A SINGLE DAY OF ETHANOL EXPOSURE DURING DEVELOPMENT HAS PERSISTENT EFFECTS ON BI-DIRECTIONAL PLASTICITY, *N*-METHYL-D-ASPARTATE RECEPTOR FUNCTION AND ETHANOL SENSITIVITY

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Abstract—To determine factors that contribute to the learning deficits observed in individuals with fetal alcohol syndrome, we examined the effects of early postnatal ethanol exposure on forms of synaptic plasticity thought to underlie memory. Treatment of rat pups with ethanol on postnatal day 7 impaired the induction of *N*-methyl-D-aspartate receptor-dependent long-term potentiation and abolished homosynaptic long-term depression in the CA1 region of hippocampal slices prepared at postnatal day 30. An *N*-methyl-D-aspartate receptor-independent form of long-term potentiation induced by very high frequency stimulation could be induced in slices from ethanol-treated rats. Defects in long-term depression correlated with a diminished contribution of ifenprodil-sensitive *N*-methyl-D-aspartate receptors to synaptic transmission and defects in a spontaneous alternation behavioral task. Rats exposed to ethanol on postnatal day 7 also exhibited diminished sensitivity of synaptic *N*-methyl-D-aspartate receptors to block by ethanol at postnatal day 30 and decreased behavioral sedation to systemic ethanol injections. These results indicate that changes in synaptic plasticity and *N*-methyl-D-aspartate receptor function are likely to provide a neural substrate for the cognitive and behavioral changes that follow developmental ethanol exposure. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: ethanol, ifenprodil, synapses, memory, plasticity, neurodevelopment.

Exposure to therapeutic or abused depressant drugs for several hours on a single day during the period of developmental synaptogenesis results in significant neuronal loss throughout the rodent forebrain (Ikonomidou et al., 1999, 2000; Jevtovic-Todorovic et al., 2003). These findings are of clinical interest because humans exposed to ethanol in utero display a cluster of physical, cognitive and behavioral abnormalities known as fetal alcohol syndrome (FAS) (Clarren and Smith, 1978) or fetal alcohol effects (FAE) when milder manifestations are observed. Children and adolescents exposed to ethanol during prenatal development have a high prevalence of neuropsychological problems including deficits in learning and memory (Olson et al., 1998; Mattson and Riley, 1999). As they mature, these individuals are also at increased risk for developing major psychiatric illnesses including substance abuse, mood and psychotic disorders (Famy et al., 1998). Thus, ethanol or other depressant drug exposure during neurodevelopment may represent an important environmental factor contributing to the pathogenesis of adult psychiatric disorders.

Although factors contributing to the spectrum of findings in individuals with FAS/FAE are uncertain, recent studies indicate that ethanol and CNS depressant drug exposure on a single day during the first postnatal week in rodents, a time corresponding to the third trimester of pregnancy and the first several years of postnatal life in humans, greatly increases apoptotic neuronal death in many brain regions (Ikonomidou et al., 1999, 2000). In cases of severe damage, this developmental neuronal loss has adverse effects on brain function and development as animals mature (Jevtovic-Todorovic et al., 2003). In particular, studies examining the effects of clinically used anesthetic drugs found changes in spatial reference learning, spatial working memory and hippocampal long-term potentiation (LTP). The most profound defects were observed in rats treated with a combination of agents that depress NMDA receptor function and that augment GABA-mediated inhibition (Jevtovic-Todorovic et al., 2003). Because ethanol is an *N*-methyl-D-aspartate receptor (NMDAR) antagonist that also augments GABAergic inhibition in some regions of the CNS and is the agent responsible for FAS, we focused the present studies on determining the effects of ethanol exposure in young postnatal rats on synaptic and behavioral outcomes as the animals matured to adolescence.

EXPERIMENTAL PROCEDURES

Ethanol treatment

Based on prior studies (Ikonomidou et al., 2000; Wozniak et al., 2004), postnatal day (P) 7 Sprague–Dawley albino rats were administered two single injections of 2.5 g/kg ethanol s.c. two hours apart. This ethanol treatment results in a transient blood ethanol concentration of about 500 mg/dl one hour after the second injection and a level of about 200 mg/dl that is sustained for several hours (Ikonomidou et al., 2000; Wozniak et al., 2004).

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Abbreviations: ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; APV, 2-amino-5-phosphonovalerate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EPSPs, excitatory postsynaptic potentials; FAE, fetal alcohol effects; FAS, fetal alcohol syndrome; LTD, long-term depression; LTP, long-term potentiation; NMDAR, *N*-methyl-D-aspartate receptor; P, postnatal day.

Control rats received similar injections of normal saline. Following recovery from acute ethanol or saline treatment, pups were placed back in their home cage with the dam where they remained until weaning.

To determine whether ethanol treatment resulted in neuronal degeneration, randomly selected pups were processed for cupric-silver staining using the de Olmos technique (Ikonomidou et al., 1999, 2000; Jevtovic-Todorovic et al., 2003) 16 hours following the second doses of ethanol/saline. Because prior studies using stereological methods have shown that this ethanol treatment paradigm induces apoptotic degeneration in the rodent forebrain (Ikonomidou et al., 2000; Wozniak et al., 2004), we were concerned primarily with determining whether ethanol treatment induced the expected neuropathological reaction. To accomplish this, silver-stained brain sections were inspected and rated by two independent observers who were unaware of the treatment status of rats from which the brain sections were taken. After light microscopic analysis of all sections from a given animal, each observer assigned a rating according to the following scale: 0: baseline levels of untreated controls; 1: a mild neurodegenerative reaction; 2: a moderate neurodegenerative reaction; 3: a severe neurodegenerative reaction. A mean of the two ratings was computed for each rat.

Pups remaining in the study were weighed and inspected for signs of illness or altered development over the period of P7–P30 and were assigned to two cohorts. One cohort served as subjects for electrophysiological and spontaneous alternation behavioral studies that were conducted at P30–P32. The other cohort was assessed on a battery of sensorimotor measures and a 1-h locomotor activity test as described below during the early post-weaning period (Jevtovic-Todorovic et al., 2003) and then spatial learning and memory capabilities were evaluated on the Morris water maze beginning at P30.

Hippocampal slice physiology

Hippocampal slices were prepared at P30-32 using standard methods (Zorumski et al., 1996). Rats were anesthetized with halothane and decapitated. Hippocampi were rapidly dissected and placed in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 2 MgSO₄, 2 CaCl₂, 1.25 NaH₂PO₄, 22 NaHCO₃, 10 glucose, bubbled with 95% O₂–5% CO₂ at 4–6 °C, and cut transversely into 450 μm slices using a vibrotome. Slices were placed in an incubation chamber containing gassed ACSF for 1 h at 30 °C. At the time of study, slices were transferred individually to a submersion-recording chamber. Experiments were done at 30 °C with continuous bath perfusion of ACSF at 2 ml/min. Extracellular recordings were obtained from the apical dendritic region of area CA1 for analysis of population excitatory postsynaptic potentials (EPSPs). NMDAR synaptic potentials were recorded in 0.1 mM Mg^{2+}, 2.5 mM Ca^{2+} and 30 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX).

Evoked synaptic responses were elicited with 0.2 ms constant current pulses through a bipolar electrode (Rhodes Medical Instruments, Tujunga, CA, USA) placed in the Schaffer collateral-commissural pathway. Synaptic responses in CA1 were monitored by applying single stimuli to the Schaffer collateral pathway every 60 s at an intensity sufficient to elicit a half maximal synaptic response. After establishing a stable baseline for at least 10 min and a control input–output curve, LTP was induced by applying a single 100 Hz×1 s tetanus using the same intensity stimulus. Long-term depression (LTD) was induced by applying single pulses at 1 Hz for 15 min. Input–output curves were repeated 20 min and 60 min following tetanic or 1 Hz stimulation. In some experiments, LTP was induced using a 200 Hz×1 s tetanus. This latter stimulation reliably induces a form of NMDAR-independent LTP in control slices.

Behavioral testing

At P30–P32, one cohort of ethanol-treated and saline-treated control rats was tested on a spontaneous alternation task using a Y-maze as described by Nakao et al. (2002). In this test, a rat is placed in the center of a maze with three arms that are 95 mm wide, 336 mm long and 120 mm deep at angles of 120° with respect to each other. Rats are allowed to explore the apparatus for up to 10 min and entry into a given arm is counted only when the hind limbs completely enter the arm. An alternation was defined as any three consecutive choices of three different arms without re-exploration of a previously visited arm. The percentage of alternations was determined by dividing the total number of alternations by the total number of choices minus 2 (Nakao et al., 2002).

Spatial learning and memory capabilities were evaluated in the other cohort of ethanol-treated and control rats at P30 using the Morris water navigation test, according to a slightly-modified version of our previously established methods (Jevtovic-Todorovic et al., 2003; Wozniak et al., 2004). However, before being tested on the water navigation task, this cohort was assessed on various sensorimotor measures and on a 1-h locomotor activity test to determine whether water maze performance was likely to be compromised by altered non-associative behavioral functions in the ethanol-treated rats. Specifically, the rats were first evaluated on the ascent test (Jevtovic-Todorovic et al., 2003; Wong et al., 2003) on P10 to determine if there were any residual sensorimotor effects induced by the ethanol treatment. The ascent test involves placing a pup on an inclined screen and timing how long it takes to reach the top of the screen and whether it falls from the screen. In addition, the rats were more thoroughly assessed on a battery of sensorimotor tests and on a 1-h locomotor activity test during the early postweaning period (P24 and P29, respectively) using methods similar to previously published protocols (Jevtovic-Todorovic et al., 2003; Wong et al., 2003) before they were evaluated on the water navigation task. The battery involved measures of balance, strength, coordination, and initiation of movement and consisted of the following tests: 1) elevated platform; 2) plank; 3) inclined (60°, 90°) and inverted screens; 4) walking initiation.

For the water navigation studies, rats were tested in a 100 cm inner diameter pool of opaque water according to a slightly modified version of our previously established methods (Jevtovic-Todorovic et al., 2003). In cued trials, rats were examined for their ability to locate a visible platform that was switched to a new location for each trial where training involved four trials a day for two consecutive days. Reference memory capabilities were evaluated during the place condition that involved finding the location of a submerged platform that remained in the same location for all trials. Acquisition training consisted of administering four consecutive trials per day for 10 days using an interval of 30 s between trials. The distance (path length) traveled and latency to escape onto the platform, and swimming speeds were derived for each trial. Retention performance was tested during probe trials (30 s in duration) at which time the platform was removed from the pool and the time spent in the pool quadrant where the platform had been located and the number of times a rat swam directly over the location where the platform had been (platform crossings) were quantified. A probe trial was administered after the last place trial on the 5th and 10th days of acquisition training.

To determine acute sensitivity to ethanol, P30 rats were administered single injections of ethanol (3.0 g/kg, i.p.). After being sedated by ethanol, rats were placed on their backs and the time required to recover the ability to right spontaneously was monitored (Yaka et al., 2003). Venous blood was sampled 30 min after ethanol injection for determination of ethanol concentrations. After deproteinization of the plasma with trichloroacetic acid, ethanol levels were determined enzymatically as an increase in absor-

bance at 340 nm resulting from the consumption of nicotinamide adenine dinucleotide (NAD) in the presence of alcohol dehydrogenase using an alcohol reagent kit (Pointe Scientific, Lincoln Park, MI, USA).

Statistical analysis

Data in the text and figures are expressed as means±S.E.M.. Student's t-test was used for comparisons between groups. Statistical comparisons in studies of synaptic plasticity were based on analysis of input–output curves at baseline and 60 minutes following tetanic or 1 Hz stimulation. P-values of less than 0.05 were considered statistically significant. The water navigation, activity, sensorimotor, and body weight data were typically analyzed using analysis of variance (ANOVA) models with Treatment and Gender as between-subjects variables and Blocks of Trials, Trials, or Postnatal Days as a within-subjects (repeated measures) variable. Pair-wise comparisons were conducted following significant effects of Treatment or significant interactions involving Treatment and other relevant variables and P-values exceeding Bonferroni corrected levels were used when appropriate to adjust alpha levels. The Huynh-Feldt statistic was used to adjust P-values to help protect against violations of compound symmetry when more than two levels of a within-subjects variable were used in an ANOVA model.

RESULTS

Neurohistological effects of P7 ethanol treatment

To determine the longer-term effects of ethanol exposure on a single day during development, we treated P7 albino rats with ethanol using a protocol that has previously been shown to produce acute apoptotic neurodegeneration in the forebrain (Ikonomidou et al., 2000), and compared ethanol-treated rats with saline-treated littermates as they matured to adolescence (P30). In initial studies, we used silver stains to confirm prior studies showing that P7 ethanol treatment produces neurodegeneration within 24 hours of exposure. Based on a 0–3 scale with excellent inter-rater reliability to rate the degree of damage ($r=0.81$, $P=0.001$), we found that P7 ethanol treatment results in a moderate degree of neurodegeneration compared with saline controls. The mean neuropathology score ($\pm$SEM) for ethanol-treated rats was and 1.69±0.28 ($N=5$) with each of the saline-treated control rats being scored as a "0" (no damage) by each rater ($N=3$). In ethanol-treated rats, somal and fiber argyrophilia was predominantly noted in the areas reported previously (Ikonomidou et al., 2000), including layers II and IV of the retrosplenial, cingulate, parietal, and frontal cortices, as well as the subiculum, anterior thalamic nuclei, globus pallidus, hippocampus and dentate gyrus.

Effects of P7 ethanol on growth

Rats treated with ethanol on P7 recover from acute ethanol exposure within 24 hours and develop relatively normally to P30. Based on measures of body weight, a Treatment
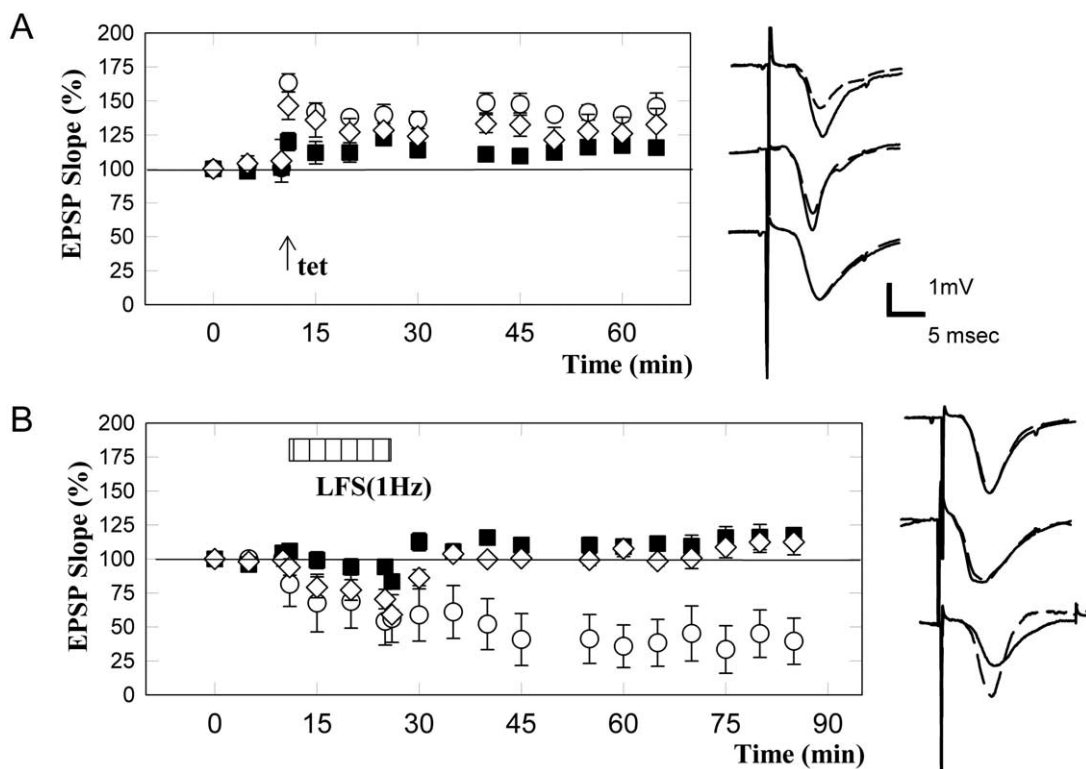

Fig. 1. Postnatal ethanol exposure on P0 or P7 has variable effects on LTP but eliminates LTD at P30. The graphs show the time course of change in EPSP slope in response to a single 100 Hz×1 s tetanus (A) or 900 pulses at 1 Hz (B). These stimuli, delivered at the times denoted in the graphs, produce robust LTP and LTD in control slices (open circles). Slices from rats treated with ethanol at P7 show diminished LTP and LTD at P30 (filled squares), while slices from P0 ethanol-treated rats show LTP but no LTD (open triangles). Traces to the left of the graph in panel A show representative EPSPs 10 min before (dashed lines) and 60 min after conditioning stimulations (solid lines) in slices from a saline-injected rat (upper) and P0 (middle) and P7 (lower) ethanol-injected rats. The traces in panel B show EPSPs from P7 ethanol, P0 ethanol and control rats (top to bottom).

by Postnatal Day interaction [$F(8,480)=5.60$, $P=0.005$] was the only significant effect over the period of P7–P15. Subsequent pair-wise comparisons conducted on each day were all non-significant including when the analysis was restricted to P7–P9, the period immediately prior to and following ethanol exposure. By P30, there were no differences in body weights between controls and ethanol-treated rats. Additionally, no obvious signs of altered development were observed in any animals.

P7 ethanol alters synaptic plasticity at P30

Because children exposed to ethanol in utero exhibit defects in learning and memory (Olson et al., 1998; Mattson and Riley, 1999), we examined the effects of P7 ethanol treatment on forms of NMDAR-dependent synaptic plasticity thought to underlie memory processing. For theses studies, we focused on synaptic transmission in the CA1 region of hippocampal slices prepared at P30, a time that corresponds to early adolescence in the rat. Based on input–output curves and paired-pulse plasticity, we observed no consistent change in baseline synaptic transmission mediated by non-NMDA glutamate receptors in slices prepared from ethanol-treated rats compared with controls. Treatment with ethanol, however, diminished the ability to induce LTP using a single 100 Hz×1 s tetanus ($+11.9\pm5.5\%$ change in EPSP slope 60 min after the tetanus in ethanol-treated rats ($N=10$) vs. $+36.9\pm4.8\%$ change in saline-treated controls ($N=14$), $P<0.01$, Fig. 1A).

LTP induced by 100 Hz×1 s stimulation requires activation of NMDARs and is blocked by the competitive NMDAR antagonist, 2-amino-5-phosphonovalerate (APV). We also examined the effects of P7 ethanol on a form of NMDA receptor-independent LTP induced by 200 Hz×1 s stimulation. This more intense stimulation resulted in a consistent and significant enhancement of synaptic responses in ethanol-treated rats ($+26.4\pm3.3\%$ change, $N=7$) that was insensitive to APV ($+25.4\pm6.8\%$ change, $N=6$). The enhancement of synaptic transmission produced by 200 Hz×1 s stimulation in ethanol-treated rats was significant compared with baseline transmission but showed only a trend level difference from the degree of LTP in saline-treated controls ($+49.0\pm15.0\%$ change, $N=5$, $P=0.09$).

In contrast to the variable effects on LTP, we found that P7 ethanol-treated rats exhibited a profound defect in the ability to generate homosynaptic LTD using repeated low frequency synaptic stimulation (900 pulses at 1 Hz) (Fig. 1B) ($-0.4\pm7.9\%$ change in ethanol-treated rats ($N=12$) vs. $-38.1\pm5.2\%$ change in controls ($N=12$), $P<0.01$). Ethanol-treated rats showed no significant change in EPSPs either during or following the 1 Hz stimulation. Interestingly, rats treated with ethanol on P0 (the day of birth) exhibited a significant and persistent enhancement of synaptic responses following standard 100 Hz×1 s stimulation ($+22.6\pm1.5\%$ change in EPSP slope, $N=4$), but a loss of ability to generate LTD at P30 ($+1.8\pm5.0\%$ change, $N=4$) (Fig. 1A, B).

P7 ethanol impairs spontaneous alternation but not reference learning at P30

To determine whether the changes in synaptic function observed in P7 ethanol-treated rats correlate with specific behavioral changes, we examined effects of early ethanol exposure on learning-related behaviors at P30–P32, the same age at which the physiology experiments were performed. Because a defect in LTD was a consistent finding in the ethanol-treated rats and prior studies have suggested that defects in hippocampal LTD correlate with behavioral abnormalities in novelty acquisition (Manahan-Vaughan and Braunewell, 1999) and one-trial learning (Zeng et al., 2001), we examined the effects of P7 ethanol exposure on spontaneous alternation behavior in a Y-maze. Deficits in Y-maze performance have previously been correlated with defects in the generation of hippocampal LTD (Nakao et al., 2002). Consistent with this, we found that P7 ethanol-treated rats exhibited impaired performance in the Y-maze compared with saline-treated controls in terms of making fewer alternations (Fig. 2). Neither control nor ethanol-treated rats showed preference for any specific region of the maze.

Defects in hippocampal LTP correlate with defects in spatial learning. Based on the variable effects of P7 ethanol on LTP generation, we used a second cohort of rats to examine behavior on a battery of sensorimotor tasks and spatial reference learning and memory in the Morris water maze. We found no differences between ethanol-treated and control animals on any sensorimotor or activity mea-
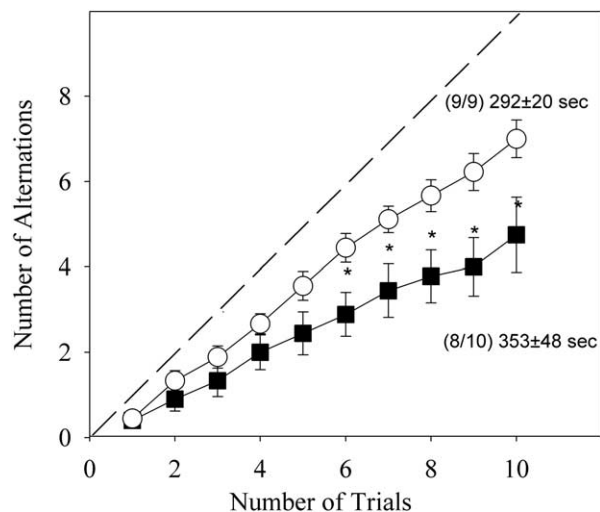

Fig. 2. P7 ethanol impairs Y-maze performance at P30. The graph shows the performance of control rats (open circles, $N=9$) and P7-ethanol-treated rats (filled squares, $N=10$) in a spontaneous alternation task using a Y-maze. The dotted line shows perfect performance in this task. The numbers in parentheses indicate the number of animals (as a ratio of all animals in the group tested) that completed 12 trials within 10 min of being placed in the maze. The times reflect how long it took the animals in that group to complete 12 trials. The number of successful alternations was calculated only for animals that completed 12 trials within 10 min. Although there were no significant differences between saline-treated and ethanol-treated rats in the times required to perform these trials, the number of successful alternations was significantly decreased in ethanol-treated rats (* denotes $P<0.05$).

sure, suggesting that P7 ethanol treatment did not compromise these functions. Consistent with this, we found that the two groups performed similarly in terms of path length (Fig. 3A), latency, and swimming speeds in the cued (visible platform) version of the water navigation task. Data from the place trials (Fig. 3B–C) suggested that, compared with controls, ethanol-treated rats showed only a mild lag in their ability to learn the position of a submerged platform, performing at control levels by the fifth block of trials and showing no differences in swimming speeds during the trials. Probe trial data showed equivalent retention performance between the ethanol-treated and control rats with regard to both time in the target quadrant and platform crossings (Fig. 3E–F). This was consistent with the finding that the two groups performed almost identically from the 5th–10th block of acquisition trials when the probe trials were administered.

P7 ethanol diminishes the contribution of ifenprodil-sensitive NMDA receptors to CA1 synaptic transmission

Most forms of long-term synaptic plasticity in the CA1 region require activation of NMDARs, although different NMDAR subtypes may contribute to LTP and LTD (Hrabetova et al., 2000; Liu et al., 2004; Massey et al., 2004). This prompted us to examine the effects of early drug exposure on synaptically-activated NMDARs, attempting to identify correlates for the changes in synaptic plasticity. Synaptic NMDARs were studied using low frequency stimulation (once per minute) in the presence of low extracellular magnesium and 30 μM CNQX to block AMPA receptors. Although slices from both ethanol-treated and control rats showed activation of synaptic NMDARs, slices from ethanol-treated rats exhibited altered NMDAR pharmacol-
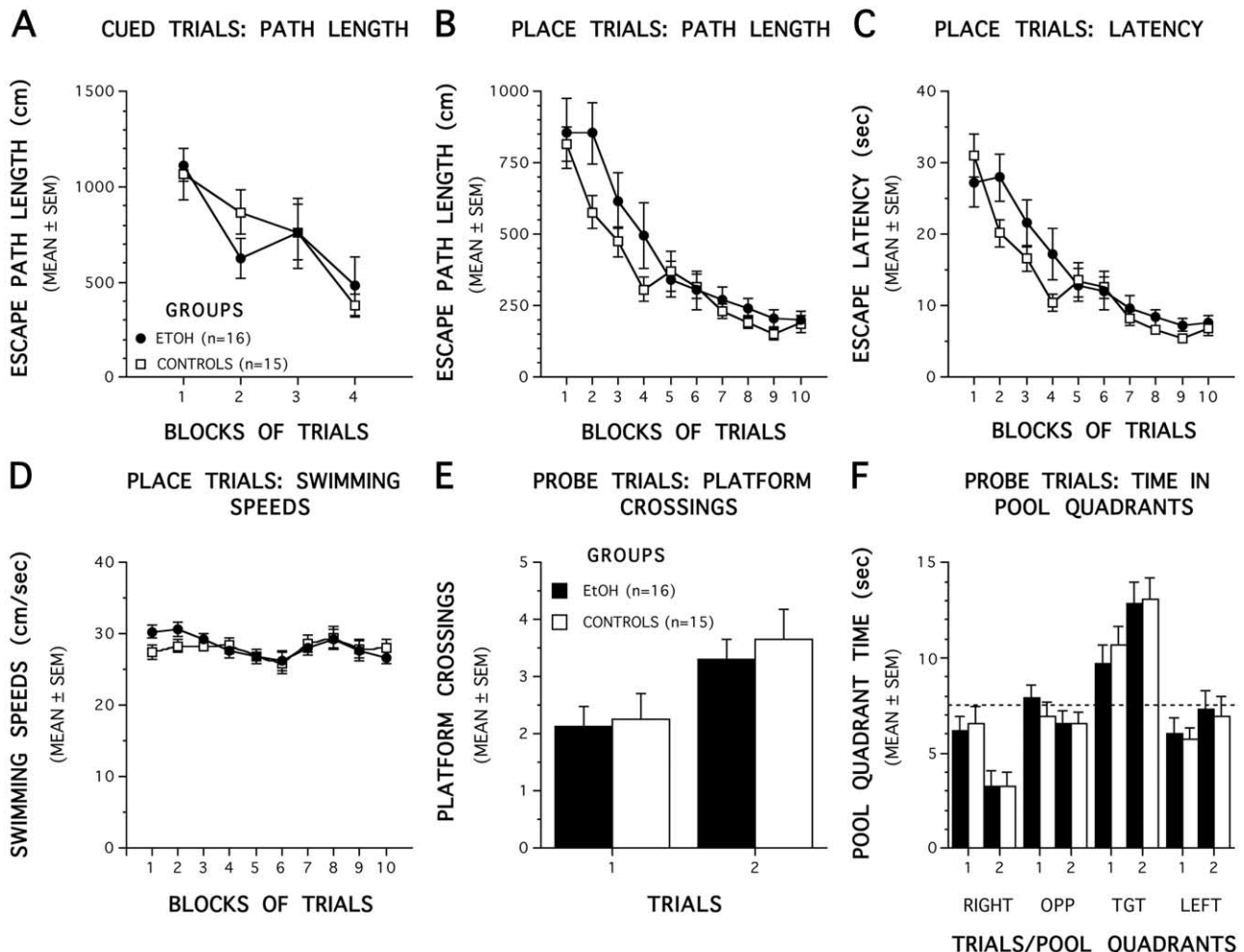

Fig. 3. P7 ethanol treatment has little effect on water maze performance. (A) The graph shows that ethanol-treated and saline-control rats performed similarly during cued (visible platform) trials in terms of the path length to escape out of the water. Performance during place (submerged platform) trials is depicted in terms of escape path length (B) latency (C), and swimming speeds (D). No significant effects were found for path length although a significant Treatment by Blocks of Trials interaction (P=0.03) was found for latency suggesting that the groups performed differently across the blocks of trials. No differences were observed between the groups in swimming speeds. Retention performance during probe trials is shown in graphs pertaining to platform crossings (E) and time spent in the target quadrant (F). No differences were observed between the groups on either variable. Note the spatial bias exhibited by both groups in terms of time spent in the target quadrant for the second probe trial as depicted in "F," demonstrating accurate retention of the platform location. Dotted line indicates amount of time expected in the pool quadrants based on chance alone.
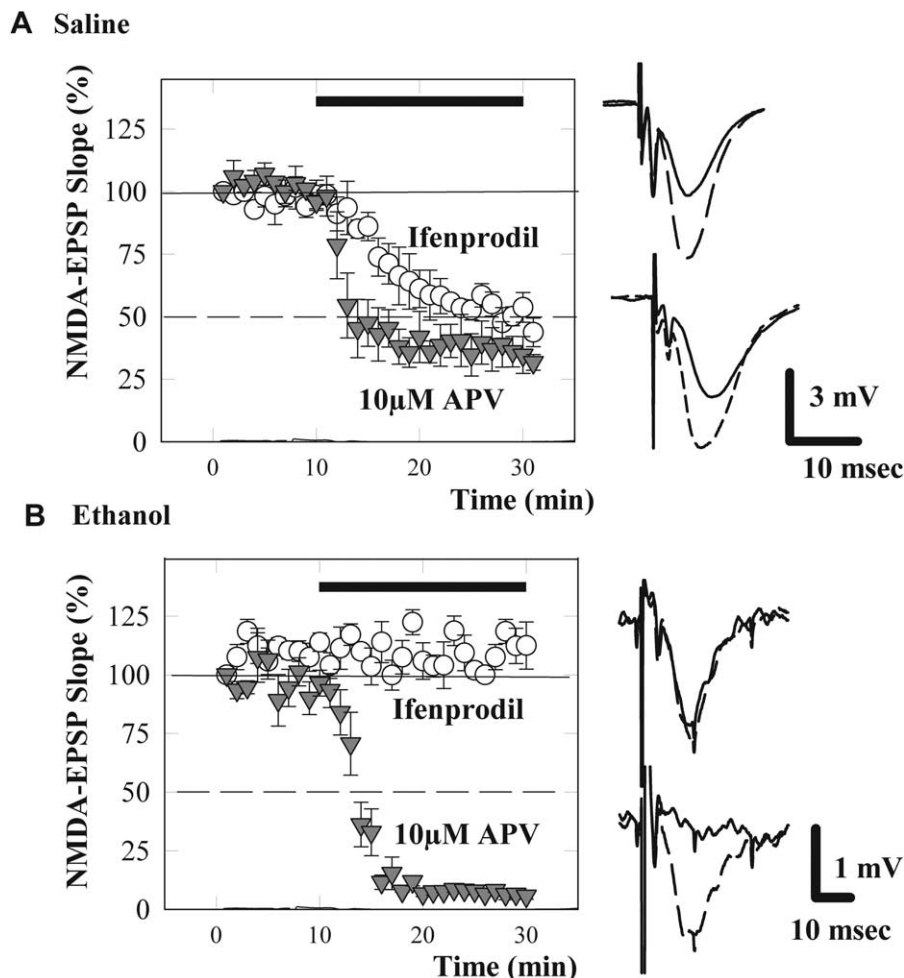
A Saline

B Ethanol

Fig. 4. P7 ethanol diminishes ifenprodil sensitivity of synaptic NMDARs at P30. (A) The graph shows the time course of change in isolated NMDAR EPSPs in slices from control rats following treatment with 10 μM ifenprodil (open circles) or 10 μM D,L-APV (gray triangles). Both agents depressed NMDAR EPSPs by about 50–60% in control slices. (B) In contrast to controls, ifenprodil had much less effect on NMDAR EPSPs in slices from rats treated with ethanol at P7 (open circles). In slices from ethanol-treated rats, APV depressed NMDAR EPSPs nearly completely (triangles). Traces to the right of the graphs show representative NMDAR EPSPs depicted 10 min before (dashed traces) and 20 min after administration (solid traces) of 10 μM ifenprodil (upper traces) or 10 μM D,L-APV (lower traces).

ogy (Fig. 4A, B). We found that 10 μM ifenprodil, a non-competitive NMDAR inhibitor with selectivity for NMDARs expressing NR1 and NR2B subunits (Williams, 1993; Priestley et al., 1995), blocked synaptic NMDARs by about 50% in P30 control slices (-46.9 ± 5.8% change, $N=5$), but was markedly less effective in slices from ethanol-treated animals (-8.6 ± 3.2% change, $N=7$, $P<0.001$). Further supporting the idea that early developmental exposure to ethanol alters synaptic NMDAR pharmacology, we found that 10 μM D,L-APV inhibited synaptic NMDARs by about 50% in control slices (-56.5 ± 9.0% change, $N=5$), but blocked synaptic NMDARs nearly completely in slices from ethanol-treated rats (-93.1 ± 1.7% change, $N=5$, $P<0.01$, Fig. 4A, B). In control slices, application of 10 μM APV in the presence of 10 μM ifenprodil also led to nearly complete block of NMDAR EPSPs (-93.3 ± 0.7% change, $N=4$), suggesting that low concentrations of APV may preferentially affect NR2A-containing NMDARs (Buller et al., 1994; Liu et al., 2004).

To determine whether changes in ifenprodil sensitivity in slices from ethanol-treated animals are relevant to defects in synaptic plasticity, we examined the effects of ifenprodil on bi-directional synaptic plasticity in slices from P30 control rats (Fig. 5A, B). At 10 μM, ifenprodil had no effect on the induction of LTP ($+42.0\pm7.8$% change in EPSPs, $N=5$) but markedly decreased LTD induction (-8.7 ± 4.5% change, $N=6$, $P<0.01$). Additionally, ifenprodil (3 mg/kg i.p.) impaired the performance of P30 control rats in the Y-maze when tested 30 min following ifenprodil injection (Fig. 5C). This dose of ifenprodil did not produce obvious signs of sedation or neurological impairment.

P7 ethanol diminishes ethanol sensitivity of synaptic NMDARs and decreases ethanol-induced sedation

Ethanol is a non-competitive NMDAR antagonist that has some selectivity for NMDARs expressing NR1 plus NR2A or NR2B subunits (Allgaier, 2002; Loftis and
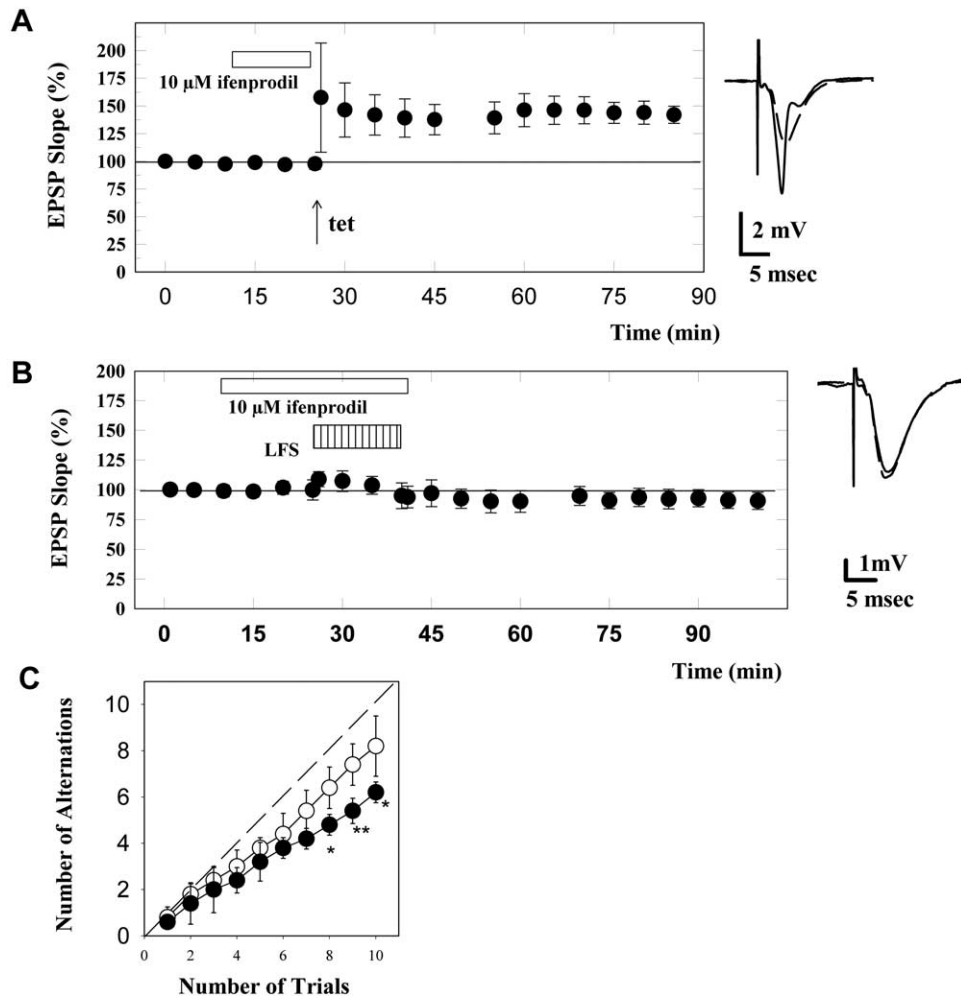
Fig. 5. Ifenprodil blocks LTD but not LTP in control slices at P30. (A) The graph shows the time course of change in EPSPs following 100 Hz×1 s tetanic stimulation in the presence of 10 μM ifenprodil (filled circles). The degree of LTP at 60 min after the tetanus did not differ from controls. (B) The graph shows the effects of ifenprodil on LTD induced by 1 Hz stimulation. The degree of change did not differ from baseline. Traces depict representative EPSPs before (dashed lines) and 60 minutes following (solid lines) stimulation. (C) The graph shows the effects of systemic treatment of control rats with a non-sedating dose of ifenprodil (3 mg/kg, i.p.) on spontaneous alternation in the Y-maze. Rats treated with ifenprodil 30 min prior to the test (filled circles, $N=5$) show impaired performance compared with saline-treated rats (open circles, $N=5$); * $P<0.05$, ** $P<0.01$ vs. control rats.

Janowsky, 2003). Some studies indicate that the ethanol sensitivity of NMDARs overlaps significantly with ifenprodil sensitivity (Lovinger, 1995; Yang et al., 1996). Based on the markedly diminished ifenprodil sensitivity of synaptic NMDARs in slices from ethanol-treated rats, we examined whether P7 ethanol exposure alters the ability of ethanol to inhibit synaptic NMDARs at P30 (Fig. 6). In control slices, 60 mM ethanol depressed synaptic NMDARs about 40–50% ($-44.4\pm8.6\%$ change, $N=5$). Ethanol was much less effective in slices from rats exposed to ethanol at P7, depressing these responses by only about 15% ($-14.2\pm2.5\%$, $N=4$, $P<0.01$).

Behaviorally, P30 rats exposed to ethanol on P7 also showed diminished sensitivity to systemic ethanol as evidenced by a decreased duration of sedation following ethanol injection. Sedation was measured as the time from ethanol administration to the time that rats were able to recover their righting reflexes. A single injection

of 3.0 mg/kg ethanol caused a loss of righting reflex that persisted for 37.5 ± 9.1 min ($N=17$) in P30 control rats. In contrast, rats treated with ethanol on P7 regained their righting reflex in 7.2 ± 4.3 min ($N=12$, $P=0.012$). Peak blood ethanol concentrations did not differ significantly between the groups (318.8 ± 34.5 mg/dl in controls and 442 ± 183.7 mg/dl in P7 ethanol-treated rats, $P>0.05$).

DISCUSSION

These studies indicate that a single day of ethanol exposure during an important period in development is sufficient to cause persistent changes in synaptic function and behavior as rats mature to adolescence. For these studies we used an ethanol-treatment protocol that has been well characterized in terms of neuropathology (Ikonomidou et al., 2000). Using this treatment regimen, P7 rats exhibit
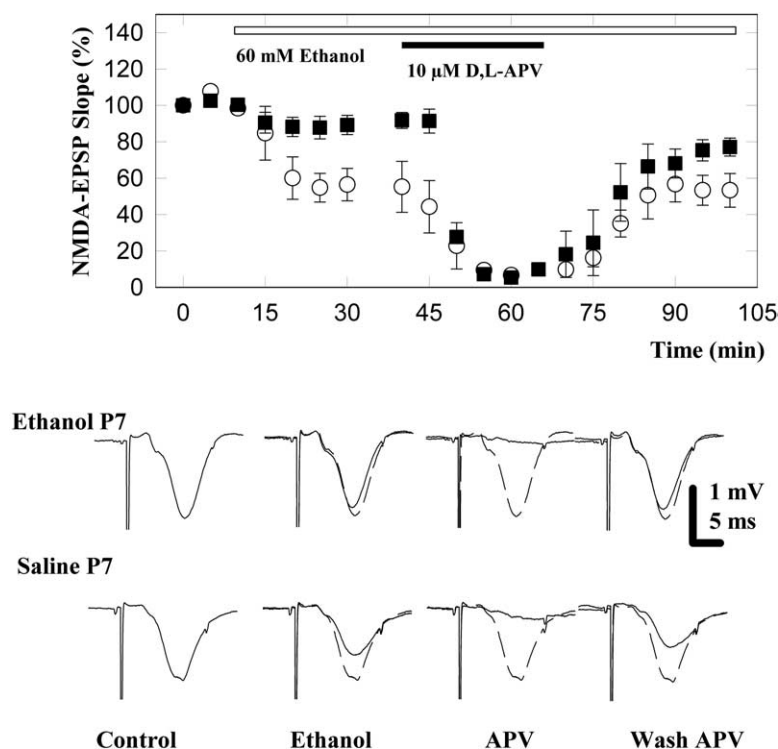
Fig. 6. P7 ethanol diminishes the acute sensitivity of synaptic NMDARs to ethanol at P30. The graph shows the time course of change in synaptic NMDAR EPSPs in the presence of 60 mM ethanol in control slices (open circles) and slices from P7 ethanol-treated rats (filled squares). Subsequent administration of 10 μM APV in the presence of ethanol nearly completely abolished the responses in slices from both groups of rats. Traces show representative NMDAR-mediated EPSPs recorded under the conditions specified in slices from a P7 ethanol-treated rat (upper) and a P7 saline-treated rat (lower).

blood ethanol levels that exceed 200 mg/dl for several hours. Twenty-four hours following ethanol treatment, rats show significant apoptotic neuronal damage throughout the forebrain. Rat pups recover from the acute ethanol exposure and grow relatively normally to adolescence based on body size and sensorimotor function. Despite the degree of neuronal damage observed at P8 (Ikonomidou et al., 2000), these rats show little impairment in spatial reference learning at P30 while exhibiting significant changes in spontaneous alternation behavior, synaptic plasticity and ethanol sensitivity. Based on the effects of ifenprodil, an agent with relative selectivity for NMDARs containing NR1 and NR2B subunits (Williams, 1993; Priestley et al., 1995), ethanol-treated rats exhibit a change in the composition or function of synaptic NMDARs, with a substantial decrease in responses mediated by ifenprodil-sensitive NMDARs. The changes in ifenprodil-sensitive NMDARs in animals with early ethanol exposure appear to correlate with defects in LTD induction based on the ability of ifenprodil to block LTD but not LTP. This makes it important to consider factors other than changes in synaptic NMDARs that account for the changes in LTP.

Based on prior studies and the known pharmacology of ifenprodil, it is possible that the changes in ifenprodil sensitivity reflect a loss or dysfunction of synaptic sites containing NR1+NR2B. Alternatively, the change in ifenprodil sensitivity could reflect the preferential formation of trihet-eromeric receptors containing NR1, NR2A and NR2B be-

cause ifenprodil shows markedly diminished potency against these receptors compared with NR1+NR2B (Tovar and Westbrook, 1999). The formation of trihetero-meric NMDARs could also account for prior observations that gestational ethanol exposure does not necessarily alter NR2B mRNA or protein expression in rodents (Hughes et al., 1998; Costa et al., 2000; Honse et al., 2003). Based on studies examining the effects of NMDAR antagonist treatment in adult rodents, it is possible that the defects in synaptic ifenprodil sensitivity reflect a loss of NR2B subunits from synapses but not extrasynaptic sites (Fujisawa and Aoki, 2003). Prior studies examining rodents exposed to ethanol throughout gestation indicate that there are decreases in the expression of NR2A and certain splice variants of NR1, with no change in NR2B in cortical membranes (Honse et al., 2003). In the hippocampus, exposure to ethanol throughout gestation and during the early postnatal period results in increased NR2A expression (Nixon et al., 2004). Thus, a complex array of changes in NMDAR subunit expression including possible changes in synaptic and extrasynaptic receptor localization may ultimately account for the altered ifenprodil sensitivity that we observed.

Ifenprodil-sensitive NMDARs are important participants in synaptic development (Loftis and Janowsky, 2003; Philpot et al., 2001). These subunits are expressed at early synapses in the hippocampus as well as at extrasynaptic loci (Tovar and Westbrook, 1999). The presence

of postsynaptic NR2B subunits, coupled with a high probability of glutamate release at developing synapses, leads to NMDAR-mediated synaptic currents with slow decay kinetics (Tovar and Westbrook, 1999; Chavis and Westbrook, 2001), a feature thought to be important in developmental plasticity (Loftis and Janowsky, 2003). During synaptic maturation, expression of NR2A subunits normally increases and NR2A becomes the major subunit expressed at synapses (Loftis and Janowsky, 2003). Coincident with this change, the ifenprodil sensitivity of synaptic NMDARs normally diminishes. By adulthood, NR2A containing receptors are the predominant synaptic NMDARs while NR1+NR2B-containing receptors continue to be expressed at extrasynaptic sites (Kirson and Yaari, 1996; Kirson et al., 1999; Li et al., 2002). Our studies indicate that ifenprodil-sensitive NR2B subunits participate significantly in synaptic transmission in the CA1 region of control P30 rats and provide a source of signals that are important for LTD induction at that age. This result is consistent with recent findings that NR1/NR2B blockers inhibit LTD but not LTP in the CA1 region of rodent hippocampal slices (Liu et al., 2004; Massey et al., 2004, but see Hendricson et al., 2002).

Although ethanol is not selective for receptors containing NR2B (Allgaier, 2002; Loftis and Janowsky, 2003), there is evidence that ethanol and ifenprodil overlap significantly in their effects on NMDARs (Lovinger, 1995; Yang et al., 1996; Engblom et al., 1997; Roberto et al., 2004). Our studies indicate that ifenprodil-sensitive NMDARs play a significant role in the effects of ethanol on synaptic NMDARs during adolescence in the rat. Furthermore, the finding that early ethanol exposure diminishes the sensitivity of synaptic NMDARs to inhibition by ethanol suggests a possible neural correlate for some of the behavioral effects of ethanol exhibited by rodents, and perhaps by humans, with early developmental ethanol exposure. In humans, there is evidence that individuals with fetal ethanol exposure show increased ethanol consumption in late adolescence and early adulthood (Yates et al., 1998; Baer et al., 2003). Interestingly, these individuals not only consume more alcohol compared with controls but also show more adverse effects of ethanol including cognitive dysfunction and physical sickness in response to ethanol intake. While diminished ethanol sensitivity of synaptic NMDARs could contribute to altered sedation from ethanol, it is clear that ethanol has complex effects on brain function and changes in a single receptor system are unlikely to account completely for altered behavioral responses.

Effects of early drug exposure on NMDARs and synaptic plasticity could contribute to defects in neuropsychological function observed in individuals with FAS (Olson et al., 1998; Mattson and Riley, 1999). Prior studies have indicated variable effects of gestational or early postnatal ethanol exposure on LTP in rodents (reviewed in Berman and Hannigan, 2000). These studies have focused primarily on effects on LTP with little information available about LTD. There are considerable study-to-study differences in the timing and degree of ethanol exposure, as well as in the stimulation paradigms and experimental conditions for inducing LTP that make these studies difficult to interpret and compare. Table 1 highlights this conclusion showing results from six studies (Bellinger et al., 1999; Chepkova et al., 1995; Krahl et al., 1999; Richardson et al., 2002; Swartzwelder et al., 1988; Tan et al., 1990) that examined the effects of developmental ethanol exposure on LTP in the CA1 region. Of these studies, three found diminished LTP while three reported intact LTP, although in some cases changes in population spikes and not EPSPs were monitored. We are unaware of other studies examining effects of developmental ethanol exposure on LTD.

Our results indicate that ethanol exposure on a single day during the first postnatal week in rats has variable effects on LTP, but consistent and profound effects on LTD. The effects on LTD appear to correlate with specific changes in NMDAR function at P30. Factors contributing to the lack of effect of P0 ethanol on LTP remain uncertain, but could reflect the fact that ethanol potentiates GABA actions in some regions and, at this time in development, GABA, has predominantly excitatory properties. Over the first postnatal week, changes in chloride gradients result in $GABA_A$ receptor activation switching from excitation to inhibition (Mladinic et al., 1999; Ben-Ari, 2002). Based on prior work, it appears that augmentation of inhibitory effects of GABA is important in determining the deleterious effects of GABA-enhancing drugs on neuronal survival during development (Xu et al., 2000).

Although the behavioral correlates of hippocampal LTD are poorly understood, recent studies suggest roles in novelty acquisition (Manahan-Vaughan and Braunewell, 1999) and spatial working memory (Zeng et al.,

Table 1. Developmental ethanol and CA1 hippocampal LTP

Study	Exposure	Age	Site	Ca/Mg	K^+	Tetanus	Intensity	LTP
Chepkova	Gestation	P26–35	CA1	2.4/2.4	4.3	100 Hz×1 s	50% Max	No (PS)
Swartzwelder	Gestation (31 mg/dl)	P50–70	CA1	1.8/1.2	3.3	60 Hz×10 s	20% Max	No (PS)
Bellinger	P4–P9 (351 mg/dl)	P45–60	CA1	2.0/1.5	3.5	100 Hz×1 s × two trains	30% Max	Yes
Tan	G8 on	P90	CA1	2.5/0.9	5.4	400 Hz×0.2 s	1 mV PS	Yes
Krahl	G8–20 (120–240 mg/dl)	P25–32	CA1	2.4/1.3	5.0	400 Hz×0.2 s	50% Max	Yes (PS)
		P63–77	CA1					Yes (PS)
Richardson	Gestation	P40–80	CA1	*In vivo guinea-pig*		100 Hz×1 s × three trains	3×threshold	No

Abbreviations: G8, eighth day of gestation; Gestation, refers to ethanol treatment throughout gestation; PS, population spike. Numbers in the Exposure column represent reported ethanol blood levels during treatment in mg/dl. Intensity refers to the stimulus intensity used during the tetanus.

2001; Nakao et al., 2002). Consistent with this, we found that P7 ethanol-treated rats are impaired in a spontaneous alternation task but exhibit only relatively minor (if any) deficits in the early phase of acquisition during the place condition in the water maze as adolescents. Because the place condition in the water maze is a reference-memory-based task and spontaneous alternation is considered by some to be a test of spatial working memory, it is possible that neonatal ethanol exposure has greater developmental effects on working memory compared with reference memory-related capabilities in the rat. Prior studies have also reported defects in spontaneous alternation, spatial working memory and spatial reference learning in rodents exposed to ethanol during gestation, although, as with the LTP literature, there are substantial differences among studies in terms of ethanol exposure, behavioral tests and age of testing (reviewed in Berman and Hannigan, 2000). Our results indicate that despite the degree of neuronal damage following ethanol exposure at P7 (Ikonomidou et al., 2000), the defects in hippocampal-mediated behaviors exhibited by these animals at P30 are relatively subtle. A recent study has shown that a similar P7 ethanol treatment protocol in C57BL/6 mice produces profound impairment in place learning and memory in the water maze at P30 although these mice show extensive recovery of function as they mature into adulthood (Wozniak et al., 2004). Thus, not only the ethanol exposure paradigm but also the species and age of behavioral testing may have significant impact on the nature of the effects that are observed. It is also important to consider the time course of changes in synaptic function in these animals. Preliminary studies indicate that altered ifenprodil sensitivity of synaptic NMDARs is already present at P15 in P7 ethanol-treated rats (data not shown), suggesting that defects are manifest relatively early following ethanol exposure. Whether differences in NMDAR function persist into adulthood remains to be determined, although it is important to note that NR1/NR2B-containing NMDARs normally play a less prominent role in synaptic function as rodents mature (Kirson and Yaari, 1996; Kirson et al., 1999; Li et al., 2002). Furthermore, while it is unknown whether the behavioral deficits observed in P30 rats diminish with maturation as found in the mouse model (Wozniak et al., 2004), it is clear that rats and mice differ greatly in the degree of spatial learning defect at P30 following similar ethanol exposure at P7 with mice showing much more profound abnormalities.

Interestingly, mice with altered expression of calcineurin also have defective LTD induction and problems with spatial working memory but relatively intact LTP and spatial learning (Zeng et al., 2001). More recently, it has been found that these mice also have abnormalities in activity, social behaviors and prepulse inhibition that may be consistent with some features observed in major psychiatric disorders like schizophrenia (Miyakawa et al., 2003). Follow-up studies in humans indicate that FAS subjects have an increased incidence of psychotic and mood disorders in adulthood (Famy et al., 1998). Thus, persisting changes in NMDAR synaptic function and synaptic plasticity may represent mechanisms contributing to the development of severe psychiatric syndromes in adults with FAS.

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