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NMDAR 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 wks of ethanol vapor exposure, and 24 hrs and 2 wks following withdrawal. After 2 wks of ethanol vapor exposure NR1, NR2A, and NR2B subunit expression was found to be increased in hippocampus of the adults. In contrast, 2 wks 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 hours and 2 wks 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 wks 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 wks 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.

Keywords

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

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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 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 development 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, 2008b). The objective of the present study was to determine whether expression of the NR1, NR2A and 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 wks. 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: no withdrawal period (chronic ethanol group), 24-hr withdrawal (WD) period (24-hr WD group) and 2-wk withdrawal period (2-wk 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 (12h light/12h dark, lights on at 06:00 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 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 were calibrated to produce moderate BALs between 175–225 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 hours of the day, ethanol vapor was not infused into the chamber. This ethanol exposure regimen continued for 2 wks. 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 blood alcohol levels (BALs) (target: 150 to 200 mg/dl). BALs were determined using the Analox micro-statGM7 (Analox Instr. Ltd., Lunenburg, MA).

Adolescent ($n = 21$) and adult ($n = 21$) ethanol-exposed rats were randomly subdivided into three groups each: the chronic ethanol treatment group (CET) ($n = 7$), the 24-hr ethanol withdrawal (WD) group ($n = 7$), and the 2-wk ethanol WD group ($n = 7$). In the CET group, rats were sacrificed and brains dissected immediately after the 2-wks of ethanol exposure. In the 24-hr WD group, brains were dissected 24 hrs after ethanol exposure. In the 2-wk WD group, brains were dissected 2 wks 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-hr WD and 2-wk WD) were maintained in the Scripps vivarium.

Tissue dissection and preparation

Figure 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 frozen until solubilization and homogenization. Brain tissue was solubilized and homogenized in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 1% deoxycholate and Complete Mini Protease Cocktail Inhibitor (Roche, Switzerland) using a tissue sonicator. The homogenate was then centrifuged for 35 minutes at 4 °C. The resulting supernatant was used as the membrane fraction. Protein concentrations were determined using a BCA assay (Pierce, Rockford, IL).

Western blotting and analysis

Samples of frontal cortical and hippocampal homogenates (5–15 ug of total protein per lane) were heated with NuPAGE 4×LDF sample buffer (Invitrogen), and then separated on 4%–8% gradient Tris-acetate minigels (Invitrogen) and electrotransferred to nitrocellulose membranes. Non-specific binding of antibodies to membranes was prevented by blocking with a solution containing 3% (w/v) nonfat dry milk, 0.05% (v/v) Tween-20 (Fisher Scientific), in Tris Buffered Saline (Fisher Scientific). Membranes were incubated with diluted primary antibodies in buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20): rabbit anti-NMDAR1 affinity-purified polyclonal antibody (1:6,000; Chemicon, Temecula, CA), rabbit anti-NMDAR2A affinity-purified polyclonal antibody (1:4,000; Chemicon), rabbit anti-NMDAR2B affinity-purified polyclonal antibody (1:3,000; Chemicon), or mouse polyclonal antibody to β -actin (1:1,000; Chemicon) overnight at 4 °C, and the –HRP conjugated secondary goat anti-rabbit IgG (1:5,000) and goat anti-mouse IgG (1:1,000) antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) incubated for one hour at room temperature. Immuno-reactive bands were visualized with the ECL Plus Western Blotting Detection System (Pierce, Rockford, IL), and band intensity quantified using Quantity One software (Bio-Rad, Hercules, CA). Protein levels are expressed as optical units obtained by normalization of NMDAR subunits against β -actin.

Data Analysis

Statistical analyses were performed by using SPSS (SPSS, Inc., Chicago, IL). ANOVAs were used to determine the effects of chronic ethanol exposure on body weight and BALs. Ethanol and control groups were compared after termination of the chronic ethanol treatment and 2-wks after withdrawal from chronic ethanol ($P < 0.05$ for significance). BALs in adolescent and adult rats exposed to ethanol were compared using one-way ANOVA ($P < 0.05$ for significance).

Brain regions (frontal cortex and hippocampus) and NMDAR subunits (NR1, NR2A and NR2B) were assessed independently. Independent analysis of variance (ANOVA) was used to assess the expression of the NR1, NR2A and NR2B NMDAR subunits in the frontal cortex and hippocampus in adolescent (PND 37–52) and adult (PND 74–88) rats. Independent ANOVAs were also used to assess the effects of CET on the expression of the NMDAR subunits in adolescent and adult rats at three different withdrawal periods following chronic ethanol exposure: 0-hr, 24-hr and 2-wk. These groups were designated as the CET, the 24-hr WD and the 2-wk WD. When appropriate, post hoc analysis of the ANOVA utilized the Fisher's Least Significance Difference test to assess group differences. ANOVAs were also used to assess developmental differences in the expression of NMDAR subunits and the NR2A/NR2B ratio in animals from control groups. To correct for multiple comparisons in the expression of NMDAR subunits, P -values for all ANOVAs were set at $P < 0.01$ to determine the levels of statistical significance. P -values for post hoc analyses were set at $P < 0.05$.

RESULTS

Body weight and BALs

Prior to the initiation of the experiment, there were no differences in body weight between the ethanol (52 ± 2 g) and control (53 ± 1 g) groups in adolescent rats. The body weight of the ethanol (355 ± 4 g) and control (362 ± 3 g) groups was also not significantly different in adult rats. Ethanol vapor exposure produced BALs averaging 193 ± 10 mg/dl in adolescent rats and 197 ± 6 mg/dl in adult rats. Following chronic ethanol exposure, no differences in body weight were observed between ethanol exposed and control adolescent rats (Ethanol exposed: 132 ± 3 g; Control: 142 ± 3 g). In contrast, differences in body weight were found

between ethanol exposed and control adult rats following chronic ethanol exposure (Ethanol exposed: 387 ± 6 g; Control: 420 ± 4 g; $F(1,41)=23.0$, $P < 0.01$). 2-wks after ethanol WD, differences in body weight were observed between ethanol exposed and control adolescent rats (Ethanol exposed: 251 ± 4 g; Control: 272 ± 5 g; $F(1,12)=10.8$, $P < 0.01$). No differences in body weight were observed between ethanol exposed and control adult rats after 2-wks of ethanol WD (Ethanol exposed: 477 ± 9 g; Control: 464 ± 8 g).

Developmental regulation of NMDAR subunit expression

We studied the expression of the NR1, NR2A and NR2B NMDAR subunits in the frontal cortex and hippocampus in adolescent (PND 37–52) and adult (PND 74–88) rats ($n = 15$ /group). The expression of the NR1 subunit in the frontal cortex was not different between adolescent and adult rats. In contrast, the expression of the NR2A and NR2B subunits in the frontal cortex was greater in adult than in adolescent rats ($F's(1,29) < 77.6$, $P's < 0.01$). The ratio of NR2A to NR2B subunit protein expression was significantly increased in frontal cortex in adult versus adolescent control animals (adolescent: 0.92 ± 0.1 ; adult: 1.75 ± 0.1 ; $F(1,29) = 31.2$, $P < 0.001$). Figures 2A–C illustrate the expression of the NR1, NR2A and NR2B NMDAR subunits in the frontal cortex in rats at PND 37, 38, 52, 74, 75 and 88.

The expression of the NR1, NR2A and NR2B subunits in the hippocampus was greater in adult than in adolescent rats ($F's(1,29) < 87.5$, $P's < 0.001$). The ratio of NR2A to NR2B subunit protein expression was significantly increased in hippocampus in adult versus adolescent control animals (adolescent: 0.40 ± 0.03 adult: 0.64 ± 0.03 ; $F(1,29) = 25.5$, $P < 0.001$). Figures 2D–F illustrate the expression of the NR1, NR2A and NR2B NMDAR subunits in hippocampus in rats at PND 37, 38, 52, 74, 75 and 88.

Effects of ethanol on NMDAR subunits expression in the frontal cortex

Use of ANOVA provided statistically significant differences in the expression of the NR1 subunit in the frontal cortex in adolescent and adult rats (Figs. 3A–B; $F's(3, 41) < 8.9$; $P's < 0.001$). Post hoc assessment revealed that the expression of the NR1 subunit in the frontal cortex was decreased in the CET group in adolescent rats, and in the CET and 24-hr WD groups in adult rats, compared to their respective control groups (Figs. 3A–B).

Ethanol exposure had no significant effect on the expression of the NR2A subunit in the frontal cortex of adolescent rats (Fig. 3C). In contrast, ethanol had a significant effect on the expression of the NR2A subunit in adult rats ($F(3,41) = 12.1$; $P < 0.001$). Post hoc assessment showed that CET significantly reduced NR2A expression, whereas the 24-hr WD group showed a significant increase (Fig. 3D). Chronic ethanol exposure had no significant effect on the expression of the NR2B subunit in the frontal cortex of adolescent rats (Fig. 3E; $F's(3,41) = 3.5$; $P > 0.01$). However, chronic ethanol exposure had a significant effect on the expression of the NR2B subunit in the frontal cortex of adult rats (Fig. 3F; $F's(3,41) = 15.8$; $P < 0.01$). Post hoc assessment revealed that the expression of the NR2B subunit in the frontal cortex was decreased in the CET and 24-hr WD groups in adult rats, compared to the control group (Figs. 3F). However, the adult 2-wk WD group showed an increase in the expression of the NR2B subunit (Fig. 3F).

Effects of ethanol on NMDAR subunits expression in the hippocampus

ANOVA revealed that ethanol had a significant effect on the expression of the NR1 subunit in the hippocampus in adolescent and adult rats (Figs. 4A–B; $F's(3, 41) < 17.1$; $P's < 0.001$). Post hoc assessment revealed that the expression of the NR1 subunit in the adolescent hippocampus was decreased in the 24-hr WD group and increased in the 2-wk WD group, compared to the control group (Fig. 4A). In contrast, the expression of the NR1 subunit in

the adult hippocampus was increased in the CET group, compared to the control group (Fig. 4B).

Ethanol exposure had a significant effect on the expression of the NR2A subunit in the hippocampus of adolescent and adult rats (Figs. 4C-D; $F(3, 41) < 18.6$; $P < 0.001$). Post hoc assessment revealed that the adolescent hippocampus showed a significant reduction in NR2A expression in the CET and 24-hr WD groups, whereas the 2-wk WD group showed a significant increase, compared to their respective controls (Fig. 4C). In contrast, the adult hippocampus showed a significant increase in the expression of the NR2A subunit in the CET group (Fig. 4D). Chronic ethanol exposure had no effect on the expression of the NR2B subunit in the hippocampus of adolescent rats (Fig. 4E; $F(3,41)=3.5$; $P > 0.01$). However, chronic ethanol exposure had a significant effect on the expression of the NR2B subunit in the hippocampus of adult rats (Fig. 4F; $F(3,41) = 34.5$; $P < 0.01$). Post hoc assessment revealed that the expression of the NR2B subunit in the adult hippocampus showed an increase in the expression of the NR2B subunit in the CET group (Fig. 4F). Figure 5 shows representative Western blots of hippocampal frontal cortex NMDAR NR1, NR2A and NR2B expression in adolescent and adult rats from the CET 24-h WD and 2-weeks WD groups.

DISCUSSION

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 wks of ethanol vapor exposure, and 24 hrs and 2 wks following withdrawal. Quantitative Western blot analysis revealed that NR1, NR2A and NR2B subunits were increased in the hippocampus of adult rats, whereas the NR2A subunit was reduced in the hippocampus of adolescent rats after 2 wks of ethanol vapor exposure. Our data also showed that while the NR1, NR2A and NR2B subunits were reduced in the frontal cortex of adult rats, only the NR1 subunit was reduced in adolescent rats after 2 wks of ethanol exposure. These findings suggest that the effects of 2 wks of ethanol exposure on the expression of NMDAR subunits were age- and region-dependent.

Western blot analysis showed no change in the expression of the NMDAR subunits in the hippocampus of adult rats during the ethanol withdrawal period. While NR1 and NR2A subunits in the hippocampus of adolescent rats were reduced after 24 hr withdrawal, expression of these subunits increased after 2 wks of ethanol exposure. These changes in the expression of NR1 and NR2A subunits in the hippocampus suggest enhanced vulnerability to ethanol withdrawal in adolescent rats. In contrast, our data indicated that in adolescents, NMDAR subunits in frontal cortex were not changed following ethanol withdrawal. Western blot analysis showed that in adult rats, NR1 and NR2B subunits were reduced after 24 hr withdrawal, while NR2A subunits were increased. NR2B subunits in frontal cortex in adult rats were also increased after 2 wks of withdrawal from ethanol exposure. These findings suggest that the effects of ethanol withdrawal on the expression of NMDAR subunits were age- and region-dependent. 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 be associated with the enhanced vulnerability of the adolescent brain to ethanol dependence. Our data also revealed that the ratio of NR2A to NR2B was significantly increased in both hippocampus and frontal cortex in adult versus adolescent control animals. Further research is needed to determine whether developmental differences in the NR2A to NR2B ratio play a role in the effects of chronic ethanol exposure and withdrawal *in vivo* on the expression of NMDAR subunits.

A number of studies have shown that NMDAR subunit expression is differentially regulated during development (Watanabe et al., 1992, 1993; Monyer et al., 1994; Zhong et al., 1995; Jin et al., 1997; Wenzel et al., 1997; Hsieh et al., 2002; Magnusson et al., 2002; Law et al., 2003; Chang et al., 2009). Most of these studies have demonstrated in the rat that NR2A expression is generally low at birth but then increases up to about P21–P28, whereas NR2B is high at birth and then peaks around P10–21. Few studies have specifically compared subunit expression between the adolescent and young adult developmental periods. In the present study, the NMDAR subunits, NR2A and NR2B, were found to be expressed at significantly lower levels in hippocampus and frontal cortex in the adolescents as compared to the adults. In one study, investigating human hippocampus, NR1, NR2A and NR2B levels did not appear to be significantly different between adolescents (n=9) and young adults (n=6) (Law et al., 2003). In another study, in Sprague-Dawley rats, little differences in expression of these subunits were seen between P25 and “adult” rats (Hsieh et al., 2002). Additionally, it has been reported that no effects of age were seen in NR1 and NR2A subunit mRNA between 3, 10 and 30 month old mice (Magnusson et al., 2006). However, highly consistent with the literature (see Monyer et al., 1994; Jin et al., 1997), we found that the ratio of NR2A to NR2B subunit protein expression levels to be significantly increased in both hippocampus and frontal cortex in adult versus adolescent control animals. Discrepancies observed between studies in subunit expression as a function of developmental trajectory may be a result of the use of different species or strains of animals and remains to be replicated in additional studies.

An abundance of data suggests that chronic ethanol exposure induces changes in the NMDA receptor (see Hoffman, 2003, for review). Early reports demonstrated that an “up-regulation” occurs in NMDA receptors in the brains of animals that have been treated chronically with ethanol (see Tabakoff and Hoffman, 1996; Kumari and Ticku, 2000). Increases in the number of NMDA receptors has been shown by ligand binding and autoradiographic technique in mice chronically fed ethanol in a liquid diet (Grant et al., 1990; Gulya et al., 1991; Snell et al., 1993). Increases in NR1 and NR2A (Snell et al., 1996) and NR2B (Narita et al., 2000) receptor subunit proteins have also been reported from studies examining brains from mice fed ethanol in a liquid diet. In rats, increases in ligand binding of NMDA receptors (Sanna et al., 1993; Rudolph et al., 1997) and increases in mRNA and/or protein levels for NR1, NR2A, and NR2B have been reported in numerous studies in vivo (Trevisan et al., 1994; Ortiz et al., 1995; Hardy et al., 1999) and in vitro (Follesa and Ticku, 1996; Hoffman et al., 1996; Hu et al., 1996; Chandler et al., 1999; Nagy et al., 2003; Hendricson et al., 2007). However, some studies have found no changes in NMDA receptor proteins in chronically ethanol treated rats (Ferreira et al., 2001).

Consistent with previously published studies we found that protein expression of the hippocampal NMDAR subunits: NR1, NR2A and NR2B, were significantly increased after chronic ethanol exposure, in adult male Wistar rats. However, we found chronic ethanol exposure decreased protein expressions of NMDAR subunits NR1, NR2B, and NR2A in frontal cortex. Thus, our study suggests that there may be regional specificity in the effects of ethanol on NMDARs in adult rats. Different findings were observed in adolescent rats where NR1 but not NR2A or NR2B subunits were found to be reduced in cortex and NR2A but not NR2B or NR1 subunits were reduced in hippocampus by chronic ethanol exposure. Findings from the present study also showed that chronic ethanol exposure had no effect on body weight in adolescent rats, but significantly reduced the body weight of adult rats. In contrast, adolescent rats, but not adult rats, showed a significant reduction in body weight following 2-wk ethanol withdrawal. These results showed age-dependent differences in the effects of chronic ethanol and withdrawal on body weight. However, these age-dependent effects of chronic ethanol exposure on body weight were not consistent with the age-dependent effects of chronic ethanol on adolescent and adult NMDAR expression. Chronic

ethanol produced changes on cortical and hippocampal NMDAR expression in adolescent rats in the CET as well as in the 2-wk WD group. While hippocampal NMDAR expression in adult rats was only altered in the CET group, cortical NMDAR expression was altered at all time points. Thus, body weight changes between the groups do not appear to be correlated with the neurochemical findings.

Only a few previous studies have investigated the effects of ethanol exposure specifically on adolescent animals. One study found down-regulation of the expression of NMDAR2B phosphorylation in adolescent but not adult rats 24 hours following a regime of intermittent I.P. (3 g/kg) ethanol treatment that occurred over a period of 14 days during adolescence in Wistar rats (Pascual et al., 2009). However in another study in Sprague Dawley rats, daily I.P. ethanol injections of 2 g/kg for five days followed by 7 days of withdrawal during adolescence was found to produce an increased in [3H] MK-801 binding and an increase in immunohistochemical measures of NR2B subunit protein in frontal cortex (Sircar and Sircar, 2006). Differences between studies may be due to length of ethanol treatment as both our study and that of Pascual et al. (2009) treated animals over a 2-wk period. Additionally, both our study and the study of Pascual et al. (2009) used Wistar rats as subjects. Another potential source of differences between studies may be related to the methods used to isolate and quantitate tissue samples, as it has been suggested that ethanol exposure may significantly affect the assembly and transport of NMDA receptors (Hughes et al., 2001).

Molecular and cellular adaptations to drug exposure are believed to lead to persistent changes in transcription, translation, synaptic morphology and function that are extremely long-lived and are analogous to the plastic processes that underlie learning and memory (Nestler, 2001; Ron and Jurd, 2005). Long-lasting adaptive changes after chronic ethanol exposure during adolescent development in NMDAR subunit expression in different brain regions may contribute to permanent changes within neural circuitry when chronic ethanol exposure is withdrawn. This has been shown in studies investigating chronic prenatal ethanol exposure where long-lasting decreases in NR2B subunit protein expression have been found in the forebrain and hippocampus of the guinea pig and rat (Hughes et al., 1998; Dettmer et al., 2003; Samudio-Ruiz et al., 2010), and reductions in NR2A and [3H] MK-801 binding density in rat (Honse et al., 2003a,b). Such changes may potentially contribute to fetal ethanol syndrome (FAS) and also to deficits observed following adolescent ethanol exposure. Although our results demonstrate that chronic ethanol exposure had no effect on the expression of the NR2B subunit in the hippocampus of adolescent rats, the NR1 and NR2A subunits were still affected after 2-wks of ethanol withdrawal. These long-lasting changes in the normal composition of NMDARs may result in long-lasting changes in the functional activity of NMDARs.

Functional physiological studies in animal models have shown that some of the consequences of chronic ethanol exposure can differ between adolescents and adults. For instance, long-term impairments in neurophysiological function, including reductions in the P3 component of the event-related potential (ERP) are observed after 10 days of ethanol exposure in adolescent (Slawecki et al., 2001), but not in adult rats (Ehlers and Chaplin, 1991). Suggesting that ethanol exposure during adolescence could result in more severe consequences relative to adult ethanol exposure. We have previously shown that administration of the NMDA antagonist MK-801 reduces the amplitude of the hippocampal P3 ERP in rats (Ehlers et al., 1992). These data suggest an important role of the glutamatergic NMDA system in the generation of the P3 ERP (Ehlers et al., 1992). More recently, we provided evidence of protracted changes in NMDA systems in cortical and hippocampal neurophysiological activity following adolescent ethanol exposure in rats (Criado et al., 2008a). In that study, we showed that MK-801 significantly reduced P3 ERP amplitude and increased P3 ERP latency in control, but not in adolescent rats exposed to

ethanol. Whether the lack of NMDA-mediated regulation of P3 amplitude is due to glutamate neurotoxicity following adolescent ethanol exposure is presently unknown, however, these results provide evidence to suggest that adolescent ethanol exposure produces long-lasting effects on the efficacy of NMDA systems regulating cortical and hippocampal ERPs. Further studies will be necessary to determine whether NMDAR subunits composition is associated with to the enhanced vulnerability of the adolescent brain to alcohol dependence.

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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
I.P	intraperitoneal
mg/dl	milligrams per deciliter
MK-801	Dizocilpine maleate
mM	millimolar
NIH	National Institutes of Health
NMDA	<i>N</i> -methyl-D-aspartate
NMDAR	<i>N</i> -methyl-D-aspartate receptor
NR1	<i>N</i> -methyl-D-aspartate receptor NR1 subunit
NR2A	<i>N</i> -methyl-D-aspartate receptor NR2A subunit
NR2B	<i>N</i> -methyl-D-aspartate receptor NR2B subunit
PND	postnatal day
WD	withdrawal

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Figure 1.
Ethanol Exposure Protocol.

Figure 2. Age-related developmental changes in the protein expression of the NMDAR NR1, NR2A and NR2B subunits

Figure 2A-F represents the protein expression levels of the NMDAR subunits in the frontal cortex (Fig. 2A-C) and hippocampus (Fig. 2D-F) from naive rats at postnatal day 37, 38, 52, 74, 75 and 88 (n=5/group). Protein levels are expressed as optical units obtained by normalization of NMDAR subunits against β -actin. Error bars= S.E.M.

Figure 3. Chronic ethanol treatment (CET) and ethanol withdrawal (WD) alters the protein expression of NMDAR subunits in adolescent and adult frontal cortical tissue

(A) In frontal cortex of CET adolescent rats, the NR1 subunit protein level is significantly decreased compared with that from naïve adolescent rats. NR1 subunit at 24-hr and 2-wk WD is returned to the protein levels of naïve adolescent rats. (B) In frontal cortex of CET adult rats, the NR1 subunit protein level is significantly decreased compared with naïve adult rats. NR1 subunit at 24-hr WD is remained decreased compared with naïve adult rats but at 2-wk WD the level of NR1 subunit returned to the level of naïve rats. (C) CET, 24-hr and 2-wk WD had no effect on protein expression of NR2A subunit in adolescent rats. (D) CET significantly decreased NR2A subunit levels as compared to naïve adult rats. At 24-hr WD, the NR2A subunit is significantly increased compared to naïve adult rats. At 2-wk WD, NR2A subunit returned to the protein levels of naïve adult rats. (E) CET, 24-hr and 2-wk WD had no effect on NR2B subunit protein expression in adolescent rats. (F) CET and 24-hr WD decreased the NR2B subunit compared to naïve adult rats. Adult NR2B subunit at 2-wk WD was increased compared to naïve rats. Protein levels are expressed as optical units obtained by normalization of NMDAR subunits against β -actin. * indicates $P < 0.05$ for significant difference from control rats. Error bars= S.E.M.

Figure 4. Chronic ethanol treatment (CET) and ethanol withdrawal (WD) alters the protein expression of NMDAR subunits in adolescent and adult hippocampal tissue
(A) At 24-hr WD, the hippocampal NR1 subunit protein level of adolescent rats is significantly decreased compared to naïve adolescent rats. At 2-wk WD, the NR1 subunit expression is increased compared to naïve adolescent rats. **(B)** In hippocampus of CET adult rats, the NR1 subunit protein level is significantly increased compared to naïve adult rats. At 24-hr and 2-wk WD, the protein expression of NR1 subunit is returned to the levels of the same-age naïve adult rats. **(C)** CET and 24-hr WD decreased the NR2A subunit compared to naïve adolescent rats. At 2-wk WD, NR2A subunit is increased as compared to naïve adolescent rats. **(D)** CET increased the NR2A subunit compared to naïve adult rats. At 24-hr and 2-wk WD, the expression of the NR2A subunit is returned to naïve adult control levels. **(E)** CET, 24-hr and 2-wk WD had no effect on NR2B subunit protein expression in adolescent rats. **(F)** CET increased the NR2B subunit compared to naïve adult rats. At 24-hr and 2-wk WD, the NR2B subunit is returned to the naïve control levels. Protein levels are expressed as optical units obtained by normalization of NMDAR subunits against β -actin. * indicates $P < 0.05$ for significant difference from control rats. Error bars= S.E.M.

Figure 5. Effect of chronic ethanol treatment (CET), 24-hr and 2-wk ethanol withdrawal (WD) on the protein expression of NMDAR subunits in hippocampus and frontal cortex of adolescent and adult rats

Representative Western blot analysis of NMDAR NR1, NR2A and NR2B subunits expression in hippocampus and frontal cortex of adolescent (top) and adult (bottom) rats