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Ethanol increases GABA release in the embryonic avian retina

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ABSTRACT

Several mechanisms underlying ethanol action in GABAergic synapses have been proposed, one of these mechanisms is on GABA release. Here, we report that *in ovo* exposure to ethanol induces an increase on GABA release in the embryonic chick retina. Eleven-day-old chick embryos (E11) received an injection of either phosphate buffer saline (PBS) or ethanol (10%, v/v, diluted in PBS), and were allowed to develop until E16. A single glutamate stimulus (2 mM) showed approximately a 40% increase on GABA release in E16 retinas when compared to controls. The effect was dependent on NMDA receptors and GAD65 mRNA levels, which were increased following the ethanol treatment. However, the numbers of GABA-, GAD-, and NR1-immunoreactive cells, and the expression levels of these proteins, were not affected. We conclude that ethanol treatment at a time point when synapses are being formed during development selectively increases GABA release in the retina via a NMDA receptor-dependent process.

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1. Introduction

The developing brain is highly sensitive to ethanol (EtOH) and a severe consequence of this drug intoxication is the fetal alcohol spectrum disorders (FASD). FASD is characterized by craniofacial malformations, growth retardation, neuropathological symptoms, and increased mortality (Chen et al., 2003). FASD is caused by maternal ingestion of EtOH during pregnancy, and is the most common and preventable cause of mental retardation.

Acute and chronic effects of EtOH in the neurochemical network of the central nervous system (CNS) have been thoroughly investigated, but progress has been relatively slow in revealing specific mechanism, which might be related to the fact that not one but several neurochemical systems seem to be affected (Zafar et al., 2000; Szot et al., 1999; Boehm et al., 2006; Olney et al., 2001; Lovinger, 2002). Compelling evidence demonstrates that in the GABAergic system, both pre and post-synaptic components are greatly affected by EtOH. For instance, $GABA_A$ receptors (Aguayo et al., 2002; Roberto et al., 2003; Shannon et al., 2004) with a specific subunit composition (Hanchar et al., 2004; Farrant and Nusser, 2005) and GABA release in rat central amydala (Roberto et al., 2003, 2004; Criswell and Breese, 2005; Breese et al., 2006) can be modulated by EtOH.

The retina is a good model for studying neurotransmission and neurotransmitter modulation in the CNS because of its accessibility, laminar arrangements and limited number of basic neuronal types (Wässle, 2004; Calaza et al., 2006). The retina contains almost every known neurotransmitter, and glutamate and GABA are distributed in a way that the former is located in the radial axis, present in the photoreceptors, bipolar and ganglion cells, whereas the latter is mainly found in the modulatory circuits composed by horizontal and most amacrine cells (Barnstable, 1993).

Between embryonic days (E) 11 and 16, the chick retina undergoes the process of synaptogenesis process; receptors for glutamate and GABA are already expressed among retinal neurons (Hughes and LaVelle, 1974; De Mello et al., 1991; Hering and Kröger, 1996; Catsicas and Mobbs, 2001; Bredariol and Hamassaki-Britto, 2001; Barros et al., 2003), and glutamate can induce GABA release (Tapia and Arias, 1982; Do Nascimento and de Mello, 1985; Reis et al., 1995; Calaza et al., 2003).

Characteristically, glutamate evoked GABA release in the retinal tissue occurs via activation of NMDA and non-NMDA ionotropic glutamate receptors, being completely blocked by application of CNQX and MK801, non-NMDA and NMDA receptor antagonists, respectively (reviewed in Calaza et al., 2006). This event involves a

Abbreviations: CNS, central nervous system; EAA, excitatory amino acids; EtOH, ethanol; FASD, fetal alcohol spectrum desorder; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GAT1, GABA transporter 1; INL, inner nuclear layer; NMDA, n-methyl D-aspartate; NR1, NMDA receptor subunit 1; IPL, inner plexiform layer.

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calcium-independent but sodium-dependent mechanism, since replacement of sodium chloride for choline chloride or lithium chloride completely eliminates the efflux of [^{3}H]-GABA evoked by excitatory amino acid stimulation (reviewed in Calaza et al., 2006).

In the brain, during normal development, neurons refine their connections to other neurons by retracting contacts to inappropriate targets while strengthening those to appropriate ones. This process is achieved by a precise fine-tuning mechanism that balances inhibition and excitation. Increased availability of GABA, induced by EtOH, might perturb this equilibrium and could explain some of the behavioral changes observed in EtOH-treated adult animals. Surprisingly, the effects of EtOH on GABA release in the developing nervous system have not been fully described. Since EtOH is associated with the modulation of $GABA_A$ and/or NMDA receptors in the retina, and *in ovo* EtOH exposure allows us to evaluate its affects during development, this study was designed to investigate the effects of *in ovo* EtOH exposure on glutamate-dependent GABA release in the embryonic chick retina.

2. Materials and methods

2.1. Materials

[^{3}H]GABA and [^{3}H]D-aspartate were obtained from Amersham Biosciences (UK). Kainate, N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid (HEPES) and NMDA were obtained from Sigma (St. Louis, MO, U.S.A.). 6,7-Dinitroquinoxaline-2,3-dione (DNQX), and MK801 were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (NY, U.S.A.). All other reagents were of analytical grade.

We used fertilized White Leghorn eggs obtained from a local hatchery, incubated at 37.8 °C and a relative humidity of 80–90%. We injected the chick embryos at E11 with 300 μL of EtOH (10%, v/v) in sterile phosphate-buffered saline (PBS). At E11, the approximate volume of the egg is 80 mL. Since 10% (v/v) EtOH is about 1.7 M, the injection of 300 μL in the viteline sac gives a dilution of approx. 266× (or 6.42 mM). The embryos injected at E11 were returned to the incubator until E16. The staging of the embryos was determined systematically using the tables of Hamburger and Hamilton (1951). Controls were obtained using eggs injected with 300 μL of sterile PBS.

2.2. [^{3}H]GABA or [^{3}H]D-aspartate release induced by glutamate

The protocol was basically the same as described elsewhere (Do Nascimento and de Mello, 1985). Briefly, pieces of intact retinas at E16 were pre-incubated with DMEM containing 0.3 μCi/mL [^{3}H]GABA or [^{3}H]D-aspartate for 90 min, at 37 °C, pH 7.35. In order to wash out the non-incorporated radioactivity, the medium was removed and the explants washed 6 times with 2 mL of Hank's solution. Then, the tissue was superfused with Hank's solution or 2 mM glutamate for 6 min in Hank's solution at 37 °C, and the radioactivity released in the medium was quantified. The amount of [^{3}H]GABA released was plotted as the percentage of the total radioactivity taken up by the cells. The total radioactivity was estimated as that released during the superfusion plus the radioactivity remaining in the cells at the end of the procedure. The remaining cellular radioactivity was determined after disruption of the cells in distilled water followed by three successive freeze–thaw cycles.

2.3. Treatment of the retinal tissue with excitatory amino acids (EAA)

Each retina was divided in two pieces taken along the superior–inferior axis. Retinal pieces under different treatments were chosen as "control" or as "experimental" following a random distribution, avoiding differences of the horizontal axis. The tissue was dissected and sectioned in Locke's solution containing the following components (in mmoles/L): 157.0 NaCl, 5.6 KCl, 2.3 $CaCl_2$, 3.6 Na_2HCO_3, 5.0 HEPES, 1.0 $MgCl_2$ and 5.6 glucose. Locke's solution was devoid of magnesium and contained 2 mM glycine and 2 mM EDTA whenever NMDA was used for stimulation of the tissue. Locke's solution was balanced with 95% O_2/5% CO_2 before use, and the pH adjusted to 7.2–7.4. The osmolarity of the saline solutions varied between 305 and 310 mOsM.

Retinal pieces were incubated in 500 μL of Locke's solution (CTR) or in Locke's solution containing one of the following glutamatergic agonists: 2 mM L-glutamate, 100 μM kainate (KA) or 100 μM NMDA at 37 °C under constant agitation for 30 min. In some experiments, retinal pieces were pre-incubated for 20 min with either DNQX (100 μM), a selective antagonist of non-NMDA ionotropic glutamatergic receptors or MK801 (10 μM), a non-competitive antagonist of NMDA receptors, followed by incubation with EAA agonists plus DNQX or MK801 for 30 min. Control retinas were incubated in Locke's solution only.

2.4. Tissue fixation

Immediately after incubation, retinal pieces were fixed by immersion in freshly prepared 4% paraformaldehyde (PA) in 0.16 M phosphate buffer (PB), pH 7.2, for 2 h. The tissue was then rinsed with PB and cryoprotected in a sequential gradient of sucrose (15 and 30%, w/v). After 24 h, retinas were mounted in OCT embedding medium (Sakura Finetek, Torrance, CA), frozen and cryosectioned. Sections perpendicular to the vitreal surface (10–14 μm) were collected on gelatinized slides. Retinal sections from control and from different treatment conditions were collected on the same glass slide. The slides were stored at −20 °C until processed for immunohistochemistry.

2.5. Immunohistochemical procedure

Slides were washed in PBS and non-specific binding sites were blocked for 1 h with 3% bovine serum albumin (BSA, Sigma). Sections were incubated overnight with primary antibodies against GABA (1:4000 dilution, raised in rabbit, Protos), NR1 (1:100 dilution, raised in mouse, Chemicon, Millipore) and GAD (1:5000 raised in sheep, Oertel, 1981). The next day, sections were rinsed in PBS and incubated with secondary biotinylated antibodies (goat-anti-rabbit, goat-anti-mouse or goat-anti-sheep, 1:200 dilution, Vector Laboratories) for 2 h. Further washes in PBS were made and the sections incubated with an avidin–biotin complex (Vectastain Elite, Vector) diluted 1:50 for 1.5 h. All reagents mentioned above were diluted in PBS plus 0.25% Triton X-100. Finally, the sections were rinsed in PBS three times for 10 min and reacted with SG cromogen (Vector) and hydrogen peroxide diluted in PBS. The reaction was allowed to proceed for 10 min, after which the sections were rinsed in PBS. After that, the slides were mounted with glycerol (40% in PB). Control sections were incubated with PBS in the absence of primary antibody, and no immunoreactivity was detected (data not shown).

2.6. Cell quantification

The quantification and comparison of the number of GABA immunoreactive cells was possible because in all experiments the controls and the experimental groups were incubated for immunohistochemistry on the same slide and treated by all reagents at the same time to minimize differences in the pattern of staining and background levels. All sections were examined, studied and photographed using differential interference contrast microscopy (Axioskop microscope, Zeiss). To quantify the number of GABA-positive cells, GABA-labeled and total unlabeled cells in retinal sections were counted and the percentage of labeled cells was calculated. Amacrine cells, labeled and unlabeled cell bodies were counted along the first two or three rows of cell bodies in the innermost part of the inner nuclear layer. The total number of amacrine cells was obtained from at least three sections for each retinal treatment from three to six animals. The data were statistically evaluated using one-way ANOVA and the Bonferroni post-test. The ANOVA was performed using the mean of the three triplicates.

2.7. Immunoblotting

Retinas from embryonic chicken were dissected out in calcium magnesium free (CMF) and homogenized with a tissue grinder in a solution composed of 20 mM Tris base, 10 mM $MgCl_2$, 600 μM $CaCl_2$, 500 μM EGTA, 1 mM DTT in the presence of a protease inhibitor cocktail (1 mM PMSF, 5 μg/mL aprotinin, 2 μg/mL leupeptin) and 0.05% Triton X-100. Protein concentration was estimated with the Bradford method. Samples were diluted in buffer composed of 10% glycerol (v/v), 1% β-mercaptoethanol, 3% SDS and 62.5 mM Tris base and boiled for 5 min. Approximately 40 μg of protein from each sample was used in 10% SDS-PAGE and transferred to nitrocellulose membranes (0.2 μm pore). The membranes were washed with 0.1% of Tween 20 Tris Buffered Saline (TTBS), blocked for 1.5 h with 5% skimmed milk and 1% BSA in TTBS, and incubated overnight with primary antibodies against GAD (1:5000 raised in sheep, Oertel) and GAT-1 (1:1000 raised in rabbit, Chemicon). The next day, membranes were rinsed in TTBS (10 min each) and incubated with specific secondary antibodies linked to peroxidase [anti-sheep (1:10,000) or anti-rabbit (1:10,000)] for 2 h. All reagents mentioned before were diluted in 1% BSA in TTBS. The immunoreaction was detected by chemiluminescence (ECL 1:40, Amersham). Blots were reprobed with anti-alfa tubulin (1:50,000 in TTBS, Sigma) or total ERK antibody for 2 h at room temperature, rinsed in TTBS and incubated with anti-mouse peroxidase conjugated secondary antibody (1:2000) for 1 h at room temperature. Following three TTBS washes (10 min each), the immunoreaction was detected with the ECL kit. Band intensities were analyzed by using Quantity One 4–6 software (Bio-Rad Laboratories Inc.). The absence of the primary antibody as a control resulted in no labeling (data not shown).

2.8. RT-PCR for GAD

The total RNA from E16 retinas was extracted with Trizol (Gibco), following the manufacturer's instructions. The RNA samples were quantified in a spectrophotometer and immediately incubated with DNAse I-RNAse free (Ambion) at 37 °C, for 10 min. After DNA digestion the material was re-extracted with a phenol–chloroform mixture and the final RNA concentration was determined again in the

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