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ALTERATIONS IN P-CREB ACTIVITY: A PATHWAY FOR FETAL ALCOHOL SYNDROME RELATED NEUROTOXICITY

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Abstract

Objective—Fetal alcohol syndrome (FAS) is the leading cause of a spectrum of preventable non-genetic learning and behavioral disorders. In adult (FAS) mice, we measured P-CREB staining in hippocampal subregions to evaluate a possible mechanism underlying FAS learning deficits.

Study Design—Pregnant C57Bl6/J mice were treated on gestational day 8 with alcohol or control (saline). After learning assessment, the offspring were perfused for immunohistochemistry, brain sections probed using SER 133 P-CREB antibody. Relative staining density was assessed using NIH Image software. Statistical analysis included ANOVA with $P < .05$ significant.

Results—In all hippocampal subregions P-CREB staining was greater in the control animals than in the alcohol treated group $p = < .0001$.

Conclusion—In utero alcohol exposure decreased P-CREB activity in hippocampal subregions of adult mice. The dentate gyrus had the most robust cumulative decrease in P-CREB staining, suggesting FAS adult learning deficits may correlate to enhanced dentate gyrus neurodegeneration.

Keywords

fetal alcohol syndrome; P-CREB; apoptosis; hippocampus; mouse

Background and Hypothesis

Prenatal alcohol exposure may result in fetal alcohol syndrome (FAS), which is characterized by mental retardation (1) craniofacial dysmorphism, growth restriction, as well as other neurobehavioral deficits (2,3). However, most children prenatally exposed to alcohol will not exhibit all classical characteristics of FAS, but instead have non-specific neurodevelopmental and behavior disorders that go under the term fetal alcohol spectrum disorder (FASD). It is estimated that one in 100 children born in the United States have fetal alcohol spectrum disorder (4), which may also include, but is not limited to, reduced brain mass, structural brain defects (5), childhood hyperactivity/attention deficit disorder (6) and depressive psychotic episodes in adulthood (7). Animal models have been developed to understand the mechanism and extent

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of damage in FAS. Gestational timing, brain structure vulnerability as well as dose and duration of alcohol exposure influence neurotoxic alcohol induced brain injury (8).

In FAS and FASD, there are known deficits in cognitive learning and spatial reference memory formation. These are thought to occur in the CA1 region of the hippocampus (9) with abnormalities in induction of long term potentiation (LTP), due to abnormal hippocampal formation. (10,11,12,13,14). We have shown that prenatal alcohol exposure induces learning deficits in adult mice using a Morris water maze to assess spatial learning and memory (15), and that the disruptive effect on learning and memory are in part mediated through the NMDA receptors(16). Other studies have demonstrated that alcohol disrupts LTP (17,18) in the hippocampus through disruption of hippocampal NMDA receptor' activation (19). Specifically, LTP is considered the cellular equivalent of long-lasting memory in paradigms of cognition and learning, (20) and it is initiated through NMDA receptor activation through phosphorylation of Cyclic AMP-response element binding protein (CREB).

The present study sought to determine if prenatal alcohol exposure alters P-CREB staining in mouse hippocampal subregions through adulthood, concomitant with impaired hippocampal-dependent spatial learning and memory,.

Materials and Methods

Breeding and prenatal ethanol treatment

C57Bl6/J female mice (Harlan Sprague-Dawley, Inc, Indianapolis, IN) were kept in a temperature controlled environment with 12-hour light/12-hour dark regimen and ad lib access to standard laboratory chow and water at all times. The mice received humane animal care in compliance with the National Institutes of Health (NIH) guidelines for care and use of experimental animals. The protocol was approved by National Institute of Child Health and Human Development (NICHD) Animal Care and Use Committee. Six-week-old females (21–24 g) were mated with C57-B16J males for 4 hours. Day 0 of the pregnancy was estimated to be initial presentation of a vaginal copulation plug. We used a well-established FAS animal model (21) that describes a high incidence of anomalies and demise in fetal mice exposed to ethanol during a critical period of organogenesis and neurogenesis. On gestational day 8, pregnant females were treated with intraperitoneal injection with 25% ethyl alcohol in saline (alcohol) or vehicle alone (saline solution) at 0.030 mL/g of body weight. This dose renders the alcohol treated animals incapacitated for approximately 6 hours after injection. To normalize for this effect food and water were removed in both groups for 6 hours post treatment. Pups delivered naturally and were reared by mothers until weaning on postnatal day 20. To ensure prenatal treatment anonymity, weaned pups were gender segregated, ear tagged and housed at four littermates per cage until the end of all experiments. We use behavioral testing that may be influenced by hormonal effects so we have utilized male mice to eliminate any potential hormonal effects encountered in female mice at this age. Learning and memory of adult male offspring was assessed through the Morris water maze on postnatal days 50–56. (22) with a learning deficit demonstrated in the alcohol exposed animals (15et al).

Tissue collection and sectioning

On post natal day 57, following Morris water maze assessment, 5 adults males from at least three different litters/treatment were anesthetized using 4% isoflurane, transcardially perfused with buffered 4% paraformaldehyde and the brains dissected and immersed in paraformaldehyde followed with equilibration in 10% then 20% sucrose. The tissue remained in 20% sucrose solution until it was sectioned. Using a cryostat, 20 um coronal sections were cut, mounted and frozen at –80° C until the immunohistochemical staining was performed.

P-CREB Immunohistochemistry

Representative slides were quenched with endogenous peroxidase activity for 30 minutes in 0.3% hydrogen peroxide in methanol. The slides were washed in PBS and blocked for 30 minutes at room temperature in 1.5% goat serum in PBS. Slides with adjacent sections were incubated overnight in 4°C in affinity purified rabbit monoclonal anti P-CREB antibody (1:50 Phospho-CREB (Ser133) (87G3) Rabbit mAb Cell Signaling Technology, Danvers, MA) and then incubated for 30 minutes at room temperature with anti-rabbit IgG biotinylated secondary antibody (1:200). Antibody localization was performed using avidin-biotin complex method (Vectastain *Elite*, Vector Laboratories, Burlingame, Calif), followed by diaminobenzidine. The sections were then dehydrated in ethanol and permanently mounted with Permount®. Alternate sections were processed with no primary antibody to detect nonspecific staining. The relative densities of staining in the dentate gyrus, CA3 and CA1 hippocampal regions were assessed using NIH Image software by 2 independent operators blinded to the treatment groups.

Absolute mean staining density from the sections of the alcohol and control, groups was used for the analysis. For each alcohol or placebo animal three separate sections were analyzed using a consistent and standard area. Four separate measurements were made and recorded collectively for each animal from hippocampal subpopulations from both hemispheres of the brains. The density of each measurement was obtained and the background density was subtracted. The absolute density of each was compared across the two groups. Statistical analysis was performed using ANOVA and a $P \leq 0.05$ was considered statistically significant.

Results

In all of the hippocampal subregions (DG, CA-1, CA-3), P-CREB-ser 133 staining was lower in alcohol treated group than in the control animals ($p < .0001$, Figure 1). In the controls, PCREB-ser 133 immunoreactivity was greatest in dentate granule cells, followed by the CA3 with the lowest staining in the in CA1 region. Although the degree of staining in every hippocampal subregion was significantly decreased in the alcohol exposed animals, the trend of staining was similar to the control, that is, dentate granule cells had the strongest staining and the CA1 area rendering the weakest staining. The dentate granule cells had the most robust cumulative decrease in P-CREB-ser 133 in control vs. alcohol staining as compared to the other hippocampal regions (Figure 2)

Discussion

We found that prenatal alcohol exposure resulted in a significant decline in P-CREB Ser 133 staining in the dentate gyrus, CA3 and CA1 regions of the hippocampus in adult offspring that also had impaired hippocampal-dependent learning. We found histological evidence of persistent hippocampal P-CREB neurochemical alterations from prenatal alcohol exposure that may underlie the impaired learning and memory throughout adulthood.

Hippocampal CREB activity is critical for long term memory formation and hippocampus-dependent learning (23). *In vitro* studies have shown that the cAMP-CREB cascade plays an important role in the differentiation and maturation of neurons during hippocampal neurogenesis (24). Similarly, *in vivo* studies demonstrated an upregulation of cell proliferation accompanied by activation of CREB in dentate gyrus granule cells (25) and a down regulation of cell proliferation and neurodegeneration both in the peripheral (26) and central nervous system (27) in CREB knockout mice.

One study demonstrated that the prolonged CREB phosphorylation on SER133 inhibited apoptosis (28) and in a perinatal hypoxic ischemic model in rats, adulthood impaired performance in learning and spatial memory task was associated with reduction in levels of

CA1 CREB (Ser 133) phosphorylation levels (29). A rat model (30) showed that P-CREB was diminished in the postnatal days 5 and 21 and adult hippocampus after 1 hour administration of alcohol; however, the long term effect, if any, of an acute binge alcohol exposure on P-CREB and/or a correlation to hippocampal dependent learning was not evaluated. Our findings are novel in that they reflect a reduction of P-CREB in every region of the hippocampus, specifically, with the dentate gyrus having the greatest decline, which may, at least in part, explain the concomitant hippocampal dependent learning deficit.

Previously we have shown that prenatal alcohol exposure alters adult NMDA receptor composition (16 et al). The NMDA receptor is a subtype of ionotropic glutamate receptor that functions as a cellular “gatekeeper”. NMDA receptor activation is the first step for Long Term Potentiation (LTP) which consists of an early phase (E-LTP) that requires covalent protein modifications, and a late phase (L-LTP), that is protein synthesis dependent, modulated by activation through phosphorylation of CREB (31). Phosphorylated CREB is essential for lasting late-phase long-term potentiation (L-LTP), which is considered to be important for the promotion of gene expression and synaptic spine formation (32, 33, 34, and 35). Thus CREB has a role in survival and differentiation during neurogenesis and that NMDA activation is coupled to P-CREB phosphorylation. P-CREB activation is a point of convergence in cascades enhancing neuronal survival, promoting proliferation and differentiation of specific neuronal populations, as well activating signaling pathways in learning and memory cascades. Persistent alterations in P-CREB may undermine these processes.

Throughout adulthood, hippocampal plasticity is enhanced through the formation and integration of newly formed neurons (36) including neural progenitor cells in subgranular zone (SGZ) of the dentate gyrus. Similar to developmental neurogenesis, adult neurogenesis is regulated by apoptosis. Chronic prenatal alcohol exposure, in a paradigm for FAS, (37) demonstrated a significant decrease in cell proliferation and neurogenesis in the dentate gyrus of adult rats (PND 57) and persistent defects in adult hippocampal neurogenesis (38) Prenatal alcohol exposure significantly compromised survival of newly generated dentate granule neurons and neuronal plasticity throughout adulthood. This is consistent with our findings of a robust decline in P-CREB in the dentate gyrus.

Prenatal alcohol exposure also has been shown to alter the number of spines from surviving CA1 pyramidal neurons which are less efficient in transmitting synaptic potentials (39,40) Given that the mossy fibers of the CA1 pyramidal field is considered the output portal for impulses traveling through the hippocampus and conductance of impulses through the hippocampus may be dampened and hindered by this “faulty wiring” resulting from altered impaired neurite arborization, this may result in impaired hippocampal-dependent learning. Therefore, it is possible that formation and incorporation of this less-than-optimal-performing neuronal circuitry compromises LTP, which is needed to ensure optimal performance of the hippocampus, and that a decline or a persistent alteration in hippocampal P-CREB levels may underlie the formation and incorporation of neurons with suboptimal circuitry or arborization.

A reduction in P-CREB may be the link between the deficits in adult hippocampal neurogenesis and/or cytoarchitectonical anomalies, and the alcohol-induced learning deficit in FAS. Impaired hippocampal dependent cognition and learning in FASD may be indicative of changes in LTP and reduced plasticity resulting from alterations or depletions in the dentate gyrus and CA1 regions, which are the respective input and output portals for information processed in the hippocampus’ synaptic circuitry.

Given the high prevalence of fetal alcohol spectrum disorder and that young adults diagnosed with FAS or FASD may have persistent mental handicap paired with the inability to sometimes live independently and with ongoing behavioral dysfunction, demonstrates that intrauterine

exposure to alcohol results in lifelong disability, which prevents young adults to reach their potential and function as independent individuals (41). These studies demonstrate the need to unravel the mechanisms and develop targeted strategies that will prevent and or treat outcomes resulting from FASD.

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Figure 1.

Absolute staining density of anti-P-CREB ser 133 in mice at PND 57 in the hippocampal subregions. *Bar* represents mean absolute staining $\pm$ SE. There is a decreased intensity of staining for anti-P-CREB ser 133r, $P < .001$, in the adult mice prenatally exposed *in utero* to alcohol as compared with control mice.

Figure 2. anti-P-CREB ser 133 immunostaining difference in **A**, 57-day-old alcohol exposed mouse hippocampus and **B**, 57-day-old control mouse hippocampus. Original magnification for anti-P-CREB ser 133 $\times 40$,