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Heterozygous deletion of NR1 subunit of the NMDA receptor alters ethanol-related behaviors and regional expression of NR2 subunits in the brain

Xiaoping Du^{a,c}, Andrea J. Elberger^a, Douglas B. Matthews^{b,d}, and Kristin M. Hamre^a

^aDepartment of Anatomy and Neurobiology, University of Tennessee, Health Science Center, 855 Monroe Avenue, Memphis, TN 38163 U.S.A.

^bDepartment of Psychology, University of Memphis, Memphis, TN 38152 U.S.A.

Abstract

NMDA receptors have been hypothesized to play a role in various aspects of ethanol-related phenotypes, notably in ethanol withdrawal. However, the role of each of the specific subunits remains unclear. To address this issue, mice that are heterozygous for the NR1 deletion, and thus have a reduction in functional NMDA receptors, were examined for ethanol consumption and acute ethanol withdrawal. Additionally, mice were examined for the level of vocalization following footshock, and behavior in an elevated plus maze, to determine their responses to stress. In these behavioral tests, NR1 heterozygous mice were shown to consume significantly higher levels of ethanol in the two bottle-choice test showing a possible role for this receptor in ethanol consumption. Analysis of acute withdrawal found that the heterozygous mice exhibit lower levels of handling-induced convulsions consistent with a role in ethanol sensitivity or withdrawal. In contrast, no effects on stress-related phenotypes were detected. Levels of NR2A-NR2D subunits of the NMDA receptor in specific brain regions were compared between NR1+/- mice and wild-type controls to assess whether the behavioral responses were specific to the diminution in NR1 expression or whether these changes could be due to secondary changes in expression of other NMDA subunits. Real-time quantitative PCR, Western blot and immunohistochemistry were used to examine expression levels in the hippocampus, neocortex, striatum and cerebellum. For the majority of the subunits, no differences were found between the wild-type and heterozygous mice in any of the brain regions. However, the NR2B subunit exhibited differences in expression of RNA in the hippocampus and protein levels in multiple brain regions, between wild-type and NR1+/- mice. These results show that NR1 plays a role, through mechanisms as yet unknown, in the expression of NR2 subunits in a region and subtype specific manner. This provides evidence of

Address Correspondence to: Kristin M Hamre, PhD., Department of Anatomy and Neurobiology University of Tennessee Health Science Center, 855 Monroe Avenue, Room 515, Memphis, TN 38163, Phone: (901) 448-2643, Fax: (901) 4487193, khamre@uthsc.edu.

^cHough Ear Institute, 3400 NW 56th St., Oklahoma City, OK 73112 U.S.A.

^dDepartment of Psychology and Neuroscience, Baylor University, Waco, TX U.S.A., and Nanyang Technological University, Department of Psychology, Singapore

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the effects of altered levels of NR1 expression on ethanol withdrawal and consumption, and suggests that concomitant changes in the levels of NR2B may contribute to that effect.

Keywords

Alcohol consumption; alcohol withdrawal; stress; expression; NR2B; brain

1. Introduction

The *N*-methyl-D-aspartate (NMDA) receptor is composed of the following subunits: NMDAR1 (NR1), four NMDAR2 subunits (NR2A-D), and NMDAR3A (NR3A). Without NR1, the NR2 subunits are non-functional (Ishii et al., 1993; Meguro et al., 1992; Vicini et al., 1998) and therefore, NR1 is essential (Blahos and Wenthold, 1996; Dunah and Standaert, 2003; Hawkins et al., 1999; Sheng et al., 1994). In the CNS, NR1 is expressed constitutively in every brain region that has been examined, with higher expression found in cortex, cerebellum, hippocampus, basal ganglia, thalamus, hypothalamus, and olfactory bulb (Monyer et al., 1992, 1994). In contrast, the various NR2 subunits are expressed in a regionally-specific manner: NR2A has the most widespread expression being primarily found in the forebrain and cerebellum, while NR2B is found predominantly in the forebrain, and NR2C in the cerebellum. NR2D is mainly expressed in the midbrain (Monyer et al., 1994). Furthermore, considerable agreement is found between the distribution patterns of NR2A and NR2B protein and mRNA encoding the subunits (Moriyoshi et al., 1991; Wang et al., 1995).

Evidence suggests that the NR1 subunit is required for normal expression of NR2 subunits in the CNS, particularly for NR2B (Forrest et al., 1994; Fukaya et al., 2003). Examination of NR1 conditional knockout mice demonstrates that expression of the NR2A and NR2B subunits is reduced in the hippocampus (Fukaya et al., 2003). However, only NR2B protein level is reduced in the whole brain tissue of the NR1^{-/-} mice (Forrest et al., 1994). It is still unknown whether partial reduction of NR1 (NR^{+/-}) would affect expression of NR2 subunits.

NMDA receptors have a role in ethanol-related behavior, most notably in ethanol withdrawal. Antagonists of NMDA receptors such as ADCI and MK-801, as well as the NR2B-specific antagonist ifenprodil, decrease or eliminate ethanol withdrawal seizures in animal models (Grant et al., 1992; Pawlak et al., 2005). Further, changes in expression of various NMDA receptors are found after ethanol withdrawal with higher levels of protein expression of NR1, NR2A and NR2B subunits found in the hippocampus (Hendricson et al., 2007; Nelson et al., 2005), and increased protein expression of NR1 and NR2B, but not NR2A, found in cultured cortical neurons (Qiang et al., 2007). Of greater significance, mRNA was reduced in selective NR1 splice variants and in NR2B in rat cortex with ethanol withdrawal, while no changes were found in expression of total NR1, NR2A and NR2C subunits (Hardy et al., 1999). However, the reason for inconsistent changes in NMDA receptor expression of mRNA and protein levels remains unclear.

NMDA receptors are also associated with ethanol consumption. Antagonists of NMDA receptors (AP5, MRZ 2/579, ifenprodil, memantine) decrease ethanol consumption in rats that are ethanol-naïve (Lin and Hubbard, 1995; Malpass et al. 2010), consuming ethanol chronically (Hölter et al., 2000), or undergoing repeated cycles of alcohol consumption and withdrawal (Vengeliene et al., 2005). However, examination of changes in expression of either protein or mRNA of various NMDA receptor subunits following chronic ethanol consumption has given a complex and often conflicting array of changes (see review by Nagy, 2008). For example, chronic ethanol consumption has not been shown to change the mRNA and protein levels of NR1, NR2A and NR2B subunits in the spinal cord (Narita et al., 2007) or the protein levels in the hippocampus (Ferreira et al., 2001) of rats. However, a recent study has demonstrated that chronic ethanol intake increases mRNA and protein expression of NR1 and NR2A in the dorsal hippocampus, although a high dose (5%) of ethanol consumption inhibits NR2B's expression (Kalev-Zylinska and During, 2007). Furthermore, only NR1, but not NR2 or NR3, mRNA levels were elevated in rat amygdala following ethanol consumption (Floyd et al., 2003). Ethanol consumption was accompanied by increased NR1 protein levels in striatum and medial prefrontal cortex (Klugmann et al., 2011), and increased NR2B protein in dorsomedial striatum (Wang et al. 2010). Therefore, more studies are needed to elucidate the role of each NMDA receptor subunit in ethanol consumption.

Several lines of evidence provide support for the role of NMDA receptors in stress responses. First, administration of NR1 antisense oligodeoxynucleotide produces anxiolytic effects in mice as shown by the increased time that the treated animals spent in the open arms of an elevated plus maze (Zapata et al., 1997). Further, administration of competitive or noncompetitive NMDA receptor antagonists, such as CGS-19755, AP-5, AP-7, CPP, MDL 100,453, and the NMDA channel blocker (MK-801) also produce anxiolytic effects in the elevated plus maze or decreased levels of separation-induced ultrasonic vocalizations (Bennett et al., 1990; Dunn et al., 1989; Kehne et al., 1991). Second, a line of NR1^{neo} -/- hypomorphic mice has been generated where the expression of the NR1 subunit has been reduced by 90–95% (Mohn et al., 1999). The low expression of the NR1 subunit is sufficient to allow these mice to survive into adulthood. The NR1 hypomorphic mice display several abnormal responses that could be interpreted as altered stress responses, including increased locomotor activity and stereotypy (Mohn et al., 1999). Third, chronic stress has been shown to alter expression of NMDA receptors, particularly in the hippocampus and amygdala (Lei and Tejani-Butt, 2010).

Evaluation of other NMDA subunits has also shown effects on stress responses. For example, transgenic NR2C-NR2B mutant mice, in which the NR2C locus was replaced by NR2B, avoid the open arms of the elevated plus maze (De Souza Silva et al., 2007). In contrast, NR2A or NR2D knockout mice have reduced spontaneous locomotor activity in novel environments and show less sensitivity to stress in the elevated plus-maze and light–dark box (Boyce–Rustay and Holmes, 2006; Miyamoto et al., 2002). While strong evidence suggests that NMDA receptors play a role in stress responses, the identification of that role or the specific subunits involved have not been conclusively demonstrated.

Using NR1 heterozygous mice to study ethanol or stress phenotypes is relevant to humans due to many forms of natural variation in the human genome, such as loss or gain of kilobases of genomic DNA in 10% of individuals examined (Iafrate et al., 2004). The mouse knockout strain of the present study is uniquely qualified to model the human condition because the changes in the NMDA receptors are present beginning with conception, in contrast to studies using short-term, localized agonists or antagonists. NR1^{-/-} mice were not able to be tested since the knockout is neonatal lethal and thus, the pups die within 15 hours after birth (Forrest et al., 1994). However, NR1 heterozygotes have been shown to exhibit intermediate prenatal and perinatal phenotypes, compared to wild types and knockouts (Deng and Elberger, 2003; Elberger and Deng, 2003; Sugiura et al., 2001). Furthermore, heterozygous gene knockouts might change associated genes' expression. For example, Df1 heterozygous deletion (Df1^{-/+}) mice have down-regulation of 12 Df1 associated genes in the hippocampus (Sivagnanasundaram et al., 2007). Additionally, heterozygous deletion of neurotrophin-3 (NT3^{+/-}) upregulates NR1 and NR2A, but not NR2B, expression in adult mice (Torres-Peraza et al., 2007).

In the present study, we compared the behavioral responses of adult NR1 heterozygous mice with wild-type controls in ethanol consumption, acute ethanol withdrawal and stress responses. Additionally, we addressed whether diminished expression of NR1 in NR1^{+/-} mice would affect mRNA expression of the NR2 subunits. Subsequent examination of protein changes was determined using Western blotting and immunohistochemistry for those subunits with altered mRNA expression. Additionally, we asked whether these changes are uniform across the brain or among the 4 subunits, or whether changes occurred in a region-specific manner. We provide evidence for the role of the NR1 subunit of the NMDA receptor in ethanol-related phenotypes, although responses to stress were not significantly different between heterozygous and wild-type mice. Further, we find altered expression of NR2B in NR1^{+/-} mice suggesting that alterations in expression of either NR1, NR2B, or both, are contributing to the altered ethanol-induced behavioral responses.

2. Materials and methods

2.1. Mice and genotyping

The original stock of NR1 mice was provided by Dr T. Curran at the Roche Institute of Molecular Biology at Hoffmann-La Roche Inc. (Nutley, NJ) (Forrest et al., 1994). NR1^{+/-} mice used in the present study were maintained at the University of Tennessee Health Science Center (UTHSC) animal care facility according to guidelines of the UTHSC IACUC. DNA analysis was used to identify the genotype of NR1^{+/-} or ^{+/+} mice. DNA was isolated from tail or liver tissue using standard protocols and the NR1 genotype was determined by PCR as described (Forrest et al., 1994). In NR1^{+/-} mice, the PCR products have both the mutant band (500 base pairs) and wild-type band (950 base pairs), while the PCR product in NR1^{+/+} control mice only has the wild-type band.

For all behavioral testing, mice were individually housed; for expression analyses, mice were group housed. Adult (over 90 days of age) mice were examined in all instances, and both males and females of each genotype were examined. Separate groups of mice were examined for behavioral testing and expression analyses. Mice were given food and water ad

libitum and maintained on a 12:12 hour light:dark cycle. An investigator who was blind to the genotype of the mice conducted all behavioral tests. For behavioral testing, a minimum of 6 mice per genotype per sex were examined. A separate group of animals was used to determine blood ethanol concentrations (BECs). For expression analyses, a minimum of 3 mice per genotype were examined, with a maximum of 6 mice if variability was greater.

2.2. Behavioral phenotyping

Several behavioral tests were conducted in both NR1+/- and wild type mice. These tests consisted of ethanol consumption using the two-bottle choice test, acute ethanol withdrawal as measured by Handling-Induced Convulsions (HICs), vocalization induced by unavoidable foot shock, and analysis of the anxiolytic response as measured in the elevated plus maze. Student's *t*-tests were used to evaluate the differences between the genotypes in all the behavioral measures. In the two-bottle choice tests, initial examinations were made between the sexes in each genotype. Because no significant differences between the sexes were found, males and females were combined in subsequent analyses.

2.2.1. The two-bottle choice test—The protocol of Whatley et al. (1999) was used to test the ethanol consumption of mice for 12 days. In this protocol, mice were initially given solely 10% ethanol (v/v) in water for 2 days, followed by 10 days of choice testing with one bottle containing 10% ethanol and the other bottle containing water. Consumption, in mls, was recorded daily and the location of the bottles, left to right, was alternated daily to avoid side preferences. Weights of the mice were recorded at the beginning and ending of testing. The grams of ethanol/kg of mice/day (g/kg/day) was computed and used in analyses to control for differences in body weights. The preference ratio, computed as the percentage of ethanol solution consumed out of the total liquid consumed, was analyzed to control for differences in levels of consumption.

2.2.2. Acute ethanol withdrawal as measured by handling-induced convulsions (HICs)—The HIC protocol is that of Buck et al. (1997). In this protocol, each mouse was held gently by the tail and spun with any resulting convulsions scored on a scale from 0 (no seizure) to 7. Mice were tested at baseline just prior to ethanol administration, and then at 4, 6 and 7 hours following an i.p. injection of 4.0 g/kg of ethanol.

2.2.3. Blood ethanol concentration (BEC)—Animals were injected with the same dose of ethanol as used to measure HICs: 4 g/kg i.p.. Blood was drawn at 1, 2 and 3 hours to assess the peak BECs, and at 7 hours to coincide with the timepoint used to assess HICs. Blood was drawn from a nick in the tail vein into heparinized capillary tubes from Analox. BECs were determined using an Analox GL5 alcohol analyser using the manufacturer's protocol.

2.2.4. Vocalization—Unavoidable footshock was used as an acute stressor and administered using shock chambers manufactured by Med Associates (St. Albans, VT) and The Med Associates, Inc Shock Titration Package for Mice. To establish the shock threshold, an initial shock of 0.05 mA was administered. Each subsequent shock was

progressively higher, increasing in increments of 0.05 mA until the animal vocalized. The shock level at which the animal vocalized was recorded for analysis (Matthews et al. 2008).

2.2.5. Elevated plus maze—The protocol for analyzing mice has been previously described (Hamre et al, 2007). The elevated maze (Med Associates, St. Albans, VT) is approximately 1 foot above the floor, and contains four 51-cm long, 11.5 cm-wide arms arranged at right angles. The closed arms have opaque walls 30 cm high, extending the length of the arm. Each arm of the plus maze contains two photobeams while the center of the maze is equipped with a single photobeam; photobeam signals are fed into a computer for automated monitoring. Each maze is located inside a cubicle which limits the view of subjects to the surroundings or to other mice undergoing behavioral testing. At the time of testing, each animal is placed in the center of the maze facing an open arm and the mouse is allowed to explore the maze for 5 minutes. The following measures were collected: amount of time in the 1) open arms, 2) closed arms and 3) middle; number of entries into: 4) open arms and 5) closed arms. Also calculated were the percentage of entries into the 6) open arms and 7) closed arms, and the percentage of time in the 8) open arms and 9) closed arms.

2.3. Brain tissue collection for NR2 subunit mRNA and protein expression analyses

Naïve adult mice were used for these analyses. For the analysis of either mRNA or protein expression, the entire cortex (Cor), cerebellum (Cb), striatum (St) and hippocampus (H) were dissected out from the unfixed brains of adult NR1+/- or +/+ mice. The fresh tissue was quick-frozen in liquid N₂ and stored at -70°C until mRNA or protein isolation.

2.4. RNA expression analysis of NR2 subunits using real time quantitative PCR

The RNA was isolated using RNA STAT-60 (Tel-Test, Inc. Friendswood, TX) following standard protocols. Homogenization was done by passing the sample through a 23g needle on a 1cc syringe until the tissue was no longer intact. Separation of the protein from the RNA was done using a Qiashredder (Qiagen Inc. Valencia, CA). The isolated RNA was treated with DNase 1 (RNase free, Roche Molecular Biochemicals, Mannheim, Germany) to eliminate DNA contamination. The purity and amount of the RNA were determined via spectrophotometry and electrophoresis. The RNA was diluted with DEPC H₂O to 0.1ug/ul and stored at -70°C.

Real time quantitative PCR was used to investigate the abundance of the NR2 subunits (NR2A-D) in the brain regions of the NR1+/- and +/+ mice as described previously (Du and Hamre, 2003). The forward and reverse primers were designed with Primer3 software (Howard Hughes Medical Institute and National Institutes of Health, National Human Genome Research Institute) and synthesized by Integrated DNA Technologies, Inc (Coralville, IA). The primer sequences are shown in Table 1. Real-time quantitative PCR was performed on the Smart Cycler (Cepheid, Sunnyvale, CA) by monitoring the increase of fluorescence by the binding of SYBR green to double-stranded DNA. Each SYBR Green reaction contained 200ng RNA (100ng RNA for β -actin). The reaction was incubated at 48°C for 15 minutes to synthesize cDNA, and at 94°C for 2 minutes for an initial denaturation, followed by 35 cycles of 94°C for 15 seconds, 56°C (NR2A), 64°C (NR2B, NR2C), or 60°C (NR2D) for 30 seconds to amplify the cDNA. Triplicate reactions of each

subunit and β -actin were run simultaneously from each region for each NR1+/- and NR1+/+ mouse. For each region, triplicates were run, with each triplicate from a separate mouse. 2^{-CT} method (Livak and Schmittgen, 2001) was used to calculate the fold changes in expression in each brain region of the NR1+/- mouse using the expression values of the actin control in the same brain region from the same mouse as the baseline value. To control for the slightly different values that could be obtained between runs, paired t-tests were used to assess whether significant differences were found between heterozygous and wild-type animals.

2.5. Protein expression analysis of NR2 subunits using Western blotting

Protein was isolated from the 4 brain regions using TRIzol Reagent (Invitrogen Co., Carlsbad, CA) following standard protocols. Quick Start Bradford Protein Assay (Bio-Rad Laboratory, Inc. Hercules, CA) was used to determine the concentration of the protein. A Mini-Protean 3 Cell (Bio-Rad Laboratory, Inc. Hercules, CA) was used in the electrophoresis and transference of the protein to a nitrocellulose membrane (Invitrogen Co. Carlsbad, CA). Anti-NR2B IgG 1:800 (Upstate, Lake Placid, NY) and an ECLTM Western Blotting System (Amersham Bioscience, Piscataway, NJ) were used to detect the NR2B subunits. The nitrocellulose membrane was incubated with primary antibody for 1 hour and then incubated with an HRP-conjugated anti-rabbit IgG (1:1000). Mouse anti β -actin IgG (Sigma-Aldrich Inc., St Louis, MO, 1:2500) was used as a control probe to confirm that equal amounts of protein from different brain regions were loaded and transferred onto the membrane. NIH *Image* (version 1.61) was used to measure the density of bands. Density in each region was normalized by the internal control (actin) in the same region. Expression change was recorded as a percentage, calculated by comparing the measured density of each brain region between heterozygous and wild-type mice.

2.6. Protein expression analysis of NR2B subunit using immunohistochemical staining

Only NR2B was examined in the analyses of protein expression because the only significant difference in mRNA expression was found in this subunit. Adult wild-type and NR1+/- mice were intracardially perfused with saline, followed by 4% paraformaldehyde in phosphate buffer (pH 7.2). The whole brain was dissected out from the skull and immersed in the same fixative overnight. The brains were sectioned in the sagittal plane on a cryostat at a thickness of 35 μ m. The antigen retrieval method of Jiao et al. (1999) and standard avidinbiotin immunohistochemical procedures were utilized to label the NR2B positive neurons in the brains. The same primary antibodies were used for Western blots and immunohistochemistry. Endogenous peroxidases were inactivated by incubating sections in PBS containing 10% methanol and 1% H₂O₂ for 10 min. The sections were then incubated with the rabbit anti-NR2B IgG (1:100) overnight. The sections were then processed using the ABC kit (Vector Laboratories, Inc. Burlingame, CA) and the immunolabeling was visualized using 3,3'-diaminobenzidine hydrochloride. Negative controls consisted of processing sections in the absence of primary antibody. Immunopositive labeled cells had a brown reaction product.

The brain regions were identified and microscopic images were collected. Two naïve investigators independently examined multiple sections and images of immunostained tissue

from each region and scored intensity/distribution of immunolabeling on a 5-point scale. Results for each label were averaged across genotypes for each brain region. The results were consistent between the two investigators. The average scoring results are shown in Table 3.

3. Results

3.1. NR1 heterozygous mice consumed more ethanol while fewer heterozygous mice exhibited ethanol withdrawal symptoms as measured by HICs

Ethanol consumption was analyzed using a standard two-bottle choice test using the protocol of Whatley et al., (1999). Comparisons of the consumption data between sexes within each genotype showed no significant sex differences (data not shown), therefore male and female data were pooled. NR1+/- mice consumed significantly more ethanol than their wild-type controls ($p < 0.05$) as shown in the grams of ethanol/kilogram body weight/day (g/kg/day) (Figure 1A). A similar trend was seen in the analyses of the preference ratios, although there were no significant differences between genotypes (Figure 1B).

Acute ethanol withdrawal sensitivity was assessed by measuring the severity of HICs at various times following ethanol exposure. At baseline, and at 4 and 6 hours after ethanol exposure, the two genotypes had equivalent HIC scores. However, at 7 hours after the ethanol injection, there was a significant difference between the two genotypes ($p < 0.05$; Figure 2). This is further exemplified by examining the percentage of mice of each genotype that demonstrated a HIC; almost 80% of the wild-type mice had a HIC while less than 50% of the heterozygous mice had a HIC. This phenotype suggests that acute sensitivity differs between NR1+/- and wild type mice and is consistent with previous data suggesting that mice that consume higher levels of ethanol exhibit lower acute ethanol withdrawal severity (Metten et al., 1998; Naassila et al., 2002).

3.1.1. Blood ethanol concentrations did not differ between genotypes—BECs were measured at 1, 2, 3 and 7 hours after ethanol injection and the values at each timepoint are shown in Figure 3. As shown, there are no differences in BECs between +/+ and NR1+/- providing evidence of similar ethanol metabolism between the two genotypes.

3.2. NR1+/- and NR1+/+ do not differ on stress and anxiety phenotypes

Sensitivity to acute footshock, which is often used as a stressor, was measured by determining the level of inescapable footshock that is required to cause vocalization; the level of anxiety was measured in the elevated plus maze. As shown in Table 2, no differences were observed in either measure between the heterozygous and wild-type mice. Equivalent levels of footshock were required to cause vocalization, indicating that these two groups of mice have similar stress-related phenotypes. In the elevated plus maze, the more time that an animal spends in the open, more-threatening arms of the maze, the less anxious it is thought to be. As can be seen in Table 2, both genotypes spent equivalent amounts of time in the open arms and made similar numbers of entries into the open arms. Thus, altering the level of NR1 appears to have no effect on these stress and anxiety phenotypes.

3.3. Region-specific alterations in mRNA expression of NR2 subunits was found in the NR1+/- mouse brain

The mRNA abundance of the four NR2 subunits was compared between the NR1+/- and wild-type mice, in four brain regions, by real time quantitative PCR. Regional differences in the levels of NR2 subunit mRNA expression in the heterozygous NR1 mice were observed. As shown in Figure 4, no differences in expression of NR2A, NR2C or NR2D were found in any brain region examined. Furthermore, no difference was detected in the NR2B subunit in the striatum. Significantly higher levels of NR2B expression were found but only in the hippocampus ($p < 0.01$). In the cortex and cerebellum there was a slight decrease in expression of NR2B although the differences were not significant. Taken together, changes in mRNA expression occur in a subunit-specific and region-specific manner.

3.4. Region-specific alterations in protein expression of NR2 subunits was found in NR1+/- mouse brain

The changes in mRNA expression of the NMDA receptors are not always reflected by changes in their protein expression (Hardy et al., 1999; Snell et al., 1996), therefore to confirm the changes observed for NR2B mRNA, the protein expression of NR2B was further investigated by Western blotting of the same four brain regions. In Western blot analysis, lower expression of NR2B protein was detected in the brain regions of the NR1+/- mice. In contrast to mRNA results, this decrease was found in all four brain regions, but in two brain regions the change was in the opposite direction (Fig. 5). This suggests that differential regulation of protein expression is occurring at the post-translational level for NR2B.

To further analyze the expression differences between the two genotypes, analysis of immunohistochemical labeling was conducted. Results of quantification of intensity and distribution of immunolabeling are shown in Table 3. Noticeably decreased expression of NR2B in NR1+/- brain was found in most regions examined, including the cortex (Fig. 6), the hippocampus (Fig. 7), the dorsal striatum and the cerebellar Purkinje cells (Fig. 8). The cortex was approximately divided into thirds anteroposteriorly for regional comparison. For cortex, neurons in the three regions separately examined and in all layers were more intensely stained in the +/+ compared to the +/- mice. For hippocampus, both pyramidal and granule cells were more intensely stained in the +/+ compared to the +/- mice. For striatum, more intensely stained neurons were found in the dorsal portion in the +/+ compared to the +/- mice; in the ventral striatum, light labeling was seen in both genotypes. For cerebellum, in all folia a subset of Purkinje cells was stained, and the staining intensity was greater in the +/+ compared to the +/- mice. In other regions of the cerebellum, no consistent staining patterns were observed in either genotype. Thus, results from the immunohistochemical staining are consistent with the results of the Western blots.

4. Discussion

The behavioral tests demonstrate that mice lacking one copy of the NR1 subunit of the NMDA receptor exhibit altered ethanol-related behaviors. NR1+/- mice consumed more ethanol and exhibited a reduction in handling-induced convulsions following acute

withdrawal, compared to NR1+/+ mice. These results demonstrate that NR1+/- mice have abnormal acute ethanol sensitivity. Furthermore, altered expression of the NR2B subunit was found in a region specific manner in NR1+/- mice, and this may contribute to the behavioral alterations in NR1+/- mice.

The neonatal lethality of the NR1-/- mice makes it impossible to assess the phenotypic responses in adult knockout animals. Nevertheless, three studies compared the 3 genotypes (NR1-/-, NR1+/- and NR1+/+) in prenatal and neonatal quantitative and qualitative phenotypes, and results consistently showed that the NR1+/- phenotype was intermediate to that of the other two, thereby showing a gene-dose effect. First, the degree of cortical development was compared among the genotypes with a number of morphologic measures obtained including thickness of the cortical plate, number of radial glial cells and extent of dendritic development. In all of these measures, the NR1+/- were intermediate to the other two genotypes (Elberger and Deng, 2003). Second, the effects of prenatal alcohol exposure on cortical morphology, such as listed above, as well as other measures of teratogenesis including incidence of malformations and survival were examined and similar gene-dose effects were observed (Deng and Elberger 2003). Third, gene expression analysis was compared using microarrays with subsequent analyses focusing on 3 of the differentially-expressed genes. For 2 of the 3 genes, expression in the NR1+/- was intermediate to that of the other groups (Sugiura et al., 2001). Taken together, these previous results provide evidence that the nature and directionality of the changes observed for NR1+/- in the present study are representative of the changes that would be seen in the NR1-/-.

4.1. The role of NMDA receptor subunits in ethanol-related behaviors

The development of physical dependence on ethanol involves up-regulation of NMDA receptors, including the NR1, NR2A and NR2B subunits, in various brain regions (Kalluri et al., 1998; Snell et al., 1996). Furthermore, increased expression of NR1 and NR2B is found following ethanol withdrawal (Hendricson et al., 2007; Nelson et al., 2005; Qiang et al., 2007) and is associated with enhanced seizure severity following ethanol withdrawal (Hendricson et al., 2007; Nelson et al., 2005; Hoffman and Tabakoff, 1994). Therefore, the lower levels of NR1 (Forrest et al., 1994) and NR2B (in the present study) protein expression in the NR1+/- mice, may explain why smaller numbers of NR1+/- mice exhibited handling-induced seizures following a bolus of alcohol. This is consistent with previous studies suggesting a role of NMDA in acute ethanol withdrawal. For example, NMDA induced significant elevation in HICs during acute withdrawal in withdrawal seizure prone mice (Crabbe et al., 1993). Additionally, administration of NMDA receptor antagonists attenuates the severity of ethanol withdrawal (see review in Bisaga and Popik, 2000; Hoffman and Tabakoff, 1994).

However, it is unclear why NR1+/- mice consume more ethanol than wild-type mice because the present results are inconsistent with previous studies using NMDA receptor antagonists. For example, several NMDA receptor antagonists, including an NR2B selective antagonist, inhibited ethanol intake in rats chronically exposed to ethanol (Hölter et al., 2000; Lin and Hubbard, 1995; Vengeliene et al., 2005). However, NMDA receptors may completely or almost completely be blocked by antagonists (Hölter et al., 2000; Lin and

Hubbard, 1995; Vengeliene et al., 2005). In contrast, 50% of the NMDA receptors are assumed to be functional in NR1+/- mice even though their expression is down-regulated (Forrest et al., 1994). Furthermore, in NR1+/- mice, the change in expression of NR1 occurs throughout life, in contrast to the temporary nature of, and sometimes localized application of, NMDA antagonists.

In addition, factors that reduce the sensitivity of NMDA to ethanol's actions contribute to ethanol tolerance. For example, clinical data suggest that functional alterations in NMDA receptors that reduce responses to ethanol may promote heavy ethanol consumption by removing the negative feedback on drinking (in review by Krystal et al, 2003). Similarly, we believe that lower NR1 and NR2B expression in the NR1+/- mice may result in lower sensitivity to ethanol and thereby increase ethanol consumption. This explanation is consistent with what is found following prenatal ethanol exposure in which there is an attenuation of ethanol's deleterious effects on dendritic outgrowth in NR1 heterozygous mice (Deng and Elberger, 2003). Consistent with our results, mice that have lower severity of alcohol withdrawal consume more alcohol in both acute and chronic ethanol exposure (Metten et al., 1998).

NR1+/- mice do not exhibit altered anxiety or stress responses as assessed in the elevated plus maze, and by vocalization following foot-shock stress. The altered anxiolytic behavior has been found when NR1 was depleted over 90% in a conditional knock out or blocked with a receptor antagonist (Bennett et al., 1990; Dunn et al., 1989; Kehne et al., 1991; Mohn et al., 1999; Zapata et al., 1997).

4.2. Heterozygous NR1 deletion alters NR2 subunit mRNA and protein expression in a regionally-specific manner

Previous experiments have suggested that NR1 is required for normal expression of NR2A and NR2B in the CNS (Forrest et al., 1994; Fukaya et al., 2003). Complete loss of NR1 alters NR2 subunit mRNA and protein levels (Forrest et al., 1994) or protein level only (Fukaya et al., 2003). Reduced protein expression was found only for the NR2B subunit while NR2A expression remained at normal levels in the brains of NR1 knockout mice (Forrest et al., 1994). However, the NR1 deletion did not affect the NR2A and NR2B mRNA expression but severely reduced the NR2A and NR2B protein expression in dendrites of CA1 pyramidal cells of the conditional NR1 knockout mouse (Fukaya et al., 2003). Consistent with previous studies, the present study has clearly demonstrated that heterozygous NR1 deletion alters NR2B expression of both mRNA and protein levels, confirming that there is an interdependence of NMDA receptor subunit expression (Forrest et al., 1994). However, the mechanism of this interaction is currently unknown. One possibility is that there is a more direct link between NR1 and NR2B expression although it is equally possible that the basis of the interaction is more compensatory in nature. In addition, there may have been alterations in NR2B expression in brain regions that were not examined in detail in the present study, and these may have contributed to the behavioral differences reported here. Future studies are needed to distinguish between these and other possibilities.

Additionally, the present study also examined the expression of NR2A, NR2C and NR2D in NR1^{+/-} mice. None of these subunits exhibited any alteration in expression, suggesting either differential relationships between these 3 subunits and NR1 or that a heterozygous deletion is insufficient to cause or detect altered subunit expression in every case. Alternatively, it is possible that these subunits may have shown alterations in expression in brain regions not examined in detail in the present study, and these changes could impact the behavioral differences observed in the heterozygotes. Taken together, the expression analysis suggests that NR2B is more sensitive to NR1 deletion than NR2A, NR2C and NR2D.

4.3. Post-transcriptional regulation is involved in the expression of NR2 subunits

The present study demonstrates that altered expression of NR1 results in altered expression of NR2B at both message and protein levels. The significant change in mRNA level was only found in the hippocampus of NR1^{+/-} mice, where expression was increased by ~4 fold compared to NR1^{+/+} mice (Fig. 4). However, the alteration in expression of NR2B at the mRNA level is not consistent with changes in protein level. For example, higher NR2B mRNA expression was found in the hippocampus of NR1^{+/-} brains, while reduced protein expression was detected in the same region (Fig. 5, 7). In contrast, in the cortex the mRNA expression of NR2B was decreased, although not significantly, with the protein expression exhibiting a larger decrease in the same region (Fig. 4–6). These results suggest a post-transcriptional regulation, which may modulate protein expression regardless of mRNA level. The inconsistency between mRNA and protein levels of NMDA receptors has also been reported in the brains of rats following chronic ethanol consumption (Snell et al., 1996; Winkler et al., 1999) or chronic morphine treatment (Bajo et al., 2006). Furthermore, discordance between protein and mRNA expression levels has been found in a number of systems including yeast (Fournier et al., 2010; Washburn et al., 2003), mouse liver (Tian et al., 2004) and NMDA receptors in rat medulla (Miki et al., 2002).

There are undoubtedly compensatory changes in the expression of other proteins in NR1^{+/-} mice that likely contribute to some of the altered phenotypic responses. Ethanol has been shown to affect a wide range of substances including various members of the GABA, dopamine and acetylcholine families, and second messenger systems (Fadda and Rossetti, 1998; Morikawa and Morrisett, 2010; Nevo and Hamon, 1995). Many of these have been shown to interact with NMDA receptors on some level including altered receptor functioning, distribution and dynamics (Cepeda and Levine, 2006; Mora et al., 2008). Moreover, ethanol has been shown to alter some of these interactions (Gremel and Cunningham, 2009; Kash et al., 2008; Ron, 2004; Stobbs et al., 2004). Given the wealth of other proteins that are known to be affected by ethanol exposure and to interact with NMDA receptors, there are a number of potential candidates for investigation in future studies.

In the present study, regional differences in expression of NR2 subunits were observed in the NR1^{+/-} brain. These data provide a baseline against which to assess influence of alcohol exposure or alcohol withdrawal on the expression of subunits of the NMDA receptor in mice that lack one copy of the NR1 subunit. Previous work has shown changes in expression of NR2 subunits following alcohol withdrawal in the hippocampus and in the cortex (Hardy et al., 1999; Hendricson et al., 2007; Nelson et al., 2005). Furthermore, changes in expression

of NMDA receptors, especially of the NR2B subunit, following chronic ethanol exposure are also region-specific. For example, up-regulation of the NR2B subunit was only found in the dorsal striatum, but not in the ventral striatum (Wang et al., 2007), and its expression was inhibited in the dorsal hippocampus (Kalev-Zylinska and Doring, 2007). Thus, the NR1+/- mouse may be a good animal model for studying ethanol related neurotoxicity or behavior following acute or chronic ethanol exposure.

5. Conclusion

In summary, NR1 heterozygous mice consume significantly higher levels of ethanol in the two bottle-choice test and exhibit lower levels of handling-induced convulsions. In contrast, no effects on stress-related phenotypes were detected by vocalization and the elevated plus maze in the NR1+/- mice. Examination of NR2 subunit expression in the brain of NR1+/- mice shows that NR1 plays a role in the expression of NR2 subunits but in a region and subtype specific manner. However, it is currently unclear whether there is a direct link between expression of these proteins or whether changes in expression of NR2B are compensatory changes due to altered levels of NR1. This study provides evidence of the effects of altered levels of NR1 expression on ethanol withdrawal and consumption, and suggests that these effects could be related to alterations in the levels of NR2B, although other unexplored molecules may also play a role.

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Highlights

- NR1 heterozygous mice consume significantly higher levels of ethanol in the two bottle-choice test
- NR1 heterozygous mice exhibit lower levels of handling-induced convulsions
- NR1 heterozygous mice exhibit normal responses in stress-related phenotypes
- NR2B subunit exhibited differences in expression of mRNA in the hippocampus and protein levels in multiple brain regions, between wild-type and NR1+/- mice; NR2A, C, D were not different

Fig. 1. NR1^{+/-} mice consume more ethanol than NR1^{+/+} mice measured in the two-bottle choice test.

A) The mean ($\pm$ SEM) g/kg/day for the two genotypes is shown. The NR1^{+/-} consume significantly higher levels ($p < 0.05$) of ethanol. B). The mean ($\pm$ SEM) preference ratio for the entire test is shown for each genotype.

Fig. 2. NR1^{+/-} mice exhibit smaller responses to acute ethanol withdrawal as measured using handling-induced convulsions (HICs).

The mean ($\pm$ SEM) HIC scores at each of the 4 timepoints are shown for each genotype. The NR1^{+/-} mice are shown by the diamonds connected with the dashed line, while NR1^{+/+} are shown by the squares connected with the solid line. At the 7 hour timepoint, the mean score for NR1^{+/-} mice is significantly lower than that for the NR1^{+/+} mice ($p < 0.05$).

Fig. 3. NR1^{+/-} mice exhibit similar Blood Ethanol Concentrations as NR1^{+/+}.

The mean ($\pm$ SEM) at each of the 4 timepoints are shown for each genotype. No significant differences between the genotypes are seen at any timepoint.

Fig. 4. mRNA expression of NR2 subunits in four brain regions of NR1^{+/-} mice. Expression was examined by real time PCR and compared between NR1^{+/-} and NR1^{+/+} mice.

Shown are the mean ($\pm$ SEM) fold changes in the NR1^{+/-} compared to the NR1^{+/+} mice. Increased expression of NR2B is found in the hippocampus of NR1^{+/-} mice (3.94 fold, $p < 0.001$, see * in the figure). No significant change in expression of NR2B is detected in cortex, striatum or cerebellum. No significant change in expression of NR2A, NR2C or NR2D is detected in any region examined.

Fig. 5. NR2B expression in the brains of NR1^{+/+} and NR1^{+/-} mice examined by Western blotting.

Anti-Beta actin was used to demonstrate that equal amounts of protein from different brain regions were loaded and transferred onto the membrane (A). NIH *Image* (version 1.61) was used to measure the density of the bands and results are shown in B. Decreased expression of NR2B (14.7–1.9%) is found in each brain region in NR1^{+/-} mice compared to NR1^{+/+} mice.

Fig. 6. Comparison of NR2B expression in the neocortex of NR1+/+ and NR1+/- mice. NR2B expression was examined by immunohistochemical staining.

Expression of NR2B in NR1+/+ mice is shown in the left column while the expression in NR1+/- mice is shown in the right column. Decreased NR2B expression was found in all 3 neocortical regions in NR1+/- mice compared to NR1+/+ mice. All neocortical regions show a similar pattern in layers II-VI. A-B) The example shown is of layers II-V in the prefrontal region of anterior cortex. C-D) The example shown is of layers II-V in the somatosensory region of middle cortex. E-F) The example shown is of layers II-V in the visual region of posterior cortex. Most neurons have more intense labeling in the NR1+/+ than in the NR1+/-; a few such cells are marked by arrows. Scale bar A-F = 50 μ m.

Fig. 7. Comparison of NR2B expression in the hippocampus of NR1+/+ and NR1+/- mice. NR2B expression was examined by immunohistochemical staining. Expression of NR2B in NR1+/+ mice is shown in the upper panel while the expression in NR1+/- mice is shown in the lower panel. The entire hippocampal formation for each genotype is shown in 5-image photomontages. In the hippocampus, the pyramidal and granular neurons show a similar pattern with most having more intense labeling in the NR1+/+ than in the NR1+/- . The pyramidal cells of CA1 are marked by an arrow, and for CA3 are marked by an arrowhead. The granule cells of the dentate gyrus are marked with an asterisk. Scale bar = 100µm.

Fig. 8. Comparison of NR2B expression in the dorsal striatum and cerebellum of NR1^{+/+} and NR1^{+/-} mice.

NR2B expression was examined by immunohistochemical staining. Expression of NR2B in NR1^{+/+} mice is shown in the left column while the expression in NR1^{+/-} mice is shown in the right column. Decreased NR2B expression was found in both regions in NR1^{+/-} mice compared to NR1^{+/+} mice. A-B) Examples of immunolabeled dorsal striatal neurons are marked by arrows. C-D) In the cerebellum the Purkinje cells show specific immunolabeling. A subset of the single layer of Purkinje cells (arrows) shows intense labeling in the NR1^{+/+} with only a few labeled Purkinje cells in the NR1^{+/-}. The superficial molecular layer (asterisk) shows no specific immunolabeling. Scale bars A-B, C-D = 50µm.

Table 1.

Primer sequences used in real-time PCR

Subunit	Forward primer	Reverse primer
NR2A	5' CTG ACT GGG TCA CAG AGC AA	5' GCA GCT CTT TTG GGT GAG TC
NR2B	5' GTG AGA GCT CCT TTG CCA AC	5' GTC AGG GTA GAG CGA CTT GC
NR2C	5' AAG GCC TTT TGT CAT TGT GG	5' CTT GAG GAT GTC GAT GCA GA
NR2D	5' TAG TGT CAG TGC GCA GAT CC	5' ACC ATG AAC CAG ACG TAG CC

Table 2.Results of behavioral tests showing mean $\pm$ sem

Behavioral test	NR1+/+	NR1+/-
Time in the open arms (mean %)	24.13 $\pm$ 6.14	22.78 $\pm$ 0.081
Arm entries (mean %)	31.56 $\pm$ 4.24	34.97 $\pm$ 0.82
Vocalization (mean level of intensity)	0.26 $\pm$ 0.033	0.25 $\pm$ 0.012

Table 3.

Analysis of intensity of NR2B immunolabeling in various brain regions in wild-type compared to NR1 +/- mice.

BRAIN REGION	SCORES	
	NR1+/+	NR1+/-
Anterior Cortex	+++	++
Middle Cortex	++++	++
Posterior Cortex	+++	++
Hippocampus (whole)	+++	++
Hippocampal Granule cells	++++	+++
CA3 pyramidal cells	++++	+++
CA1 pyramidal cells	++++	+++
Dorsal striatum	++	+
Ventral striatum	+	+
Cerebellar Purkinje cells	+++	++
Cerebellar Molecular layer	^a -	^a -

^aDenotes area without specific immunolabeling.