American Journal of Obstetrics and Gynecology (2006) **194**, 681–6

ELSEVIER

American Journal of Obstetrics & Gynecology

www.ajog.org

Prevention of alcohol-induced learning deficits in fetal alcohol syndrome mediated through NMDA and GABA receptors

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Received for publication December 7, 2005; revised January 5, 2006; accepted January 7, 2006

KEY WORDS
Fetal alcohol syndrome
Alcohol
NMDA
GABA
Learning
Neurotrophic factors

Objective: Vasoactive intestinal peptide (VIP)-related peptides prevented the learning deficit in the offspring in a model for fetal alcohol syndrome. We evaluated whether the mechanism of the peptide protection included NR2B, NR2A, and $GABA_A\alpha_5$.

Study design: Timed, pregnant C57BL6/J mice were injected on gestational day 8 with alcohol (0.03 mL/kg), placebo, or alcohol plus peptides. Embryos were harvested after 6 hours, 24 hours, and on gestational day 18. Some of the litters were allowed to deliver, and the adult brains harvested after the offspring were tested for learning. Calibrator-normalized relative real-time polymerase chain reaction (PCR) was performed using primers for NR2B, NR2A, and $GABA_A\alpha_5$ with GAPDH standardization. Statistic: analysis of variance (ANOVA) and Fisher PLSD, $P < .05$ was considered significant.

Results: In the embryo, the peptides prevented NR2B rise ($P < .001$) at 6 hours, NR2B down-regulation ($P = .002$), and $GABA_A\alpha_5$ decrease ($P < .01$) on gestational day 18. In the adult, the peptides prevented NR2B down-regulation ($P = .01$) and NR2A up-regulation ($P < .001$).

Conclusion: VIP-related peptides prevented alcohol-induced changes in NR2B, NR2A, and $GABA_A\alpha_5$. This may explain, at least in part, the peptides' prevention of alcohol-induced learning deficits.

Prenatal alcohol exposure is the foremost preventable cause of neurobehavioral and developmental abnormalities.[1] Women who drink during pregnancy place themselves at risk for having a child with fetal alcohol syndrome (FAS) or fetal alcohol spectrum disorders (FASD), the latter characterized by the presence of learning deficits without the cranio-facial dysmorphology

Presented at the 26th Annual Meeting of the Society for Maternal Fetal Medicine, Miami, FL, January 30-February 4, 2006.

This work was supported by the Division of Intramural Research of National Institute of Child and Human Development.

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0002-9378/$ - see front matter © 2006 Mosby, Inc. All rights reserved.
doi:10.1016/j.ajog.2006.01.003

observed in FAS.[2] No level of alcohol consumption during pregnancy has been determined safe,[3] yet approximately 10% of pregnant women drink alcohol, and 2% engage in binge drinking or frequent use of alcohol during pregnancy.[4]

One electrophysiological mechanism for learning is long-term potentiation (LTP), which consists in a long-lasting enhancement of synaptic transmission. An essential first step for LTP induction is the activation of a subclass of post-synaptic glutamatergic receptors, the N-methyl-aspartate (NMDA) receptors. These are composed of different subunits that determine their functionality; among them, the NR2B subunit is the most critical for LTP induction and most sensitive to ethanol effects. While NR2B is prevalent during development and is associated with a higher plasticity of the brain circuits, the NR2A subunit is associated with aging and less prone to trigger LTP.[5]

The γ-aminobuyiric acid (GABA) receptor subclass $GABA_A$ plays a regulative role on LTP via inhibition of glutamate transmission and NMDA triggering LTP. The subunit $GABA_A\alpha_5$ plays an important role in the cognitive process of spatial learning.[6] In a mouse model for FAS, we have previously shown that during embryogenesis prenatal alcohol administration down-regulates NR2B and $GABA_A\alpha_5$, which are both excitatory and fundamental for central nervous system (CNS) development and morphogenesis. In adult brains exposed to alcohol *in utero*, NR2B down-regulation persists, while there is an up-regulation in NR2A.[5] Concomitantly, $GABA_A\alpha_5$ that in the adult matures inhibitory proprieties is up-regulated.[7] The net effect of these alterations is likely an inhibition of LTP, which may explain the learning impairment observed in alcohol-exposed mice.

Previously, we have shown that prenatal administration of vasoactive intestinal peptide (VIP)-related neuropeptides, NAP and ADNF-9, prevents alcohol-induced fetal death, growth restriction, microcephaly, and learning deficits in adulthood.[8,9] Our objective was to evaluate if the mechanism of the peptide protection was through prevention of the alcohol-induced changes in the NMDA and GABA receptors in a mouse model for FAS.

Material and methods

C57Bl6/J female mice (Harlan Sprague-Dawley, Inc, Indianapolis, IN) were kept in a 12-hour light/12-hour dark regimen, with food and water available at all times. The mice received humane animal care in compliance with the National Institutes of Health Guidelines for Care and Use of Experimental Animals. The protocol was approved by NICHD Animal Care and Use Committee. Six-week-old females (21-24 g) were mated with C57B16J males for 4 hours. The presence of vaginal plug was considered day 0 of pregnancy.

A well-described model for FAS was used, based on an acute high exposure to alcohol resulting in many of the characteristic features including growth restriction, teratogenicity, and embryo lethality.[10] On gestational day 8 (a typical mouse gestation is 21 days long), pregnant mice were treated (intraperitoneal) with 25% (0.03 mL/kg) ethyl alcohol, saline (placebo), or alcohol + peptides (peptides 30 minutes before alcohol). Pretreatment was performed with VIP-derived peptides activity-dependent-neurotrophic-protein (ADNP) and activity-dependent-neurotrophic-factor (ADNF), whose active fragments are, respectively, NAP (20 μg) and ADNF-9 (20 μg) (SynPep, Dublin, CA). NAP was diluted in 50 μL of dimethyl sulfoxide and diluted in filtered Dulbecco's phosphate-buffered saline (DPBS); ADNF-9 was dissolved and diluted in filtered DPBS. Because the animals receiving alcohol were incapacitated for approximately 6 hours after injection, food was withheld in all groups for 6 hours.

Embryos were explanted using microdissection from the uterus after 6 hours, 24 hours, and 10 days (embryonic day 18, E18) and placed in phosphate-buffered saline solution (PBS) and frozen in liquid N_2. Each gestational time-point included at least 3 samples, each sample included at least 2 to 3 embryos derived from 3 to 5 different litters.

To evaluate long-term alterations in expression, adult brain was obtained after evaluation of learning and memory. At least 3 litters per treatment group were allowed to deliver naturally; they were weaned on day 20 and adult male offspring were tested in the Morris Watermaze (control = 12, alcohol = 11, and alcohol + peptides = 15), as previously described.[9] Three adult brains per treatment of the same animals that underwent testing, each from a different litter, were collected separately and immediately frozen in liquid N_2.

For RNA extraction, samples were homogenized by using disposable micropestles to avoid cross-contamination. The samples were processed with SV Total RNA Isolation System (Promega, Madison, WI). A 5-μL aliquot was taken for spectrophotometric determination of RNA content. The remaining sample was stored at −80°C. Using 5 μg of total RNA, the reverse transcriptase (RT) reaction was performed (Perkin-Elmer Corp, Branchburg, NJ) in a final volume of 150 μL. Each RNA sample was run in duplicate. Negative controls included reverse transcription (RT) reactions omitting RNA or reverse transcriptase.

For polymerase chain reaction (PCR) application NR2B, NR2A, and GAPDH primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA). NR2B (GenBank accession number NM_008171) primer pair was designed using Primer3 software and. NR2B primer sequence was 5′-CCG CAG CAC TAT TAGA GAA CA-3′ (sense) and 5′-ATC CAT GTG TAG CCG TAG CC-5′ (antisense). NR2A (GenBank