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Research report

Melanin-concentrating hormone expression in the rat hypothalamus is not affected in an experiment of prenatal alcohol exposure

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ABSTRACT

Alcohol consumption during pregnancy can cause a "fetal alcoholic syndrome" (FAS) in the progeny. This syndrome is characterized by important brain defects often associated to a decreased expression of the morphogenic protein sonic hedgehog (Shh). The goal of this study was to verify if a FAS could modify the differentiation of hypothalamic neurons producing MCH. Indeed, the expression of this peptide and neurons producing it are dependent of a Shh controlled genetic cascade in the embryo. To address this question, female rats received a 15% ethanol solution to drink during pregnancy and lactation. Higher abortion rate and smaller pups at birth confirmed that descendants were affected by this experimental condition. MCH expression was analyzed by RT-qPCR and immunohistochemistry in embryos taken at E11 and E13, or in pups and young adults born from control and alcoholic mothers. MCH expression level, number of MCH neurons or ratio of MCH sub-populations were not modified by our experimental conditions. However, Shh expression was significantly lover at E11 and we also observed that hindbrain serotonergic neurons were affected as reported in the literature. These findings as well as other data from the literature suggest that protective mechanisms are involved to maintain peptide expressions and differentiation of some specific neuron populations in the ventral diencephalon in surviving embryos exposed to ethanol during pregnancy.

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1. Introduction

Ethanol consumption by the mother during pregnancy can lead to the onset of a fetal alcohol syndrome (FAS) in the progeny. FAS is the most severe state caused by early alcohol exposure. It involves facial dysmorphology, growth restriction and central nervous system disorders (Sokol et al., 2003). Indeed, the development of multiple brain structures is affected by the prenatal exposure to ethanol. Deficits include cortical thinning, ventricle enlargement, reduction in cerebellum, ganglion eminence, diencephalon, septal nucleus, hippocampus and amygdala volume (Zhou et al., 2004). These developmental perturbations have behavioral consequences, including alteration in spontaneous locomotor activity or deficient memory processing (Becker et al., 1996). This syndrome is a significant social problem and represents a major health issue in many countries, but the action mechanisms of ethanol on brain development are poorly understood.

A fetal morphogen protein named sonic hedgehog (Shh) plays an important role in cell proliferation, differentiation and embryonic patterning (Hammerschmidt et al., 1997). It is suggested that morphological changes associated to ethanol exposure during development resemble to that produced by aberrant Shh signaling pathway (Aoto et al., 2009). Indeed, Shh signal is essential for the prechordal plate cell survival and these cells are important for craniofacial development, one of the structures modified by FAS. Several studies have shown that Shh expression is modified by prenatal ethanol exposure during the early stage of development. Indeed, reduction of Shh expression in E11 embryos was found after ethanol injection to E9 pregnant mice (Chrisman et al., 2004). Furthermore, administration of ethanol to chick embryos affects expression of Shh and Shh administration partially remedies to the detrimental effects of fetal ethanol exposure (Ahlgren et al., 2002). Exposure of zebrafish embryos to ethanol during gastrulation modifies the hedgehog signal transduction. This alteration leads to developmental defects close to those observed for FAS (Li et al., 2007). During the development, Shh is expressed in the ventral midline and notochord and is necessary to induce the

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differentiation of several very important cell populations in the neural tube (Marti et al., 1995; Briscoe and Ericson, 2001). For instance, Shh is necessary for the production of serotonergic cells in the rhombencephalon (Ye et al., 1998). Serotonin neurons are also affected by alcohol exposure in the embryo (Zhou et al., 2001, 2005). Within the hypothalamus, differentiation of the melanin-concentrating hormone (MCH) neurons is also the result of a Shh-controlled genetic cascade (Szabo et al., 2009; Croizier et al., 2011). The distribution of these neurons is stereotyped in rodents, and their chemical phenotype as well as their projection patterns are determined by early developmental processes (Brischoux et al., 2002; Cvetkovic et al., 2004; Croizier et al., 2010, 2011).

Most children with FAS or fetal alcohol spectrum disorders (FASD) have growth retardation, hyperactivity, trouble eating and sleeping. MCH neurons are involved in each of these functions. For example, mice lacking MCH or MCHR1 are lean and hyperactive, while MCH neurons play a role in sleep/wake behavior (Shimada et al., 1998; Takase et al., 2014). Therefore, these neurons may be affected by alcohol exposure during development.

The goal of the present study was thus to investigate the impact of prenatal ethanol consumption by the mother on MCH expression and MCH neuron differentiation in the progeny.

2. Materials and methods

2.1. Animals

All animal use and care protocols were in accordance with institutional guidelines, and investigators authorized. All experiments were approved by the institutional ethic committee (protocol number: 2012/006-PYR). Sprague-Dawley rats were obtained from Janvier, Le Genest-Saint-Isle, France. They were housed under 12 h light: 12 h dark cycle at a constant room temperature and had free access to standard laboratory diet and water, except when indicated.

2.2. Alcohol treatment

The experimental procedure was based on Shilko's study (Shilko et al., 2010). The female rats were divided in two groups. The experimental group consisted of 11 rats receiving *ad libitum* 15% ethanol solution instead of water during one month before mating and during pregnancy and lactation. The control group consisted of 11 rats receiving water during the same period. Ingested volumes as well as body weight gain were monitored. After 30 days of adaptation, male rats were slipped into the female cages during the whole 12 h dark period, every day until all female be pregnant. The time of conception was documented by sperm-positive vaginal smear examined on the morning following the mating night (embryonic day 0, E0). Timed-pregnant rats were anesthetized with an intraperitoneal injection (IP) of pentobarbital (50 mg/kg, CEVA) and embryos at E11 and E13 (corresponding to stages of MCH neurons genesis and differentiation) were taken. Furthermore, the descendants of three experimental and three control females were born and litters reduced at seven per female after birth. However, six descendants of one experimental litter died before brain extraction. Ten weanling (1 month, 6 controls and 4 experimented) and 13 adult rats (2 month, 8 controls and 5 experimented) were anesthetized with IP injection of pentobarbital and fresh brains were extracted. Finally, 13 adult rats (2 month, 7 controls and 6 experimented) were perfused with 0.9% NaCl followed by ice-cold 4% paraformaldehyde (PFA, Roth) fixative in 0.1 M phosphate buffer (PB) pH 7.4. All the adult rats were weighted at day 42, after the separation of rat with their mother, and at day 62, before the brain perfusion.

2.3. Real-time PCR and western blotting

For western blot and real-time PCR experiments, fresh whole heads of E11 or E13 embryos or the fresh brains of weanling and adult rats were frozen by immersion in isopentane at −74 °C using the Snap-Frost™ system (Excilone, France).

2.3.1. Western blot

The whole head of embryos at E11 and E13 were ground and lysed using a lysis buffer [10 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 10 mM $MgCl_2$, 0.5% NP-40, 1% Triton X-100, protease inhibitor cocktail (Sigma)]. Then 50 μg of tissue lysates were resolved on a 12.5% polyacrylamide gel in running buffer (25 mM Tris base, 200 mM glycine and 0.1% SDS) at 20 mA using Biorad Power Pack 1000. Proteins were transferred onto Immun-Blot PVDF 0.2 μm membranes (Biorad) for 1 h 30 min at 4 °C at 200 mA in Western Blot transfer buffer (25 mM Tris base, 200 mM glycine and 10% methanol). Membranes were subsequently blocked with 5% skim milk powder in Tris-buffered saline with 0.1% Tween 20 (TBS-T). Membranes were blotted in anti-Shh (goat polyclonal, Santa Cruz Biotechnology, 1:200) and anti-Actin (rabbit polyclonal, Sigma, 1:10,000) antibodies diluted in TBS-T supplemented with 0.5% skim milk. The secondary antibodies (horseradish peroxydase-conjugated anti-rabbit IgG or anti-goat IgG, 1:10,000) were diluted in TBS-T containing 0.5% skim milk powder. Binding of antibodies to membranes was detected by Enhanced Chemiluminescence Plus Reagent (ECL Plus, GE Healthcare Life Sciences), according to the manufacturer's protocol. Image Lab software (Bio-Rad Laboratories) was used to quantify protein band density.

2.3.2. RT-qPCR

The whole head of embryos at E11 and E13 and the brain of weanling and adult rats descending from alcoholic or control mothers were ground and total RNAs were extracted using TRIzol® Reagent (Life Technologies) with DNAse (Fermentas) treatment following the manufacturer's instruction. Then, cDNAs were synthesized from 1 μg total RNA using oligo(dT), dNTP and MMLV Reverse Transcriptase (Fermentas). Real-time PCR was performed with gene specific primers for pMCH (Pissios et al., 2007). A 1 μL aliquot of cDNAs (diluted 1:10) was subjected to real-time RT-PCR using SyBR Green One step RT-PCR reagents (Applied Biosystems) and TaqMan® Universal Master Mix (Applied Biosystems). All reactions were run in triplicate, and results were normalized over β-actin expression. StepOne software v2.1 (Applied Biosystems) was used to quantify RNA.

2.4. Histological analysis

Perfused adult brains were post-fixed in a 4% PFA solution while embryonic heads were fixed in a 1% PFA overnight at 4 °C. Both tissue samples were then cryoprotected by saturation in a 15% sucrose solution (Sigma) in 0.1 M PB and frozen as stated above. Embryonic whole heads were cut in serial sections (10 μm thick) using a cryostat microtome (Microm), placed on gelatin-coated slides (Roth) and stored at −40 °C. Adult brains were cut in coronal 30 μm thick serial sections, collected in a cryoprotector solution (1:1:2 glycerol/ethylene glycol/phosphate buffered saline or PBS) and stored at −20 °C.

2.4.1. In situ hybridization

The Shh RNA probe was given by Dr C.C. Hui (Nieuwenhuis et al., 2007). Frozen sections of E11 embryos were post-fixed in 4% PFA during 10 min at room temperature and rinsed briefly in 1× PBS then in 5× SSC (standard sodium citrate buffer). Slides were incubated for 4 h in prehybridization buffer at 56 °C and

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