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N-METHYL-D-ASPARTATE RECEPTOR SUBUNIT EXPRESSION IN ADULT AND ADOLESCENT BRAIN FOLLOWING CHRONIC ETHANOL EXPOSURE

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Abstract—Substantial evidence suggests that glutamatergic neurotransmission is a critical mediator of the experience-dependent synaptic plasticity that may underlie alcohol dependence. Substance abuse typically begins in adolescence; therefore, the impact of alcohol on glutamatergic systems during this critical time in brain development is of particular importance. The N-methyl-D-aspartate receptor (NMDAR) is involved in developmental mechanisms underlying neuronal differentiation and synaptogenesis and as such may be a target system for alcohol effects during adolescence. In the present study quantitative biochemical determinations were made of the relative abundance of different protein expressions of NMDAR subunits in adolescents and adults after 2 weeks of ethanol vapor exposure, and 24 h and 2 weeks following withdrawal. After 2 weeks of ethanol vapor exposure N-methyl-D-aspartate receptor NR1 subunit (NR1), N-methyl-D-aspartate receptor NR2A subunit (NR2A), and N-methyl-D-aspartate receptor NR2B subunit (NR2B) subunit expression was found to be increased in hippocampus of the adults. In contrast, 2 weeks of ethanol exposure resulted in no significant changes in NR1 and NR2B subunits and a reduction NR2A subunit expression in hippocampus in adolescents. Twenty-four h and 2 weeks following withdrawal from ethanol vapor NR1 and NR2A subunit expression in hippocampus was decreased in adolescents, whereas in adults it had returned to control levels. In frontal cortex, 2 weeks of chronic ethanol exposure produced decreases in NR1 subunit expression in both adults and adolescents but also produced decreases in NR2A and NR2B subunit expression in adults that returned or exceeded control levels by 2 weeks following withdrawal from ethanol vapor. These results demonstrate that NMDAR subunit composition can be modulated differentially between adolescents and adults by chronic ethanol exposure and withdrawal. These developmental differences in NMDAR subunits composition may also be associated with the enhanced vulnerability of the adolescent brain to ethanol dependence.

Key words: NR1, NR2A, NR2B, alcohol withdrawal, hippocampus, frontal cortex.

Adolescence is a critical stage of brain development when humans are initially exposed to potentially toxic external stimuli such as ethanol and other drugs of abuse (Johnston, 1995). Ethanol is one of the most abused drugs during adolescence and can produce detrimental effects on brain development (Spear, 2000; Crews et al., 2007). Given that the brain continues to develop throughout the adolescent period (Markus and Petit, 1987; Sowell et al., 1999), early ethanol exposure may have unique deleterious consequences.

Studies characterizing the cellular and molecular mechanisms underlying the enhanced vulnerability to ethanol exposure during adolescence have frequently focused on studying glutamatergic neurotransmission (see Fadda and Rossetti, 1998; Crews et al., 2002), and in particular the N-methyl-D-aspartate (NMDA) type of glutamate receptor. Substantial loss of synapses, especially the excitatory glutamatergic inputs to the forebrain, occurs during adolescence (Huttenlocher, 1984; Zecevic et al., 1989) and may be vulnerable to ethanol exposure. In the hippocampus, the exuberant outgrowth of excitatory axon collaterals and synapses that occur earlier during young ages are morphologically remodeled and branches within dendritic arbors are pruned during adolescent maturation with most synaptic pruning involving glutamatergic receptors (Swann et al., 1999).

Evidence has shown that NMDA receptor subunit expression is developmentally regulated during brain maturation (Watanabe et al., 1992, 1993; Jin et al., 1997; Wenzel et al., 1997; Barria and Malinow, 2002; Magnusson et al., 2002; Law et al., 2003; Chang et al., 2009). This regulation, which has been demonstrated both *in vivo* and *in vitro*, leads to changes in the functional and pharmacological properties of N-methyl-D-aspartate receptor (NMDAR). Several studies suggest that NMDA receptors influence synaptogenesis, neuronal growth and plasticity, as well as the fine-tuning of neuronal connections (see Kloda et al., 2007). One of the fundamental changes that occur in NMDA receptor subunit composition during devel-

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Abbreviations: ANOVA, analysis of variance; BALs, blood alcohol levels; BCA assay, bicinchoninic acid assay; C, degree Celsius; CET, chronic ethanol treatment; ERP, event-related potential; FAS, fetal alcohol syndrome; g, grams; g/kg, grams per kilogram; IgG, immunoglobulin G; mg/dl, miligrams per deciliter; MK-801, dizocilpine maleate; mM, millimolar; NIH, national institutes of health; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; NR1, N-methyl-D-aspartate receptor NR1 subunit; NR2A, N-methyl-D-aspartate receptor NR2A subunit; NR2B, N-methyl-D-aspartate receptor NR2B subunit; PND, postnatal day; WD, withdrawal.

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opment is a gradual reduction in NR2B levels, with a concomitant increase in NR2A levels (Monyer et al., 1994; Jin et al., 1997).

The NMDAR has also been shown to be an important site for ethanol actions (see Lovinger et al., 1989; Peoples and Weight, 1999; Schummers and Browning, 2001; Hoffman, 2003) and to play a role mediating ethanol dependence, tolerance, and withdrawal (Eckardt et al., 1998; Kalluri et al., 1998; Krystal et al., 1998; Darstein et al., 2000). Studies have shown that acute ethanol exposure inhibits the expression of NMDAR subunits. There is considerable evidence to suggest that the NR2A- and NR2B subunits are the most sensitive NMDAR subunits to ethanol actions (Masood et al., 1994; Trevisan et al., 1994; Grant and Lovinger, 1995; Allgaier, 2002; Nixon et al., 2004; Hendricson et al., 2007). However, the consequences of adolescent ethanol exposure on the expression of NMDAR subunits in the cortex and hippocampus *in vivo* are just beginning to be understood (Sircar and Sircar, 2006; Pascual et al., 2009). Characterizing the effects of adolescent versus adult ethanol exposure on the expression of NMDAR subunits could provide important information on the unique mechanisms that may mediate the neurophysiological consequences of adolescent ethanol exposure previously shown in our laboratory (Slawecki et al., 2004; Criado et al., 2008a, b). The objective of the present study was to determine whether expression of the N-methyl-D-aspartate receptor NR1 subunit (NR1), N-methyl-D-aspartate receptor NR2A subunit (NR2A) and N-methyl-D-aspartate receptor NR2B subunit (NR2B) subunits in the hippocampus and frontal cortex is influenced by chronic ethanol exposure and/or withdrawal in adolescent versus adult rats. Adolescent and adult rats were exposed to intermittent chronic ethanol vapor for a period of 2 weeks. Expression protein levels of the three different NMDA receptor subunits (NR1, NR2A, and NR2B) were quantified in the frontal cortex and hippocampus at three different time points following chronic ethanol treatment (CET): no withdrawal period (chronic ethanol group), 24-h withdrawal (WD) period (24-h WD group) and 2-week withdrawal period (2-week WD group).

EXPERIMENTAL PROCEDURES

Subjects

Male Wistar rats at postnatal day (PND) 23 (n=42; Charles River, USA) and at PND 60 (n=42; Charles River, USA) were used in this study. Adolescent (PND 23) and adult (PND 60) rats were housed four and two per cage respectively in standard cages for the duration of the experiment. Animals were kept in a light/dark (12-h light/12-h dark, lights on at 6:00 A.M.) and temperature-controlled environment except for during the vapor exposure (see below). Food and water were available *ad libitum* throughout the experiment, except where noted. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Scripps Research Institute and were consistent with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

Ethanol vapor exposure

Ethanol vapor exposure has been shown to reliably allow for the titration of blood alcohol levels (BALs) that are sufficient for inducing ethanol physical dependence as indicated by signs of withdrawal (Roberts et al., 1996, 2000; O'Dell et al., 2004). The ethanol vapor inhalation procedure and the chambers used in this study were previously described (Rogers et al., 1979; Slawecki et al., 2001; Slawecki, 2002). Ethanol vapor chambers (La Jolla Alcohol Research Inc., La Jolla, CA, USA) were calibrated to produce moderate BALs between 175 and 225 mg/dl. In brief, adolescent (n=42) and adult (n=42) rats were divided into two groups each (ethanol-exposed group, n=21; control group, n=21). Ethanol-exposed rats were housed in sealed chambers, which were infused with vaporized 95% ethanol from 8 PM to 10 AM. For the remaining of the 10 h of the day, ethanol vapor was not infused into the chamber. This ethanol exposure regimen continued for 2 weeks. At the start of the ethanol exposure, adolescent rats were 23-days old and the exposure continued until they were 37-days old. Adult rats were 60-days old and exposure continued until they were 74-days old. Age-matched controls were handled identically to ethanol-exposed rats. Food and water were always available. Blood samples were collected from the tip of the tail three times per week to assess BALs (target: 150–200 mg/dl). BALs were determined using the Analox micro-statGM7 (Analox Instr. Ltd., Lunenberg, MA, USA).

Adolescent (n=21) and adult (n=21) ethanol-exposed rats were randomly subdivided into three groups each: the CET group (n=7), the 24-h ethanol WD group (n=7), and the 2-week ethanol WD group (n=7). In the CET group, rats were sacrificed and brains dissected immediately after the 2 weeks of ethanol exposure. In the 24-h WD group, brains were dissected 24 h after ethanol exposure. In the 2-week WD group, brains were dissected 2 weeks after ethanol exposure. Each ethanol group was compared to its respective control group. When ethanol exposure ended, rats from groups assigned to the withdrawal periods (24-h WD and 2-week WD) were maintained in the Scripps vivarium.

Tissue dissection and preparation

Fig. 1 shows graphical representation of the experimental protocol used to harvest adolescent (PND 37–52) and adult (PND 74–88) rat brains. Frontal cortex and hippocampus were dissected and

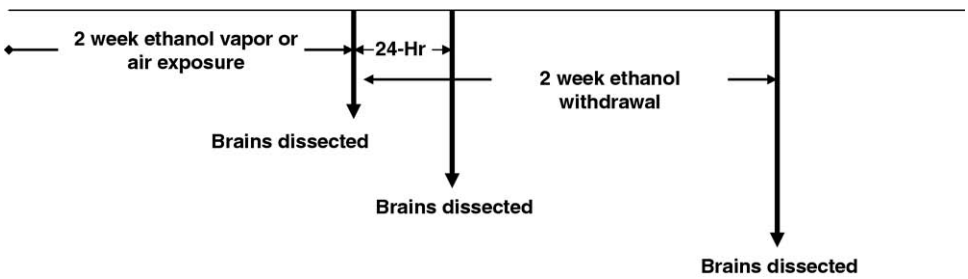

Fig. 1. Ethanol exposure protocol.