Fetal Alcohol Exposure Alters Neurosteroid Modulation of Hippocampal NMDA Receptors


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SUMMARY

The actions of ethanol on brain ligand-gated ion channels have important roles in the pathophysiology of alcohol-related neurodevelopmental disorders and fetal alcohol syndrome. Studies have shown that N-methyl-D-Aspartate (NMDA) receptors are among the ligand-gated ion channels affected by prenatal ethanol exposure. We exposed pregnant dams to an ethanol-containing liquid diet that results in blood ethanol levels near the legal intoxication limit in most states (0.08 %). Primary cultures of hippocampal neurons were prepared from the neonatal offspring of these dams and NMDA receptor function was assessed by patch-clamp electrophysiological techniques after 6-7 days in culture in ethanol-free media. Unexpectedly, we did not detect any changes in hippocampal NMDA receptor function at either the whole-cell or single-channel levels. However, we determined that fetal alcohol exposure alters the actions of the neurosteroids pregnenolone sulfate and pregnenolone hemisuccinate, which potentiate NMDA receptor function. Western immunoblot analyses demonstrated that this alteration is not due to a change in the expression levels of NMDA receptor subunits. Importantly, in utero ethanol exposure did not affect the actions of neurosteroids that inhibit NMDA receptor function. Moreover, the actions of pregnenolone sulfate on type A γ-aminobutyric acid and non-NMDA receptor function were unaltered by ethanol exposure in utero, which suggests that the alteration is specific to NMDA receptors. These findings are significant because they provide, at least in part, a plausible mechanistic explanation for the alterations in the behavioral responses to neurosteroids found in neonatal rats prenatally exposed to ethanol and to other forms of maternal stress (Zimmerberg and McDonald, Pharmacol Biochem Behav 55: 541-547, 1996).
INTRODUCTION

Ingestion of ethanol during pregnancy can have profound effects on normal fetal development. These effects range from isolated alcohol-related birth defects to a combination of abnormalities that characterize the Fetal Alcohol Syndrome (1). This syndrome is characterized by alterations in growth, facial and skull development, and central nervous system function. Fetal ethanol exposure produces long-lasting and debilitating neurobehavioral and neurophysiological dysfunctions such as deficits in learning, memory, information processing and problem solving skills (2-5). Therefore, there is considerable interest in understanding the consequences of the teratogenic actions of ethanol in the central nervous system.

Research from a number of laboratories suggests that the actions of ethanol on ligand-gated ion channels have important roles in the pathophysiology of alcohol-related neurodevelopmental disorders (For a review see (6)). Experimental evidence indicates that glutamate receptors of the N-Methyl-D-Aspartate (NMDA) subtype are among the ligand-gated ion channels affected by fetal exposure to ethanol. Studies have shown that fetal and/or neonatal ethanol exposure alters ligand binding to NMDA receptors and expression of NMDA receptor subunits (7-11). Reductions in NMDA receptor function have also been detected in neuronal preparations from both the neonatal and the adult offspring of rats exposed to ethanol during pregnancy (6, 12-15). Since the normal functioning of NMDA receptors is critical for growth, proliferation, differentiation, migration, plasticity and programmed death of neurons (16-23), the effects of ethanol
on fetal NMDA receptors could seriously affect normal neurodevelopment and have long-lasting consequences later in life.

The participation of the NMDA-Rs in complex neurodevelopmental and neurobehavioral processes requires precise regulation of the function of these channels. Among the molecules that regulate NMDA receptors are the neurosteroids, which produce rapid effects on the function of these receptors by nongenomic mechanisms. Compounds such as dehydroepiandrosterone (DHEA) and pregnenolone sulfate (PS) enhance NMDA-R function (24-28). This neurosteroid-mediated enhancement of NMDA-R function was shown to have a role in axonal elongation in developing neurons (25). DHEA and PS were also shown to enhance cognitive performance and to have anxiogenic effects in rodents (29-32). Importantly, Zimmerberg and collaborators found that prenatal ethanol exposure reduced the anxiogenic effect of PS in neonates subjected to maternal separation-induced stress (33). The authors of this study postulated that alterations in the sensitivity of ligand-gated ion channels to neurosteroids could mediate these effects of prenatal ethanol exposure. It is noteworthy, however, that the mechanism by which prenatal alcohol exposure produces these alterations in the behavioral responses to neurosteroids has yet to be determined.

We have investigated the effects of fetal ethanol exposure on NMDA-R function and modulation by neurosteroids. Rats consumed a liquid diet that produces blood ethanol levels of ~0.08 %, which are near the legal intoxication limit in many states. We then
prepared primary cultures of hippocampal neurons from the offspring of these rats and used patch-clamp electrophysiological techniques to assess NMDA-R function after 6-7 days in culture. We found that exposure to ethanol during pregnancy affects the sensitivity of NMDA-Rs to neurosteroids.
EXPERIMENTAL PROCEDURES

_Ethanol liquid-diet paradigm_ – Details of the breeding colony procedures have been described previously (34). Five-month-old Sprague-Dawley rat dams (Harlan Industries, Indianapolis, IN) were individually housed in plastic cages in a temperature-controlled room (22°C) on a 16 hr dark:8 hr light schedule (lights off from 5:30 P.M. to 9:30 A.M.). Beginning on day 1 of gestation, rat dams were assigned to one of the three diet groups. Two of the three diets consisted of a liquid diet based on the Lieber-DeCarli (35) formulation, which provides 1 Kcal/ml (BioServ, Frenchtown, NJ). These groups received 110 ml of liquid diet at 5:30 P.M. each day. The feeding tubes were removed 16 hr later (at 9:30 A.M. on the next morning). The fetal ethanol-exposed group received a liquid diet containing no ethanol for the first 2 days of gestation for adjustment to the liquid diet. The animals in this group were then given 110 ml of a liquid diet containing 2% (v/v) ethanol for the gestational days 3-4, 3% (v/v) for the next two days, and thereafter 5% (v/v) ethanol (26% ethanol-derived calories) until they gave birth. This diet produces blood alcohol levels of ~0.08% (34). The other liquid diet group, serving as pair-fed control, was given a 0% ethanol liquid diet (isocalorically equivalent to the 5% ethanol diet) each day throughout the gestation. A third diet group had continuous access to Purina breeder block chow and water _ad libitum_ and served as control for the paired feeding technique. At birth, all litters were weighed and counted.
**Primary cultures of hippocampal neurons** – Cultures were prepared from 3-4 day-old rats. Meninge-free hippocampi were microdissected in cold sterile PBS plus 15 mM HEPES, 27 mM glucose, 17.5 mM sucrose, pH 7.4, with a final osmolarity of 320-335 mOsm. Isolated hippocampi were incubated for 10 min at 37 °C in 0.05% trypsin-EDTA (GIBCO-BRL, Gaithesburg, MD), transferred to Neurobasal A media (GIBCO-BRL) containing 10% FBS, and then gently triturated with a Pasteur pipet. Trituration was repeated with a Pasteur pipet flamed to half its original opening size. Cells were then plated at a density of 15-20 x 10^3/ml of media in culture dishes containing coverslips coated with polylysine and collagen, and maintained at 37 °C with 5% CO₂ in a humidified atmosphere. Cells were initially plated in Neurobasal A media (GIBCO-BRL) containing 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 25 μM glutamate. After 24 hours, fetal bovine serum was substituted with the B27 supplement (GIBCO-BRL) and after 3 days in culture, glutamate was removed from the culture media. Cells were used for experiments after 6-7 days in culture. Neurons used for recordings were large and had pyramidal shape and well-defined dendritic processes.

**Patch-clamp electrophysiological recordings**

*Whole-cell configuration* – Immediately prior to recording, coverslips were transferred to a perfusion chamber (Warner Instruments, Hampden, CT) and neurons were visualized under a Zeiss inverted microscope equipped with Varel contrast optics, or under an Olympus microscope equipped with Hoffman modulation optics. Membrane potentials were clamped at -60 mV with an Axopatch 200B amplifier (Axon Instruments,
The resting membrane potential for the recorded neurons was approximately –60 mV. Recording pipettes (borosilicate capillaries with filament, O.D. 1.5 mm, Sutter Instruments, Novato, CA) were prepared with a two-step puller (Narishige Instrument Co, Tokyo, Japan) and had resistances between 5-9 MΩ. Series resistance was not compensated. The external solution (all chemicals from Sigma, St. Louis, MO) contained in mM: 130 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES (pH 7.3), 11 glucose and 300 nM tetrodotoxin. For NMDA recordings, this solution was nominally Mg²⁺-free. The internal solution contained in mM (all chemicals from Fluka, Milwaukee, WI): 130 KCl, 10 HEPES, 0.1 CaCl₂, 1 EGTA, 2 ATP, and 0.2 GTP. For recordings of kainate and GABA_A receptor-mediated currents, glass microelectrodes were front filled with internal solution containing in mM (all chemicals were from Fluka, Milwaukee, WI): 155 KCl, 10 HEPES (pH 7.3), 5 EGTA, and 1 MgCl₂, adjusted to 285 mOsm. Pipettes were then backfilled with the same internal solution containing 50 U/ml of creatinine phosphokinase, 22 mM phosphocreatine, and 4 mM Mg²⁺-ATP (300 mOsm). Drugs were applied with a fast-exchange flow-tube perfusion system driven by motor (Warner Instrument Co.). Agonists were applied at 30-60 sec intervals. The neurosteroids, PS (5-pregnen-3β-ol-20-one sulfate), pregnenolone hemisuccinate (PHS; 5-pregnen-3β-ol-20-one hemisuccinate), 5β-pregnan-3α-ol-20-one sulfate (3α, 5β-PS), and 5β-pregnan-3α-ol-20-one hemisuccinate (3α, 5β-PHS) were obtained from Steraloids Inc. (Newport, RI). Steroid solutions were prepared as 100-250 mM stocks in dimethyl sulfoxide (DMSO). The final DMSO concentration of all recording solutions was between 0.01% to 0.05%. Identical concentrations of DMSO were added to control solutions. All experiments were performed at room temperature (23-25 °C).
Data were acquired and analyzed with either the Neuropro software (RC Electronics, Santa Barbara, CA) or pClamp7 (Axon Instruments). Modulation of NMDA, kainate and GABA_\text{A} responses by neurosteroids is presented as percent change, \([(I'/I) – 1] \times 100\%\), where I is the average of control responses obtained before application and after washout of steroid, and I' is the average of agonist-induced responses obtained from the same cell in the presence of steroid.

**Single-channel measurements** – Single-channel recordings were obtained in the cell-attached configuration to avoid alterations in intracellular modulators of NMDA-R function. The patch solution contained 10 \(\mu\)M NMDA and 1\(\mu\)M glycine in a solution containing in mM: 70 NaCl, 70 Na_2SO_4, 10 HEPES, 1.2 Ca_2Cl, 5 Cs_2SO_4, 33 glucose, pH 7.4 and 300 mOsm. The pipette potential was +20 mV. Events were recorded with an Axopatch 200B amplifier (Axon Instruments) and stored on digital tape using a DAT recorder (Panasonic SV3800). Pipettes were pulled from borosilicate glass, lightly fire-polished, and coated with Sylgard (Dow Corning, MI). Recordings were played back and acquired and analyzed using the pClamp7 program (Axon Instruments). Single-channel currents were initially filtered at 2 kHz and sampled at 5 kHz. Only patches with stable basal activities were used. The single channel open probability was determined from the ratio of the time spent in the open state to the duration of recording: \(P_0=(t_1 + t_2 + \ldots + t_n)/Nt_{tot}\), where \(t\) is the amount of time that \(n\) channels are open and \(N\) is the maximum number of levels observed in the recording. Open times were obtained with the pStat 6.0 computer program (Axon Instruments) by fitting plots of event number vs open time to exponential functions using the Marquardt algorithm.
Closed times were obtained in the same manner from plots of event number vs log of closed time.

*Western Immunoblots* – Hippocampi from two P3 neonates, each from 3-6 different litters per diet group, were homogenized by sonication and analyzed by SDS-PAGE and Western immunoblot assays. Anti-NR2A, 2B, 2C, NR1-N1, NR1-C1, and NR1-C2 antibodies were produced, purified, and characterized in the laboratory of Dr. Browning at the University of Colorado HSC. Anti-NR1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Detection of bound antibodies was performed by chemiluminescence using a kit from Boeringer Manheim. Densitometric analysis of Western blot chemiluminescence X-ray films were performed using an Image-Pro® Plus image analysis system (Media Cybernetics, L.P., Silver Spring, MD). In all cases, a protein standard curve was generated from a single homogenate of adult rat hippocampal tissue kept at –80 °C. This standard curve was included in the same membrane (using 15-well combs) as samples from control and ethanol-treated animals. This curve was used to calculate relative units of protein concentrations. We selected the appropriate bands and measured relative optical density with respect to these standards. To correct for variations in protein loading, concentrations of all samples were normalized against β-tubulin levels (Anti-β-tubulin monoclonal antibody was from Sigma, St. Louis, MO). Relative protein concentrations are expressed as µg protein/µg tubulin.

*Statistical Analysis* – The potentiating or inhibiting effects of neurosteroids were quantified with respect to the average of control and washout responses. Neurosteroid
dose/response curves were fitted to four-parameter logistic equations (sigmoid) using
GraphPad Prizm Computer Program (San Diego, CA). Statistical comparison of the effect
of neurosteroids among the three diet groups was performed by ANOVA followed by
Bonferroni’s post hoc test. Effect of multiple concentrations of PS on neurons from all
three diet groups were analyzed by two-way ANOVA. Effects of PS on non-NMDA and
GABA<sub>A</sub> receptor function in cells from the pair-fed vs. fetal ethanol-exposed groups were
analyzed by t test. In all cases, a p<0.05 was considered to indicate statistical
significance.
RESULTS

Fetal Ethanol Exposure Paradigm – Table 1 summarizes the results of the feeding paradigm used in this study (values are mean ± S.E.M. of 34-43 dams / diet group). No statistical differences were found in diet consumption, total number of newborns, live newborns, or the average mean weight of live newborns among the three diet groups. Furthermore, no gross anatomical abnormalities were noted at birth in the fetal ethanol-exposed animals.

Effects of Prenatal Ethanol Exposure on Basic NMDA-R Function – Shown in Fig. 1 are NMDA dose/response curves recorded in the whole-cell patch-clamp configuration for neurons from the ad-libitum control, pair-fed control, and fetal ethanol-exposed groups. Fig. 1A shows examples of currents obtained by increasing concentrations of NMDA in the presence of a constant glycine (1 µM) concentration. Fig. 1B shows a summary graph of NMDA dose/response curves obtained from neurons from the three feeding groups (n=6-9 neurons from 2-3 different litters per diet group). Data were normalized with respect to maximum NMDA (100 µM) currents. The EC₅₀ concentrations were 11 ± 1, 12 ± 1, and 13 ± 1 µM for neurons from the ad libitum control, pair-fed control, and the fetal ethanol-exposed groups, respectively. The Hill slope was ~1.4 for all treatment groups. It should be noted that we did not find a difference in NMDA-R maximum current densities (pA/pF) (Fig. 1B, Inset). Current densities were 11± 1, 11 ± 1, and 13 ± 1 pA/pF for neurons from the ad libitum control,
pair-fed control, and the fetal ethanol-exposed groups, respectively (n=21-23 neurons from 3-4 different litters per diet group).

We also measured dose/response curves for 7-chlorokynurenate (7-CKA), a competitive antagonist of the glycine co-agonist site of NMDA-Rs (Fig. 1C), in the presence of constant NMDA (100 µM) plus glycine (1 µM) concentrations (n = 4-5 neurons from 2-3 litters per diet group). The IC$_{50}$ concentrations were 83 ± 18, 57 ± 17 and 93± 29 nM for neurons from the ad libitum control, pair-fed control, and the fetal ethanol-exposed groups, respectively. Statistical analysis did not reveal significant differences among these diet groups.

We also assessed the effect of fetal ethanol exposure on NMDA-R function at the single channel level. Fig. 2 shows sample tracings obtained from neurons from pair-fed and fetal ethanol-exposed groups in the cell-attached configuration. The patch solution contained 10 µM NMDA and 1µM glycine. Single channel parameters are summarized in Table 2. We did not detect any statistically significant differences in any of these parameters among the three diet groups. Since resting membrane potentials cannot be determined in the cell-attached mode, single-channel conductance was not calculated.

**Positive modulation of NMDA-R by neurosteroids** – Sample tracings illustrating the effects of 50 µM PS on currents induced by application of 50 µM NMDA on cultured hippocampal neurons from ad-libitum, pair-fed, and fetal ethanol-exposed neonatal rats are shown in Fig. 3A. PS produced a significant potentiation of NMDA-R mediated currents in cells from the ad-libitum and the pair-fed groups but not in cells from the
fetal ethanol-exposed group (Table 3). The reduction in the effects of PS was evident at different concentrations of this neurosteroid (Fig 3B).

Shown in Fig. 4A are sample traces illustrating the effect of PHS on NMDA-R mediated currents in all the three diet groups. In neurons from ad libitum controls, pair-fed controls and fetal ethanol-exposed rats, the amplitude of these currents in the presence of 50 µM PHS corresponded to 53 ± 9 % (n=6 neurons from 3 different litters), 55 ±13 % (n=6 neurons from 3 different litters), and 17 ± 8 % (n=8 neurons from 2 different litters) of control, respectively (Fig. 4B).

Statistical analysis indicated that the effect of both PS and PHS was significantly different in neurons from ethanol-exposed rats vs. ad libitum and pair-fed controls (p<0.02 by ANOVA followed by Bonferroni’s test). The results of these experiments suggest that modulation of NMDA-Rs by neurosteroids that potentiate receptor function is impaired by prenatal ethanol exposure.

Modulation of NMDA-R by negative modulating neurosteroids—Shown in Fig. 5A are sample tracings illustrating the effect of 3α5βPS, a negative neurosteroid modulator of NMDA-Rs. In neurons from ad libitum, pair-fed controls and fetal ethanol-exposed rats, the amplitude of these currents in the presence of 100 µM 3α5βPS were decreased by 52 ± 12 % (n = 9 neurons from 1 litter), 44 ±15 % (n=7 neurons from 2 different litters), and 40 ± 4 % (n = 6 neurons from 2 different litters) in comparison to control, respectively (Fig 5B). The amplitude of NMDA-R currents in neurons from the same diet groups in the presence of 100 µM of 3α5βPHS were decreased to 60 ± 23 % (n = 9 neurons from 2 different litters), 78 ±17 % (n=7 neurons from 3 different litters),
and 73 ± 23 % (n = 7 neurons from 3 different litters) of control values (Fig. 5C).

Statistical analysis indicated that the effect of both 3α5βPS and 3α5βPHS was not significantly different in neurons from ethanol-exposed rats vs. ad libitum and pair-fed controls (p>0.05 by ANOVA followed by Bonferroni’s test).

Modulation of non-NMDA and GABA<sub>A</sub> receptors by neurosteroids. PS has been shown to selectively modulate other classes of ionotropic receptors. PS inhibits AMPA/kainate and GABA<sub>A</sub> receptors (27,36,37). Thus, we performed experiments to investigate possible alterations in the neurosteroid modulation on the function of these receptors in neurons from animals prenatally exposed to ethanol. In neurons from the pair-fed control and fetal ethanol-exposed rats, application of 50 µM PS inhibited to the same extent currents elicited by 100 µM kainate (Table 3). 50 µM PS also inhibited by the same magnitude currents gated by 50 µM GABA (Table 3) in neurons from the pair-fed control and fetal ethanol-exposed neonatal rats. Statistical analysis (t test) indicated that the effect of PS was not significantly different in neurons from ethanol-exposed rats vs. pair-fed controls (Table 3).

Expression Levels of NMDA Receptor Subunits. Fig 6 shows examples of Western immunoblot analyses of NMDA receptor subunit expression in hippocampal homogenates from P3 neonates from the ad libitum, pair-fed and fetal ethanol groups. These examples illustrate that the levels of these subunits are not affected by fetal ethanol exposure. NR2A subunit levels were 0.46 ± 0.13, 0.39 ± 0.09 and 0.28 ± 0.13 µg protein/µg of tubulin, respectively (n = 3 homogenates, each from a different litter). NR2B subunit levels
were $0.34 \pm 0.06$, $0.37 \pm 0.10$ and $0.38 \pm 0.05 \mu g$ protein/\(\mu g\) of tubulin, respectively (\(n = 6\) homogenates, each from a different litter). NR2C subunit levels were $0.27 \pm 0.02$, $0.29 \pm 0.03$ and $0.29 \pm 0.03 \mu g$ protein/\(\mu g\) of tubulin, respectively (\(n = 6\) homogenates, each from a different litter). NR1 subunit levels were $0.76 \pm 0.12$, $1.1 \pm 0.22$ and $0.92 \pm 0.14 \mu g$ protein/\(\mu g\) of tubulin, respectively (\(n = 6\) homogenates, each from a different litter). Levels of NR1 subunits containing the N1 cassette (NR1-N1) were $1.04 \pm 0.15$, $1.03 \pm 0.18$ and $0.86 \pm 0.13 \mu g$ protein/\(\mu g\) of tubulin, respectively (\(n = 5\) homogenates, each from a different litter). Levels of NR1 subunits containing the C2 cassette (NR1-C2) were $0.61 \pm 0.08$, $0.71 \pm 0.11$, and $0.65 \pm 0.03 \mu g$ protein/\(\mu g\) of tubulin (\(n = 5\) homogenates, each from a different litter). Expression of NR1 subunits containing the C1 cassette (NR1-C1) was not detected (\(n = 6\) homogenates, each from a different litter).
DISCUSSION

Contrary to expectations, we found that the basic parameters of NMDA-R function were unaffected by our fetal ethanol exposure paradigm. We expected to detect alterations in NMDA-R function because a number of studies have consistently found reductions in NMDA-R-mediated responses in neurons from the offspring of ethanol-exposed dams. One of these studies showed that exposure of pregnant rats to blood alcohol levels of ~0.04 % resulted in a long-term reduction of NMDA-dependent responses in CA1 hippocampal pyramidal neurons in slices from the adult offspring of these rats (38). Studies using dispersed neurons have also demonstrated prenatal ethanol exposure-induced reductions in NMDA-R function. Fetal ethanol exposure, to blood alcohol levels ranging between 0.03-0.15 %, was shown to reduce NMDA receptor-mediated increases in intracellular Ca^{2+} levels in acutely-dissociated neurons from either the whole brain (12,13) or specific central nervous system regions such as the hippocampus, forebrain and cerebellum of newborn rats (14). In contrast to these studies, we did not detect any changes in hippocampal NMDA-R function at neither the whole-cell or single-channel levels (Fig 1-2). There are multiple factors that could account for the differences between our results and those of the Ca^{2+} imaging studies discussed above. First, it must be considered that, for some of those studies (13,14), pregnant rats were exposed to higher blood alcohol levels (0.12-0.15 %) than those that are achieved with our diet (~0.08 %) (34). Second, for the Ca^{2+} imaging studies, NMDA-R function was assessed in neurons acutely dissociated from newborn rats within 24 hrs after birth; i.e. during the immediate period of withdrawal from in utero ethanol
exposure (12-14). In contrast, we measured NMDA receptor function in hippocampal neuronal cultures prepared 3-4 days after withdrawal from fetal ethanol exposure and we performed electrophysiological recordings after culturing these neurons for an additional 6-7 days in ethanol-free media. Finally, it must be kept in mind that Ca$^{2+}$ imaging techniques do not directly assess NMDA-R function but measure the change in intracellular Ca$^{2+}$ levels in response to NMDA-R activation. NMDA-R-induced changes in intracellular Ca$^{2+}$ levels have been shown to be complex, involving, for example, the release of Ca$^{2+}$ from intracellular stores (39) and the activation of voltage-gated Ca$^{2+}$ channels (40). Thus, the findings of these Ca$^{2+}$ imaging studies must be interpreted cautiously considering that prenatal ethanol exposure could have affected NMDA-R-dependent Ca$^{2+}$ elevations at a point downstream of the NMDA-R itself. Despite these technical differences between studies, it should be emphasized that an important conclusion of our study is that prenatal ethanol exposure may not produce long-lasting changes in hippocampal NMDA-R function in all cases.

A positive finding of our study was that prenatal ethanol exposure affects the sensitivity of NMDA-R to neurosteroids (Fig 3-4 and Table 3). In agreement with previous reports (26-28,41), we found that both PS and PHS produced significant potentiation of NMDA-R-mediated whole-cell currents in cultured hippocampal neurons from control rats. In contrast, the NMDA-R potentiating actions of these neurosteroids were significantly reduced in neurons obtained from neonates prenatally exposed to ethanol. Interestingly, the actions of neurosteroids that inhibit NMDA-R function, such as 3α5βPS and 3α5βPHS, were not significantly affected by prenatal ethanol exposure
Based on competition studies, Park-Chung et al. (26) determined that positive and negative neurosteroid modulators of NMDA-R function act at specific, extracellularly directed sites that are distinct from one another. Thus, prenatal ethanol exposure appears to selectively affect the site of action of positive neurosteroid modulators of NMDA-Rs. The mechanism underlying this selective effect of prenatal ethanol exposure is unclear at the present time because the mechanism of the modulatory actions of neurosteroids on NMDA-R has yet to be determined. It is noteworthy, however, that studies with recombinant receptors suggest that subunit composition is an important determinant of the sensitivity of the NMDA-R to neurosteroids (28). However, we did not detect any changes in the expression levels of NMDA-R subunits in hippocampal homogenates from fetally ethanol-exposed neonates. Consequently, another mechanism must underlie this decrease in sensitivity of NMDA-Rs to positive neurosteroid modulators. A possible mechanism could involve alterations in NMDA-R phosphorylation. Although the role of phosphorylation on neurosteroid’s actions on NMDA-R has not been studied, changes in protein phosphorylation do regulate the sensitivity to neurosteroids of voltage-gated Ca\(^{2+}\) channels (42) and GABA\(_A\) receptors (43). Therefore, it is possible that the mechanism of action of prenatal ethanol exposure involves a change in the phosphorylation state of the NMDA-R and we are currently examining this possibility experimentally.

It should be noted that prenatal ethanol exposure was recently shown to alter modulation of another ligand-gated ion channel by neurosteroids. Allan et al. (34) reported that neurosteroid modulation of GABA\(_A\) receptors is altered in the adult
offspring of dams exposed to a diet of identical composition to the one used in our study. Prenatal ethanol exposure induced a reduction in the modulatory effects of alphaxalone and PS on GABA-stimulated $^{36}$Cl$^{-}$ flux into membrane vesicles prepared from the medial frontal cortex (34). In hippocampal membrane vesicles, prenatal ethanol exposure did not change the effects of PS but enhanced the positive modulatory effects of alphaxalone (34). The results of these experiments with hippocampal microsacs are consistent with our finding that the inhibitory effect of PS on GABA$_{A}$ receptors is unaltered in cultured hippocampal neurons from the fetal ethanol-exposed group. Thus, prenatal ethanol exposure appears to selectively target neurosteroid modulation of NMDA receptors in the hippocampus. Our finding that fetal ethanol exposure did not alter neurosteroid modulation of kainate-induced currents in hippocampal neurons further supports this conclusion.

The findings of the present study are significant because they provide, at least in part, a plausible mechanistic explanation for the alterations in the behavioral responses to neurosteroids of prenatally ethanol-exposed animals (44,45). PS was shown to increase the number of ultrasonic vocalizations in pups subjected to maternal separation-induced stress (33) and this anxiogenic effect of PS was found to be reduced in pups from ethanol-exposed dams (33). We postulate that this decrease in the behavioral responses to exogenous PS could be due, in part, to alterations in the sensitivity of NMDA receptors to this neurosteroid in the hippocampus and other brain regions involved in the stress response. It would be important to determine if
modulation of the stress response by endogenous neurosteroids is also altered by fetal ethanol exposure.

In conclusion, we found that prenatal exposure to ~0.08 % blood alcohol levels do not produce detectable alterations in NMDA receptor function in neonatal hippocampal neurons after 6-7 days in culture. However, we found that exposure to ethanol in utero alters the actions of neurosteroids that positively modulate NMDA-R function and that this effect is not due to an alteration in the expression levels of NMDA-R subunits. A challenging task for future research will be to determine the mechanism and the precise contribution of these alterations to the pathophysiology of learning disabilities associated with Fetal Alcohol Syndrome and alcohol-related neurodevelopmental disorders.
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FIGURE LEGENDS

Figure 1. NMDA dose/response curves recorded in the whole-cell patch-clamp configuration from cultured hippocampal neurons. (A) Sample currents from ad libitum control and fetal ethanol-exposed groups obtained by increasing concentrations of NMDA in presence of a constant glycine (1 µM) concentration (scale bar represents 100 pA and 200 msec). (B) Summary graph of NMDA dose/response curves obtained from all three feeding groups (n = 6-9 neurons from 2-3 different litters per group). Data were normalized with respect to maximum NMDA (100 µM) currents. The inset in panel B shows the NMDA receptor maximum current densities (pA/pF) for neurons from the ad libitum control, pair-fed control, and the fetal ethanol-exposed groups, respectively (n = 21-23 neurons from 3-4 different litters per group). (C) Summary graph of 7-CKA competition curves obtained from all three feeding groups (n = 4-5 neurons from 2-3 different litters per group) in the presence of a constant concentration of NMDA (100 µM) and glycine (1 µM).

Figure 2. Sample tracings from cell-attached single-channel recordings obtained from neurons from pair-fed and fetal ethanol-exposed neonatal rats. The patch solution contained 10 µM NMDA and 1µM glycine. The pipette potential was +20 mV. Single channel parameters are summarized in Table 2. Scale represents 10 pA and 40 msec.
Figure 3. Fetal alcohol exposure affects modulation of NMDA-R by PS. (A) Sample tracings showing the effect of 50 μM PS on currents gated by 50 μM NMDA in cultured hippocampal neurons from ad libitum control, pair-fed control and fetal ethanol-exposed neonatal rats (scale bar is 100 pA and 200 msec). (B) Prenatal ethanol exposure significantly ($p < 0.02$ by two-way ANOVA) reduces the action of different concentrations of PS on currents gated by 50 μM NMDA in neurons from the fetal ethanol-exposed group (for each point, $n = 4$-$7$ neurons from 2-8 different litters per group).

Figure 4. (A) Sample tracings showing the effect of 50 μM PHS on currents gated by 50 μM NMDA in cultured hippocampal neurons from ad libitum control, pair-fed control and fetal ethanol-exposed neonatal rats (scale bar is 100 pA and 200 msec). (B) Summary of the effect of 50 μM PHS in the three diet groups (for each point, $n = 6$-$8$ neurons from 2-3 different litters per group; *$p < 0.02$ by one-way ANOVA followed by Bonferroni’s post hoc test).

Figure 5. Actions of negative modulating neurosteroids 3α5βPS and 3α5βPHS are unaffected by fetal ethanol exposure. (A) Sample tracings showing the effect of 100 μM 3α5βPS on currents gated by 50 μM NMDA in cultured hippocampal neurons from ad libitum control, pair-fed control and fetal ethanol-exposed rats (scale bar is 100 pA and 200 msec). (B) Summary of the effect of 100 μM 3α5βPS ($n = 6$-$7$ neurons from 1-2 different litters per group) (C) Summary of the effect of 100 μM 3α5βPHS ($n = 7$-$9$ neurons from 2-3 different litters per group).
Figure 6. Expression of NMDA-R subunits in hippocampal homogenates from neonates belonging to the *ad libitum*, pair-fed and fetal ethanol-exposed groups. Shown are examples of Western immunoblots for the indicated NMDA-R subunits, including NR1 subunits containing the N1, C1 and C2 cassettes. Also shown are samples of immunoblots for β-tubulin, which were used to correct for variations in protein loading. Each of the bands shown per group is from a homogenate prepared from the hippocampi of two neonates (P3-4). Each homogenate was obtained from animals belonging to different litters. Shown on the left are protein standard curves generated from a single homogenate of adult rat hippocampal tissue of known protein concentration. The protein amounts loaded per standard curve lane were 5, 10, 15, and 20 µg. This curve was used to calculate relative units of protein concentrations. Note the expression of NR1-C1 subunits in the adult homogenates but not in the neonatal homogenates. See text for details on the methodology and the results of densitometric analyses.
Table 1. Comparison of liquid diet consumption and the effects of diets on offspring at birth

<table>
<thead>
<tr>
<th>DIET GROUP</th>
<th>AVERAGE # OF NEWBORNS/LITTER</th>
<th>AVERAGE # OF LIVE NEWBORNS/LITTER</th>
<th>AVERAGE MEAN WEIGHT OF LIVE NEWBORNS (g)</th>
<th>AVERAGE DAILY FOOD CONSUMPTION (Kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD LIBITUM</td>
<td>12 ± 1.0</td>
<td>12 ± 1.0</td>
<td>6.7 ± 1.0</td>
<td>Undetermined</td>
</tr>
<tr>
<td>PAIR-FED</td>
<td>12 ± 1.0</td>
<td>12 ± 1.0</td>
<td>6.5 ± 0.1</td>
<td>85 ± 0.3</td>
</tr>
<tr>
<td>FETAL ETHANOL</td>
<td>11 ± 1.0</td>
<td>11 ± 1.0</td>
<td>6.3 ± 0.1</td>
<td>91 ± 1.7</td>
</tr>
</tbody>
</table>

*Values are mean ± S.E.M. of 36, 43, and 40 dams for the ad libitum, pair-fed and fetal ethanol groups, respectively.*
### Table 2. NMDA Receptors Single Channel Parameters (Cell-Attached Mode)\(^a\)

<table>
<thead>
<tr>
<th>DIET GROUP</th>
<th>OPEN PROBABILITY ((n = 12-14)^b)</th>
<th>OPEN TIME (msec) ((n = 11-12)^b)</th>
<th>CLOSED TIME (msec) ((n = 5-7)^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\tau_1)</td>
<td>(\tau_2)</td>
<td>(\tau_1)</td>
</tr>
<tr>
<td>AD LIBITUM</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>PAIR-FED</td>
<td>0.08 ± 0.01</td>
<td>0.10 ± 0.03</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>FETAL ETHANOL</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\)Values are mean ± S.E.M.

\(^b\) \(n\) = number of neurons recorded, which were obtained from 8-12 different litters per diet group.
Table 3. Effect of Fetal Ethanol Exposure on Modulation of Ligand-gated Ion Channel Currents by PS (50 µM)$^a$.

<table>
<thead>
<tr>
<th>DIET GROUP</th>
<th>NMDA$^b$</th>
<th>NON-NMDA$^c$</th>
<th>GABA$^d_A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD LIBITUM</td>
<td>72 ± 10 % (12)</td>
<td>Not Determined</td>
<td>Not Determined</td>
</tr>
<tr>
<td>PAIR-FED</td>
<td>67 ± 11 % (12)</td>
<td>-22 ± 12 % (8)</td>
<td>-82 ± 2 % (7)</td>
</tr>
<tr>
<td>FETAL ETHANOL</td>
<td>-4 ± 8 % (11)$^e$</td>
<td>-20 ± 7 % (6)</td>
<td>-88 ± 2 % (6)</td>
</tr>
</tbody>
</table>

$^a$Values are mean + S.E.M. Numbers of neurons tested are given in parentheses. Neurons were obtained from 5-8 different litters per diet group for NMDA experiments and from one litter per diet group for the non-NMDA and GABA$^d_A$ experiments.

$^b$Currents were elicited by 50 µM NMDA in Mg$^{2+}$-free external solution.

$^c$Currents were elicited by 100 µM kainate.

$^d$Currents were elicited by 50 µM GABA.

$^e$Significantly different from ad libitum and pair-fed groups (P < 0.0001 by one-way ANOVA followed by Bonferroni’s post hoc test).
**FIGURE 1**

A. Comparison of AD LIBITUM and FETAL ETOH (NMDA) µM levels.

B. Graph showing normalized current with [NMDA], M concentration in AD LIBITUM, PARI-FED, and FETAL ETOH groups.

C. Graph depicting % from control with [7-CKA], M concentration in AD LIBITUM, PARI-FED, and FETAL ETOH groups.
FIGURE 2

PAIR-FED

FETAL ETOH
FIGURE 3

A

AD LIBITUM

PAIR-FED

FETAL ETOH

NMDA

NMDA + PS

NMDA

NMDA + PS

NMDA

NMDA + PS

B

% CHANGE FROM CONTROL

150

100

50

0

10^{-6} 10^{-5} 10^{-4}

[PREGNENOLONE SULFATE], M

• AD LIBITUM

□ PAIR-FED

○ FETAL ETOH
FIGURE 4

AD LIBITUM
PAIR-FED
FETAL ETOH

NMDA
NMDA
NMDA + PHS
NMDA + PHS
NMDA + PHS

% CHANGE FROM
PHS 50 µM
CONTROL

AD LIBITUM
PAIR-FED
FETAL ETOH
FIGURE 5

A

AD LIBITUM                PAIR-FED                FETAL ETOH

NMDA +3α5βPS

NMDA

NMDA +3α5βPS

NMDA

B

% CHANGE FROM
CONTROL

[3α5β PS]

AD LIBITUM        PAIR-FED        FETAL ETOH

C

% CHANGE FROM
CONTROL

[3α5β PHS]

AD LIBITUM        PAIR-FED        FETAL ETOH
<table>
<thead>
<tr>
<th>Protein</th>
<th>STANDARD CURVE</th>
<th>AD LIBITUM</th>
<th>PAIR-FED</th>
<th>FETAL ETHANOL</th>
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<tbody>
<tr>
<td>NR2A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR2B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR2C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NR1 - N1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR1 - C1</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>NR1 - C2</td>
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<td></td>
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<tr>
<td>β-Tubulin</td>
<td></td>
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</tbody>
</table>

**FIGURE 6**
Fetal alcohol exposure alters neurosteroid modulation of hippocampal NMDA receptors

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