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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

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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 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.

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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 the 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 proteins 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.

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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 the striatum and medial prefrontal cortex (Klugmann et al., 2011), and increased NR2B protein in the 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 has 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 h 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 the expression of 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