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AMPA Receptor-mediated Synaptic Transmission in the CA1 Hippocampal Region of Neonatal Rats: Unexpected Resistance to Repeated Ethanol Exposure

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Abstract

AMPA glutamatergic receptors (AMPA) mediate the majority of fast excitatory synaptic transmission in mature neurons. In contrast, a number of developing synapses do not express AMPARs; these are gradually acquired in an activity-driven manner during the first week of life in rats, which is equivalent to the third trimester of human pregnancy. Neuronal stimulation has been shown to drive high conductance Ca^{2+} -permeable AMPARs into the synapse, strengthening glutamatergic synaptic transmission. Alterations in this process could induce premature stabilization or inappropriate elimination of newly formed synapses and contribute to the hippocampal abnormalities associated with fetal alcohol spectrum disorder. Previous studies from our laboratory performed with hippocampal slices from neonatal rats showed that acute ethanol exposure exerts potent stimulant effects on CA1 and CA3 neuronal networks. However, the impact of these *in vitro* actions of acute ethanol exposure is unknown. Here, we tested the hypothesis that repeated *in vivo* exposure to ethanol strengthens AMPAR-mediated neurotransmission in the CA1 region via an increase in synaptic expression of Ca^{2+} -permeable AMPARs. We exposed rats to ethanol vapor (serum ethanol concentration ~40 mM) or air for 4 hr/day from postnatal day (P) 2 to 6. In brain slices prepared at P4–6, we found no significant effect of ethanol exposure on input-output curves for AMPAR-mediated field excitatory postsynaptic potentials (fEPSPs), the contribution of Ca^{2+} -permeable AMPARs to these fEPSPs, or the acute effect of ethanol on fEPSP amplitude. These results suggest that homeostatic plasticity mechanisms act to maintain glutamatergic synaptic strength and ethanol sensitivity in response to repeated developmental ethanol exposure.

Introduction

Ethanol exposure during development can result in fetal alcohol spectrum disorder (FASD), which represents a broad range of clinical alterations (Green, 2007). These include learning and memory deficits that are, in part, a consequence of damage to the hippocampal formation (Berman and Hannigan, 2000). Approximately 10% of pregnant women consume ethanol and ~2% binge drink (Centers for Disease Control and Prevention (CDC), 2009). In addition, about half of pregnant women who consume ethanol continue to do so through the third trimester, a period of intense glutamatergic synaptic formation and refinement (Khazipov et al., 2001; U.S. Department of Health and Human Services, 1998). Ionotropic glutamatergic signaling is

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largely mediated by α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPA) and N-methyl-D-aspartate receptors (NMDARs) (Dingledine et al., 1999). AMPARs mediate synaptic transmission at the resting membrane potential and contribute to removal of Mg^{2+} block from the NMDAR (Dingledine et al., 1999). The AMPAR is a tetrameric receptor composed of various combinations of subunits (GluR1-4) and the functional properties of the receptor are highly dependent on its subunit composition. Receptors containing GluR1, 3 and/or 4 subunits are Ca^{2+} -permeable and susceptible to polyamine blockade (Donevan and Rogawski, 1995). When AMPARs contain a GluR2 subunit, they become Ca^{2+} -impermeable as a result of the presence of a positively-charged arginine in the trans-membrane 2 domain, which blocks Ca^{2+} entry and prevents blockade by polyamines (Bassani et al., 2009).

Increases in spontaneous neuronal activity in hippocampal slice cultures were shown to induce synaptic insertion of Ca^{2+} -permeable (GluR4-containing; higher conductance) AMPARs into CA1 pyramidal neurons, which are then gradually replaced in an activity independent manner by Ca^{2+} -impermeable (GluR2-containing; lower conductance) AMPARs (Zhu et al., 2000). Work from our laboratory indicates that during the neonatal period of rat development, which is equivalent to the third trimester of human pregnancy, ethanol paradoxically acts as a potent stimulant of neuronal activity both in the CA3 and CA1 hippocampal regions (reviewed in Valenzuela et al., 2008). Acute ethanol application in brain slices increased spontaneous network activity of CA3 hippocampal pyramidal neurons, which provide glutamatergic input to CA1 hippocampal pyramidal neurons (Galindo et al., 2005). Moreover, acute ethanol exposure of slices from postnatal day (P) 3–5 rats induced the production and/or release of a pregnenolone sulfate-like neurosteroid retrograde messenger that decreases failures of AMPAR-mediated excitatory postsynaptic currents (EPSCs) at Schaffer collateral-to-CA1 pyramidal neuron synapses under conditions of minimal stimulation (Mameli et al., 2005; Mameli and Valenzuela, 2006). Based on these results and those of Zhu et al. (2000), we hypothesized that repeated *in vivo* exposure to ethanol would strengthen AMPAR-mediated neurotransmission in the CA1 region via an increase in synaptic expression of Ca^{2+} -permeable AMPARs.

To test this hypothesis, we used field recording techniques in the CA1 hippocampal region in brain slices from animals developmentally exposed to ethanol vapor or air. We measured AMPAR-dependent field excitatory postsynaptic potential (fEPSP) input-output curves to assess changes in synaptic strength, and used a pharmacologic inhibitor of Ca^{2+} -permeable AMPARs to assess changes in the functional expression of these receptors. We also re-examined the acute effects of ethanol on AMPAR-mediated synaptic responses and tested whether these acute effects were affected by repeated ethanol vapor exposure.

Experimental Procedures

Ethanol Vapor Chamber Exposure Paradigm

Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center and conformed to National Institutes of Health guidelines. Timed-pregnant Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Neonatal rat pups and dams were exposed to ethanol vapor as previously described (Galindo and Valenzuela, 2006). Starting on P2, animals were transported to a room housing the ethanol vapor chamber apparatus and weighed prior to exposure. Air tight lids were then placed on the animals' home cages (La Jolla Alcohol Research Inc, La Jolla, CA) and were perfused with air (control group) or an air/ethanol vapor mixture (ethanol group). Ethanol was vaporized using a heating flask that receives a constant drip of 95% liquid ethanol (Tarr LLC, Phoenix, AZ) regulated with a peristaltic pump. Ethanol vapor (or air) was continuously removed by an exhaust hose connected to a vacuum line, maintaining constant

ethanol vapor levels. Litters were culled to 10 pups on P2 and exposed daily for 4 hrs per day until P6 (Fig. 1A). Exposures were started at 07:00 hrs (lights on at 06:00 hrs and lights off at 18:00 hrs). The P2-6 developmental period was chosen because it encompasses the time frame where the acute stimulatory actions of ethanol were detected in the CA1 and CA3 hippocampal regions (Galindo et al., 2005; Mameli et al., 2005; Mameli and Valenzuela, 2006).

Tissue preparation and solutions

Unless indicated, chemicals were from Sigma (St. Louis, MO) or Tocris Cookson (Ellisville, MO). Both male and female rat pups were deeply anesthetized with 250 mg/kg of ketamine and euthanized by decapitation immediately after the 4 hr exposure on P4-6. Trunk blood samples were allowed to coagulate and centrifuged at $2.3 \times g$ for 10 min to obtain serum. Serum ethanol concentrations were determined using an alcohol dehydrogenase-based assay involving the reduction of NAD⁺ (β -NAD, free acid, grade I, Roche, Indianapolis, IN). NADH absorbance was read at a wavelength of 340 nm. Coronal brain slices (400 μ m) were prepared using a vibratome, as previously described (Mameli et al., 2005). After a recovery period of 45 min at 35–36°C, slices were stored for 1–8 hr at room temperature. Artificial cerebrospinal fluid (ACSF) contained the following (in mM): 126 NaCl, 2 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 26 NaHCO₃, 2 CaCl₂, 10 glucose, and 0.01 gabazine (also known as SR-95531) equilibrated with 95% O₂/5% CO₂. When indicated, the ACSF also contained 100 μ M 1-naphthylacetyl spermine trihydrochloride (NASPM), 50 μ M D, L-2-amino-5-phosphonovaleric acid (AP5), 50 μ M GYKI-53655 and/or 10 μ M 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f] quinoxaline-2,3-dione (NBQX).

Electrophysiological recordings

Recordings were performed in the CA1 *stratum radiatum* at 32°C with a perfusion rate of 2 ml/min using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Recording micropipette glass electrodes had resistances of 3–5 M Ω and were filled with ACSF. AMPAR fEPSPs were evoked using a concentric bipolar electrode (inner pole 25 μ m; outer pole 125 μ m; Frederick Haer Company, Bowdoinham, ME) placed in vicinity of the Schaffer collateral fibers. Input-output curves were measured at the start of all recordings, and, for subsequent experiments, the stimulation intensity was set at 40–50% of the intensity required to elicit maximal responses. Stimulus duration was 75 μ s and stimuli were delivered at 0.033 Hz.

Data Analysis

Data were acquired and analyzed with pClamp 9 (Molecular Devices, Sunnyvale, CA) and GraphPad Prism 4.0 (San Diego, CA). The short time interval between the presynaptic volley and fEPSP prevented accurate measurement of the slope in most experiments. Therefore, data were analyzed using the fEPSP amplitude. For each experiment, baseline was defined as the average of 20 fEPSPs immediately before drug application. For experimental conditions, 10 fEPSPs immediately preceding the start of the ethanol washout or the application of NBQX or GYKI-53655 were averaged for analysis. Statistical analyses of pooled data were performed by Student's *t* test, one sample *t* test vs. a theoretical mean of zero or 100%, or repeated measure two-way ANOVA. A $p \leq 0.05$ was considered to be statistically significant. Data are presented as mean $\pm$ SEM with the number of determinations (*n*) representing the number of recordings. Data shown in Figs. 2–3 were obtained in slices from 9–10 pups from 5 different litters per treatment condition. Data shown in Fig. 4 were obtained in slices from 2 pups from 2 different litters. Data shown in Fig. 5 were obtained in slices from 4 pups from 1 litter each for the control and ethanol groups.

Results

To model exposure to ethanol during the third trimester of human pregnancy, rats were exposed to ethanol vapor during the neonatal period of development (Fig. 1A). Dams and rat pups were exposed to ethanol vapor daily for 4 hr per day with the average chamber ethanol concentration ranging from 1.51 to 1.65 g/dL (Fig. 1B). This exposure resulted in elevated ethanol concentrations in neonatal pups, and very low levels in dams (Fig. 1C). Daily litter average pup weights were not different between the control and ethanol groups ($n=6$ for control and $n=6$ for ethanol; not significant (N.S.) by repeated measures two-way ANOVA; Fig. 1D).

In brain slices from control and ethanol-exposed animals, we recorded AMPAR-mediated fEPSP input-output curves in the presence of gabazine (10 μ M) and AP5 (50 μ M) to measure changes in synaptic strength. There was no significant difference between the control and ethanol-exposed groups (N.S. by repeated measure two-way ANOVA; Fig. 2).

We then tested the effects of NASPM (a blocker of Ca^{2+} -permeable AMPARs) on AMPAR-mediated fEPSPs in slices from control and ethanol exposed animals. Figs 3A-B show that NASPM reduced amplitudes of AMPAR-mediated fEPSPs to a similar extent in the control and ethanol groups. As expected, the events were abolished by the AMPAR antagonist, GYKI-53655 (50 μ M). The residual potential recorded in the presence of GYKI-53655 (or NBQX; data not shown) corresponds to the presynaptic volley, which was not significantly different between the control (0.12 ± 0.02 mV; $n=12$) and ethanol (0.12 ± 0.02 mV; $n=14$) groups (N.S. by unpaired t-test). The time course of the effect of NASPM is shown in Fig. 3C, where after a stable baseline was recorded in the presence of gabazine (10 μ M) and AP5 (50 μ M), NASPM (100 μ M) was applied for 15 min. In a subset of experiments, NASPM application was followed by application of GYKI-53655 or the non-NMDA antagonist, NBQX (10 μ M) ($n=12$ for control and $n=14$ for ethanol) confirming that the fEPSPs were AMPAR-mediated. The percent of functional Ca^{2+} -permeable AMPARs (i.e. those lacking GluR2) corresponds to the magnitude of inhibition of fEPSPs by NASPM, and was $21 \pm 1.9\%$ ($n=19$) for the control and $17.8 \pm 1.5\%$ for the ethanol ($n=21$) groups (N.S. by unpaired t-test). NASPM did not differentially inhibit the AMPAR-mediated fEPSPs at P4, P5 or P6 (i.e. 3, 4, or 5 ethanol exposures; N.S. by two-way ANOVA; Fig. 3D). In addition, the effect of NASPM was not correlated with the serum ethanol concentration (by linear regression; $R^2=0.02$; slope not significantly different from zero; Fig. 3E).

Mameli et al. (2006) showed previously that acute exposure to ethanol, at concentrations as low as 15 mM, significantly decreased the failure rate of AMPAR-mediated EPSCs in CA1 pyramidal neurons from P3-4 rats under conditions of minimal stimulation; this effect was dependent on the release of a pregnenolone sulfate-like neurosteroid. Here, we re-examined the acute effect of ethanol on AMPAR-mediated synaptic transmission in the developing CA1 region using extracellular recording techniques. Ethanol was bath-applied after a stable baseline was obtained in the continued presence of gabazine (10 μ M); note that AP5 was omitted because the actions of the pregnenolone sulfate-like neurosteroid are NMDAR dependent (Mameli and Valenzuela, 2006). Unexpectedly, we found that ethanol (40 mM) induced a small but significant inhibition of the AMPAR-mediated fEPSP ($-7.53 \pm 1.49\%$ with respect to the average of baseline and washout; $p<0.01$; by one sample t-test v. zero, $n=6$; Fig. 4A-B).

Finally, we investigated whether ethanol vapor exposure affected the acute sensitivity to ethanol of AMPAR-mediated fEPSPs. Since the acute effect of 40 mM ethanol was small, we tested the acute effect of a higher ethanol concentration (80 mM). In the presence of gabazine (10 μ M) and AP5 (50 μ M), ethanol (80 mM) was bath-applied for 10 minutes (Fig. 5A-B). Acute ethanol application significantly inhibited AMPAR-mediated fEPSPs by $13.07 \pm 1.35\%$

and $10.02 \pm 2.29\%$ (from the average of baseline and washout) in slices from the control and ethanol groups, respectively ($n=8$; $p<0.01$ by one sample t-test from zero for both conditions). These values were not significantly different from each other (N.S. by unpaired t-test).

Discussion

A number of studies have demonstrated that developmental ethanol exposure produces persistent alterations in AMPAR function in CA1 hippocampal and medial septum/diagonal band neurons (Bellinger et al., 1999; Hsiao and Frye, 2003; Wijayawardhane et al., 2007). However, to the best of our knowledge, this is the first characterization of the effect of *in vivo* third trimester-equivalent ethanol exposure on AMPAR function in the CA1 hippocampal region of neonatal rats. We used a vapor chamber ethanol exposure paradigm that models repeated moderate-to-heavy maternal ethanol use during the third trimester of human pregnancy. An advantage of this exposure paradigm is that serum ethanol concentrations in the dams are low, resulting in undetectable alterations in maternal care (Galindo and Valenzuela, 2006). Averaged individual pup weight was not different between control and ethanol-exposed litters suggesting that this exposure paradigm models FASD more closely than fetal alcohol syndrome, which involves growth retardation. This exposure paradigm focuses on a critical period of brain development, termed the brain growth spurt, where most glutamatergic synapses are generated and refined. A limitation of our study is that it models a relatively short period of the third trimester-equivalent of human pregnancy. Maternal ethanol use typically spans all three trimesters and pregnant women rarely start drinking during the third trimester (Centers for Disease Control and Prevention (CDC), 2009). However, this study was undertaken to selectively assess the impact of repeated ethanol exposure during the third trimester-equivalent because of the critical importance of this period for neuronal circuit maturation. Future studies should re-examine the impact of ethanol exposure during all three trimesters on glutamatergic synaptic transmission in the developing CA1 region.

Contrary to our hypothesis, the strength of AMPAR-mediated synaptic transmission did not differ between control and ethanol-exposed animals. This is surprising in light of the previous finding from our laboratory that acute exposure to 15–75 mM ethanol induced the production and/or release of a pregnenolone sulfate-like neurosteroid retrograde messenger that strengthened AMPAR-mediated synaptic transmission in the CA1 region of neonatal rats (Mameli and Valenzuela, 2006). However, when we re-examined this effect of ethanol using field recording techniques, we found that AMPAR-mediated fEPSPs were not potentiated, but rather inhibited, by acute exposure to ethanol. Inhibitory effects of ethanol on AMPARs have previously been observed in both the CA3 and CA1 hippocampal regions of neonatal rat pups (Mameli et al., 2005; Puglia and Valenzuela, Submitted). Therefore, it is possible that the acute effects of ethanol reported by Mameli and Valenzuela (2006) can only be observed under certain experimental conditions *in vitro* —i.e. under minimal stimulation in the whole-cell voltage-clamp configuration with K^+ channel blockers present in the internal solution. These conditions could favor the detection of the neurosteroid-dependent stimulatory actions of ethanol because of two reasons. First, excitatory postsynaptic currents evoked under conditions of minimal stimulation are likely mediated by AMPARs located in the proximal dendrites and/or soma of CA1 pyramidal neurons and these receptors could be affected by ethanol differently than the more distal receptors sampled in the fEPSP recordings. Second, K^+ channel inhibition could facilitate ethanol-induced dendritic release of the pregnenolone sulfate-like neurosteroid. It is also possible that the stimulatory effects of ethanol on CA3 network activity that were previously reported by Galindo et al. (2005) are also dependent on the experimental conditions. However, this possibility is unlikely given that these effects were observed with whole-cell electrophysiological recordings in single neurons, and also with Ca^{2+} imaging techniques in ensembles of CA3 neurons.

An alternative explanation for the lack of an effect of neonatal ethanol vapor exposure on AMPAR-mediated transmission in the CA1 region is that ethanol stimulates CA1 and CA3 neuronal activity *in vivo*, but neurons adapt to these effects; i.e., homeostatic plasticity mechanisms may act to restore synaptic strength (Carpenter-Hyland and Chandler, 2006). Our finding that the acute effect of ethanol on AMPAR-mediated fEPSPs is not affected by vapor chamber ethanol exposure suggests that these homeostatic mechanisms do not involve the development of AMPAR tolerance to ethanol. This conclusion is also supported by the finding of Galindo et al. (2006) that CA3 pyramidal neurons did not develop tolerance to the excitatory actions of ethanol after repeated ethanol vapor exposure. Moreover, lack of development of AMPAR tolerance to the acute effects of ethanol was also observed in a study with medial septum/diagonal band neurons from P15–25 and P32–35 rats that were exposed to ethanol on P4–9 (blood ethanol level = 352 ± 9 mg/dL) (Hsiao and Frye, 2003).

It was also unexpected that third trimester-equivalent ethanol exposure did not alter the functional expression of Ca^{2+} -impermeable AMPARs. In hippocampal slice cultures from neonatal rats, it has been shown that increased spontaneous excitatory activity induces synaptic trafficking of Ca^{2+} -permeable, GluR4-containing AMPARs (Zhu et al., 2000). Consistent with this, we predicted that our ethanol exposure paradigm would repeatedly excite CA1 and CA3 neuronal networks, thus resulting in increased expression of Ca^{2+} -permeable AMPARs at CA1 pyramidal neuron synapses. As discussed above, repeated ethanol exposure could initiate homeostatic plasticity mechanisms that would prevent changes in synaptic expression of Ca^{2+} -permeable AMPAR (Lissin et al., 1998; Watt et al., 2000). Alternatively, it is possible that the paradigm of slice preparation immediately after ethanol exposure led to GluR4 insertion and subsequent exchange of this subunit with the Ca^{2+} -impermeable GluR2 subunit. Although the activity-independent exchange of GluR4 with GluR2 has indeed been documented, this possibility is unlikely because this is an activity-independent slow process that can take up to 30 hrs and we measured expression of Ca^{2+} -permeable AMPARs less than 8 hrs after ethanol exposure (Zhu et al., 2000). Moreover, if this exchange process had taken place, we should have observed an increase in AMPAR fEPSP input-output curves mediated by increased synaptic expression of GluR2-containing receptors.

In conclusion, our findings show that the *in vitro* acute stimulatory actions of ethanol on developing CA1 pyramidal neurons occur under some, but not all, experimental conditions. If these actions of ethanol take place *in vivo*, the long-lasting impact on AMPAR-mediated synaptic transmission in the CA1 region remains unknown. Moreover, AMPAR-mediated responses can be inhibited by acute ethanol exposure in this region and this effect is not affected by long-term ethanol vapor exposure. This finding confirms that AMPAR-mediated responses are important targets of the developmental actions of ethanol and that these responses are not subject to the acquisition of ethanol tolerance. Future studies should examine whether ethanol alters the normal functioning of other neurotransmitter receptors or intracellular signaling pathways during the third trimester of pregnancy, as these could have deleterious consequences on the maturation of hippocampal neuronal networks and play a role in the learning and memory alterations that characterize FASD.

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Figure 1.

Third trimester-equivalent ethanol exposure paradigm. (A) Schematic representation of the exposure paradigm where rat pups and their respective dams were repeatedly exposed to ethanol vapor for 4 hr per day. Exposure(s) started at P2 and continued to P6. Brain slices for electrophysiology studies were prepared from P4, P5 or P6 rats, immediately after the 4 hr exposure period. (B) Ethanol concentrations in the exposure (vapor) chamber. (C) Serum ethanol concentrations measured from trunk blood samples collected immediately after euthanasia. Line represents the legal intoxication limit (17.4 mM). (D) Average pup weight of control and ethanol vapor-exposed pups as a function of postnatal day.

Figure 2.

Third trimester-equivalent ethanol exposure does not alter synaptic efficacy in the CA1 hippocampus of P4-6 rat pups. (A) Representative traces of AMPAR fEPSPs recorded at increasing stimulus intensities. (B) Pooled data of control and ethanol exposed animals.

Figure 3.

Third trimester-equivalent ethanol exposure does not alter functional expression of Ca^{2+} -permeable AMPARs. Representative traces of fEPSP recordings from the control (A) and ethanol (B) groups obtained in the presence of gabazine (10 μM) and AP5 (50 μM), followed by NASPM (100 μM) and then by GYKI-53655 (50 μM). (C) Time course of the effect of NASPM application followed by GYKI-53655 (or NBQX 10 μM) application. Letters above the time course correspond to letters in sample traces (a, b and c). (D) NASPM-mediated inhibition of fEPSP amplitude as a function of postnatal day. (E) NASPM-mediated inhibition of fEPSP amplitude as a function of the serum ethanol concentration.

Figure 4. Acute Ethanol (40 mM) inhibited fEPSP responses in slices from naïve P4 rats. (A) Sample traces from fEPSP recordings and (B) time course. Recordings were performed in the presence of gabazine (10 μ M) and fEPSPs were inhibited by NBQX (10 μ M). Letters above the time course correspond to letters in sample traces (a, b, c, and d).

Figure 5.

Third trimester-equivalent ethanol vapor exposure does not induce tolerance to the acute inhibitory effects of ethanol on the amplitude of AMPAR-mediated fEPSPs. Ethanol (80 mM) was bath-applied to brain slices from P4-6 rat pups. (A) Sample traces from fEPSP recordings and (B) time course. Recordings were performed in the presence of gabazine (10 μ M) and AP5 (50 μ M). Letters above the time course correspond to letters in sample traces (a, b, and c).