mice and may underlie the lack of hyper-responsiveness to pain, as well as the lack of ASR. Based on the hot plate test where Nr2e1frc mice were not tested under restraint and showed a significant reduction in time to lick their paws, we concluded that Nr2e1frc mice had increased pain sensitivity. However, in the tail flick test, Nr2e1frc mice were placed in a restrainer, and we concluded that under this stressor the expected hyper-responsive phenotype of Nr2e1frc mice was masked by the atypical stress response caused by restraint. Alternatively, the discrepancy in pain sensitivity of Nr2e1frc mice in the two tests could be the result of different neurocircuits that are activated by the different tests (Davidovalo et al. 2009; Fields & Heinricher 1985; Jasmin et al. 1997; Lane et al. 2005; Morgan & Clayton 2005).

We chose to evaluate the effect of lithium treatment on Nr2e1frc mice for four reasons: (1) lithium has been shown to attenuate symptoms of mania in patients with BP (Shastry 2005); (2) lithium reduces genetically- and amphetamine-induced hyperactivity in rodents (Gould et al. 2001, 2007; Yuskaitis et al.); (3) lithium has induced neural stem cell proliferation in the mouse DG both in vitro and in vivo assays (Wada et al. 2005); and (4) lithium is thought to act through multiple key neurological pathways (Jope 1999), thus increasing the probability that lithium would effect Nr2e1frc behavioral phenotypes compared to drugs with restricted modes of action.

In this study, we showed that adult lithium treatment was ineffectively in attenuating any of the abnormal behavioral phenotypes observed in Nr2e1frc mice including the extreme hyperactivity in the home cage, the habituation deficit in the open field test and the lack of ASR. Despite the fact that lithium can induce neurogenesis in vitro and in vivo (Kim et al. 2004) and that the introduction of Nr2e1 can rescue quiescent stem cells from Nr2e1-null brains in vitro (Shi et al. 2004), here we showed that lithium administration to adult Nr2e1frc mice was unable to trigger an increase in cell proliferation in the SVZ and DG. The lack of lithium effect on Wt-cell proliferation was initially surprising given the evidence for increased hippocampal neurogenesis in normal mice treated with lithium (Chen et al. 2000; Kim et al. 2004). However, there were key experimental differences between our analysis of Ki67+ cells and studies demonstrating an increase in cell proliferation with lithium treatment. Cells analyzed by these studies are labeled with bromodeoxyuridine (BrdU) via consecutive days of injections, thereby labeling not only currently dividing cells, but their progeny as well. Our Ki67+ cell counts would more accurately mimic results from single-day BrdU injections, which would only label currently dividing cells. It has been showed that under this condition, lithium treatment was unable to increase cell proliferation in Wt mice (Eom & Jope 2009), which is consistent with our observation.

One might be tempted to speculate that with an increased number of mice we might have detected an effect of lithium on hyperactivity. However, based on the literature of other genetically- and psychostimulant-induced hyperactivity in mice, lithium treatment was able to reduce the hyperactivity phenotype by at least half, if not returning activity level to that seen in Wt controls (Gould et al. 2001, 2007; Yuskaitis et al.). Therefore, because Nr2e1frc mice exhibit an ~eightfold increase in locomotor activity compared to Wt controls, the number of mice tested in the lithium experiment would have had sufficient power to detect lithium effect given the anticipated reduction in locomotor activity. For detection of a lithium effect on startle reactivity, we had no apriori hypothesis for the number of subjects required to detect this effect because a lack of startle reactivity is a novel finding.

The development of a totally appropriate mouse model for complex disease, such as mental illness, is challenging for reasons of environmental factors, minor multiple gene effects and appropriate pharmacological responsiveness. However, many single gene mouse models, such as Gsk3b overexpressing mice, nitric oxide synthase (NOS-III) and nNOS knockout mice, and DISC1 mutant mice (Flint & Shifman 2008; Kato et al. 2007; Prickaerts et al. 2006; Reif et al. 2006; Tanda et al. 2009), have proven valuable as they exhibit aspects of complex disorders. We have now added Nr2e1frc mice to this group. We have shown here that Nr2e1frc mice show the behavioral traits of hyperactivity and deficit in habituation and learning tasks, which are commonly used in genetic models of BP.

However, because Nr2e1frc mice failed to respond to the adult lithium treatment used here, they have not currently met the criteria of pharmacological validity as a model for BP (Kato et al. 2007). Given the extreme level of hyperactivity in Nr2e1frc mice, the treatment regiment used in other studies, which was adopted here, may not be sufficient in reducing hyperactivity. We hypothesize for future consideration that Nr2e1frc mice should be examined using higher doses or longer administration of lithium, or different combinations of mood-stabilizing and antipsychotic drugs to attenuate their behavioral phenotypes; the latter would more accurately mimic treatment regimens commonly prescribed to patients with BP. We also acknowledge that the genetic components of BP are likely to be multiple mutations of minor effect; furthermore, the phenotype of the Nr2e1 heterozygous mouse is too weak for behavioral detection (Roy et al. 2002). Therefore, we hypothesize that mice carrying subtle mutations, or patient variants, in trans across from an Nr2e1 deletion might more closely represent the human condition.

References


men with bipolar I disorder or schizophrenia and normal control subjects. *Biol Psychiatry* **56**, 560–569.


and increased D1-mediated dopaminergic signaling in neuronal nitric oxide synthase knockout mice. Mol Brain 2, 19.


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