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High postnatal susceptibility of hippocampal cytoskeleton in response to ethanol exposure during pregnancy and lactation

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ABSTRACT

Ethanol exposure to offspring during pregnancy and lactation leads to developmental disorders, including central nervous system dysfunction. In the present work, we have studied the effect of chronic ethanol exposure during pregnancy and lactation on the phosphorylating system associated with the astrocytic and neuronal intermediate filament (IF) proteins: glial fibrillary acidic protein (GFAP), and neurofilament (NF) subunits of low, medium, and high molecular weight (NFL, NFM, and NFH, respectively) in 9- and 21-day-old pups. Female rats were fed with 20% ethanol in their drinking water during pregnancy and lactation. The homeostasis of the IF phosphorylation was not altered in the cerebral cortex, cerebellum, or hippocampus of 9-day-old pups. However, GFAP, NFL, and NFM were hyperphosphorylated in the hippocampus of 21-day-old pups. PKA had been activated in the hippocampus, and Ser55 in the N-terminal region of NFL was hyperphosphorylated. In addition, JNK/MAPK was activated and KSP repeats in the C-terminal region of NFM were hyperphosphorylated in the hippocampus of 21-day-old pups. Decreased NFH immunocontent but an unaltered total NFH/phosphoNFH ratio suggested altered stoichiometry of NFs in the hippocampus of ethanol-exposed 21-day-old pups. In contrast to the high susceptibility of hippocampal cytoskeleton in developing rats, the homeostasis of the cytoskeleton of ethanol-fed adult females was not altered. Disruption of the cytoskeletal homeostasis in neural cells supports the view that regions of the brain are differentially vulnerable to alcohol insult during pregnancy and lactation, suggesting that modulation of JNK/MAPK and PKA signaling cascades target the hippocampal cytoskeleton in a window of vulnerability in 21-day-old pups. Our findings are relevant, since disruption of the cytoskeleton in immature hippocampus could contribute to later hippocampal damage associated with ethanol toxicity.

Introduction

Alcohol use is characterized by central nervous system (CNS) intoxication symptoms, impaired brain activity, poor motor coordination, and behavioral changes (Gohlke, Griffith, & Faustman, 2008). These symptoms are largely ascribed to impaired CNS activity due to alcohol's effect on synthesis (Pietrzykowski et al., 2008), release (Roberto et al., 2006), and signaling (Wilkie et al., 2007) of neurotransmitters, including serotonin (LeMarquand, Pihl, & Benkelfat, 1994), glutamate (Prosser, Mangrum, & Glass, 2008), gamma-aminobutyric acid (Wallner, Hanchar, & Olsen, 2006), endocannabinoids (Perra, Pillolla, Luchicchi, & Pistis, 2008), and their receptors.

Clinical and experimental studies have demonstrated that the developing brain is particularly vulnerable to alcohol, and that drinking during pregnancy induces a wide spectrum of adverse effects in offspring, the most extreme of which is fetal alcohol syndrome (FAS), a condition characterized by microcephaly, neurologic abnormalities, facial dysmorphology, and pre- and postnatal growth retardation (Manzo-Avalos & Saavedra-Molina, 2010; Streissguth, Landersman-Dwyer, Martin, & Smith, 1980).

Neuropathologic abnormalities in FAS include neuronal–glial heterotopias, cerebellar dysplasia, hydrocephalus, microcephaly, and agenesis of the corpus callosum (Clarren, Alvord, Sumi, Streissguth, & Smith, 1978). Rat models of FAS have greatly contributed to unraveling some of the molecular dysfunctions

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related to the deleterious actions of ethanol in the brain. However, most of the underlying mechanisms remain elusive. The evidence indicates aberrant migration, decreased proliferation, and death of neuronal cells. However, during development the different brain regions present different windows of vulnerability to ethanol, depending on the growth spurt, ethanol levels, and duration of the insult. In this context, prenatal exposure to ethanol affects neuronal plasticity (Medina & Krahe, 2008) and the early postnatal expression of death-related proteins in the cerebral cortex of pups (Mooney & Miller, 2001). Accordingly, perinatal ethanol exposure results in the occurrence of depressive and anxiety-like behaviors in adult rats, and produces oxidative stress in the hippocampus and cerebellum of ethanol-exposed rats (Clarren et al., 1978). In addition, effects of ethanol on the function of glutamate receptors in the hippocampus, amygdala, and striatum have been described. Inhibition of the glutamatergic system may explain the consequences of severe ethanol intoxication, such as impairment of motor performance and the memory system, and may have a role in causing FAS (Möykkynen & Korpi, 2012).

Neurons are highly specialized in the transmission and processing of electrical and chemical signals, and their unique structural characteristics are provided by a neuron-specific cytoskeleton. The largest family of cytoskeletal proteins in mammalian cells is the superfamily of the intermediate filaments (IF). The IFs of mature neurons are mainly represented by the neurofilaments (NF). They assemble from three subunit polypeptides of low, medium, and high molecular weight, NFL, NFM, and NFH, respectively, and are responsible for determination of axonal diameter and transport of essential substances to nerve terminals (Holmgren, Bouhy, & Timmerman, 2012). This process is finely regulated via phosphorylation of lysine-serine-proline (KSP) repeats in the carboxyl-terminal domain of NFM and NFH. The majority of KSP repeats in rat/mouse NF tail domains are phosphorylated by mitogen-activated protein kinases (MAPK) (Veeranna et al., 1998), glycogen synthetase kinase 3 (GSK3) (Guan, Khatra, & Cohlberg, 1991), p38MAPK (Ackerley et al., 2004), and c-Jun N-terminus kinase 1 and 3 (JNK1/3) (Brownlees et al., 2000). Otherwise, phosphorylation sites located on the amino-terminal domains of the three NF subunits are the targets of second messenger-dependent protein kinases, such as cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase (PKCaM), and Ca^{2+}/diacylglycerol-dependent protein kinase (PKC) (Sihag & Nixon, 1990).

Glial fibrillary acidic protein (GFAP) is the IF of mature astrocytes, and vimentin is expressed in cultured or immature astrocytes (de Almeida, Funchal, Gottfried, Wajner, & Pessoa-Pureur, 2006). Phosphorylation of GFAP and vimentin located on the N-terminal head domain regulates assembly of GFAP and vimentin into filaments (Inagaki, Nakamura, Takeda, Nishimura, & Inagaki, 1994). GFAP expression is essential for normal white matter architecture and blood–brain barrier integrity, and its absence leads to late-onset CNS dysmyelination (Liedtke et al., 1996).

Disruption of the homeostasis of the endogenous phosphorylating system associated with the IFs has been substantially described by us in different brain structures of developing rat brain following exposure to neurotoxicants and metabolites (Heimfarth et al., 2013; Loureiro et al., 2010; Pierozan et al., 2012; Zamoner, Heimfarth, & Pessoa-Pureur, 2008; Zamoner, Pierozan, et al., 2008; Zanatta et al., 2012). Interestingly, Heimfarth, Reis, et al. (2012) have described that *in vivo* exposure of dams to the neurotoxin diphenyl ditelluride during pregnancy and lactation provoked misregulation of the cellular signaling targeting the cytoskeleton of striatum and cerebellum in their offspring, suggesting that placental transmission of a toxin could disrupt the cytoskeleton of developing brain.

The susceptibility of the cytoskeleton to acute (Loureiro et al., 2011) or chronic (Loureiro et al., 2012) ethanol exposure in cultured glial cells has been previously described by us. However, despite the increasing efforts to understand the molecular basis of ethanol neurotoxicity, little is known about the effects of long-term exposure to ethanol during pregnancy and lactation on the homeostasis of the cytoskeleton of immature brain. Therefore, in an attempt to better understand the toxicity of ethanol on the developing brain, we used an animal model of exposure to ethanol during pregnancy and lactation, which was able to provoke systemic toxicity. We evaluated the activity of the endogenous phosphorylation system associated with the IF-enriched cytoskeletal fraction of cerebral cortex, cerebellum, and hippocampus of the developing pups. We describe the higher susceptibility of hippocampal cytoskeleton of 21-day-old pups to the deleterious effects of ethanol and the possible role of MAPK and PKA pathways in this effect.

Materials and methods

Radiochemical and chemical compounds

[^{32}P]-Na_2HPO_4 was purchased from Comissão Nacional de Energia (CNEN), São Paulo, Brazil. Benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, and *bis*-acrylamide were obtained from Sigma (St. Louis, MO, USA). The chemiluminescence ECL kit peroxidase and the conjugated anti-rabbit IgG (A0545) were obtained from Amersham (Oakville, Ontario, Canada). Anti-NFL, anti-NFM (clone NN-18), anti-NFH (clone N52), and anti-phosphoNFH (clone NE14) monoclonal antibodies were purchased from Sigma Chemical Company (St. Louis, MO, USA). Anti-Erk (#9102), anti-phosphoErk, anti-pSAP/JNK (clone 98F2), anti-p38MAPK (clone A-12), anti-phospho p38MAPK, anti-PKAcα, anti-PKCaMII, anti-KSP repeats (clone NP1), anti-pSer55NFL, and anti-pSer57NFL antibodies were obtained from Cell Signaling Technology (USA). All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

Animals

Adult female Wistar rats (150–160 g) obtained from Central Animal House of the Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, were used in the experiments. The animals were maintained on a 12:12-h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room, with food and water or ethanol solution *ad libitum*. The experimental protocol followed the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) and was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul.

In vivo ethanol treatment

Female Wistar rats (32 animals) were randomized into two groups: control (drinking water) and alcohol (drinking water with ethanol) *ad libitum*. Ethanol treatment was carried out as previously described (Ojeda, Delgado-Villa, Llopis, Murillo, & Carreras, 2008). In brief, alcohol-fed adult females were started on tap water containing 5% v/v alcohol in the first week (initial time). The alcohol concentration was increased to 10% in the second week, 15% in the third week, and 20% in the fourth week. This solution was maintained for 4 weeks. After this period, females were mated. Pregnant females were placed in individual cages and were again provided 20% ethanol in drinking water and food *ad libitum* during pregnancy and lactation. The experiments were performed on the offspring at