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Intrauterine Ethanol Exposure Results in Hypothalamic Oxidative Stress and Neuroendocrine Alterations in Adult Rat Offspring.

Korami Dembele¹, Xing-Hai Yao¹, Li Chen¹,³ and B. L. Grégoire Nyomba¹,².

Departments of Internal Medicine¹ and Physiology², University of Manitoba, Winnipeg, Manitoba, Canada and ³Department of Pharmacology, Jilin University, Changchun, China.

Short Title: Hypothalamic Oxidative Damage in Ethanol Exposed Rat offspring.

Key Words: Fetal growth restriction, neuropeptides, protein carbonyls, lipid peroxides, superoxide dismutases

Correspondence: B. L. G. Nyomba, MD, Ph.D., FACE
Diabetes Research Group, University of Manitoba
715 McDermot Avenue Room 834
Winnipeg, Manitoba, Canada R3E3P4
Tel: (204) 789-3962.
Fax: (204) 789-3987.
E-mail: bnyomba@cc.umanitoba.ca
ABSTRACT

Prenatal ethanol (EtOH) exposure is associated with low birth weight, followed by increased appetite, catch-up growth, insulin resistance and impaired glucose tolerance in the rat offspring. Because EtOH can induce oxidative stress, which is a putative mechanism of insulin resistance and because of the central role of the hypothalamus in the regulation of energy homeostasis and insulin action, we investigated whether prenatal EtOH exposure causes oxidative damage to the hypothalamus, which may alter its function. Female rats were given EtOH by gavage throughout pregnancy. At birth, their offspring were smaller than those of non-EtOH rats. Markers of oxidative stress and expression of neuropeptide Y and pro-opiomelanocortin (POMC) were determined in hypothalami of postnatal day-7 (PD7) and 3-months old (adult) rat offspring. In both PD7 and adult rats, prenatal EtOH exposure was associated with decreased levels of glutathione and increased expression of MnSOD. The concentrations of lipid peroxides and protein carbonyls were normal in PD7 EtOH-exposed offspring, but were increased in adult EtOH-exposed offspring. Both PD7 and adult EtOH-exposed offspring had normal neuropeptide Y and POMC mRNA levels, but the adult offspring had reduced POMC protein concentration. Thus, only adult offspring pre-exposed to EtOH had increased hypothalamic tissue damage and decreased levels of POMC, which could impair melanocortin signaling. We conclude that prenatal EtOH exposure causes hypothalamic oxidative stress, which persists into adult life and alters melanocortin action during adulthood. These neuroendocrine alterations may explain weight gain and insulin resistance in rats exposed to EtOH early in life.
INTRODUCTION

It is now well accepted that adverse events during pregnancy are associated with obesity, insulin resistance and type 2 diabetes in adult offspring. This association was first suspected in epidemiologic studies (5, 50, 53) and confirmed in various animal models of intrauterine growth restriction (IUGR) (6, 58, 65, 66), the best known of which uses protein malnutrition (47). Ethanol (EtOH) consumption during pregnancy can lead to a spectrum of defects that include fetal alcohol syndrome (FAS) and less severe abnormalities known as fetal alcohol effects, the characteristics of which include various degrees of IUGR, abnormal facial features, and central nervous system anomalies (17). The prevalence of FAS is elevated in populations with lower socioeconomic status (7), where type 2 diabetes is also common (43). We know of only one study in humans where glucose intolerance was associated with FAS (10). In this study, 3 out of 7 prepubertal children with FAS had abnormal oral glucose tolerance tests with increased plasma insulin response. We and others have shown that EtOH ingestion during pregnancy in amounts corresponding to human chronic drinking (63, 69) can lead to IUGR and is associated with insulin resistance, hyperlipidemia, and glucose intolerance in adult rat offspring (12, 13, 23, 40, 48). At the cellular level, these rats have increased intramuscular and intrahepatic triglycerides (14) and impaired insulin signaling with reduced muscle protein kinase Cζ activation (15), glucose transporter-4 translocation (12, 13) and glucose uptake (23), and increased liver expression of gluconeogenic genes (16, 68).

In parallel with insulin resistance, rats born with IUGR undergo a period of catch-up growth or fat deposition. Catch-up fat deposition is associated with increased food intake
(12, 14, 30) and diminished energy expenditure (19). Remarkably, rats undergoing catch-up growth can be insulin resistant at a time point when their body fat is comparable to that of controls. This has been attributed to suppressed thermogenesis (i.e. a more efficient energy use) for the purpose of sparing glucose for catch-up fat, via a coordinated induction of muscle insulin resistance and adipose tissue insulin sensitivity (11, 19). Hypothalamic peptides, which regulate appetite and energy homeostasis, also regulate insulin action and play an important role in glucose metabolism (11, 34). Recent studies suggest that oxidative stress is an important factor contributing to obesity, insulin resistance and type 2 diabetes, and rats with IUGR have been shown to have increased systemic oxidative stress with damage to liver and skeletal muscle (27, 49, 57). Because heavy EtOH exposure causes oxidative stress (18), we hypothesized that rats exposed to EtOH in utero may have oxidative damage to the hypothalamus, altering hypothalamic neuropeptides, which may provide an explanation for catch-up fat deposition and insulin resistance.

**METHODS**

**Materials**

Ethanol was obtained from pharmaceutical services of the Health Sciences Centre (Winnipeg, MB, Canada). Trizol, SuperScript reverse transcriptase, Taq DNA polymerase and Oligo(deoxythymidin)e primers were obtained from Life Technologies (Rockville, MD) or purchased from GIBCO BRL (Gaithersburg, MD, USA). cDNA primers were synthesized by Life Technologies. Electrophoresis and electroblotting consumables were from BioRad (Hercules, CA). Antibodies were from Santa Cruz
Biotechnology (Santa Cruz, CA) or Stressgen (Vancouver, BC). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Penzberg, Germany). ECL chemiluminescence kit was obtained from Amersham Pharmacia (Piscataway, NJ). Isopropyl alcohol and methanol were from Fisher Scientific (Nepean, ON, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

**Animals**

Rat offspring exposed to EtOH in utero were generated as described before (12-14) with minor modifications. Briefly, time-mated Sprague-Dawley rats were randomly divided into 3 weight-matched groups. One group (EtOH) was given EtOH, 2 g/kg (36%) by gavage twice daily from day 1 to 22 of gestation, and the other 2 groups were given the same volume of water instead of EtOH. Among the latter, one group (PF) was pair-fed the amount of chow consumed by the EtOH group, whereas the other group (GAV) was given free access to chow. With this method, we have obtained a peak alcoholemia of 115 mg/dl and 70 mg/dl at 2 and 4 hours after ingestion, respectively (12), similar to levels found in sober alcohol users (63). Feed intake of EtOH-treated dams was <10% that of controls, but weight gain during pregnancy, litter size, and perinatal mortality were similar to controls. Our model differs from the model used by Pennington and colleagues (23, 48) in that they administered EtOH in a liquid diet, and pups were surrogate fostered to non-treated dams. Cross-fostering is used to prevent a delay in pups weight gain while suckling from their own undernourished mothers or to study the effects of chronic alcoholism during pregnancy or lactation separately (67). We did not use cross-fostering because dams in our model show no signs of malnutrition compared with
normal (12, 14). Furthermore, it has been suggested that fostering may confound the effects of prenatal EtOH exposure (28). Because of similar litter sizes between groups, we also refrained from culling pups as litter size manipulation has been shown to alter neuronal activity and the level of nutrition received during lactation (20).

At postnatal day 7 (PD7) and at 12 weeks (adult) of age, one to two offspring, randomly taken per litter per treatment group were fasted for 2 h and 15 h, respectively, and they were sacrificed. Because of reports of detrimental effects of the gavage procedure on offspring development (60, 62), a group of normal adult male rats were used as non-gavaged controls (NORM). All the animal studies were approved by the Committee for Animal Use in Research and Teaching of the University of Manitoba.

Collection of the Hypothalamus

The hypothalamus was collected as described by Hanson et al (31). After decapitation, the brain was removed within 60 seconds and placed in a prechilled brain matrix (Harvard Instruments). A 3-mm coronal section was cut using the caudal optic chiasm as the rostral boundary of the section. The sectioned brain piece was placed on a prechilled glass plate with the rostral side up, and the medial hypothalamus was dissected using the top of the 3rd ventricle as the dorsal boundary and the lateral hypothalamic sulci as the lateral boundaries. This hypothalamic block was then cut horizontally in half, and the basal portion corresponding to the medial-basal hypothalamus was used.

Preparation of Tissue Homogenate
Hypothalami were homogenized in an ice cold 1 ml buffer containing 20 mM Tris, pH 7.4, 140 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM Na$_3$VO$_4$, 2 mg/ml benzamidine, 1 mM PMSF, and a protease inhibitors cocktail (1 tablet/10 mL). Tissue lysates were centrifuged for 10 min at 12,000xg. The supernatant was used for studies described below. Protein was measured using BioRad protein assay method.

**Western Blotting**

Homogenates (50 µg protein/lane) were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. The blots were blocked with 5% dry milk and incubated overnight at 4°C with the following antibodies: anti-MnSOD (0.85 µg/ml), -Cu/ZnSOD (1 µg/ml), -POMC (1 µg/ml), -MC3R (1 µg/ml), and -MC4R (1 µg/ml). The blots were then washed in Tris-buffered saline (TBS)-Tween for 15 min, incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody at 1:3000 for 1 h at room temperature, and washed in TBS-Tween for 15 min. Immune complexes were detected using the ECL chemiluminescent detection kit after exposing the blots to a Kodak X-OMAT AR (XAR-5) film. Protein contents were quantified by densitometry using NIH Image software and the reading was corrected for that of the positive control used as standard (12).

**Immunoprecipitation**

Tissue homogenates (500 µg of protein) were immunoprecipitated overnight at 4°C with 15 µg/ml of anti-hydroxynonenal (HNE) antibody and protein A-sepharose beads.
The immunoprecipitates were washed twice with phosphate-buffered saline, redissolved in 20 µl of electrophoresis buffer, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The blots were incubated with anti-POMC, -MC3R or -MC4R antibodies to detect HNE-modification of these proteins. After washing, the blots were incubated with a second antibody and immunoreactive bands were visualized and quantified as described. To further ensure equal protein loading, the blots were stripped in a Tris-buffer, pH 6.7, containing 2% SDS and 100 mM mercaptoethanol, and reprobed with the appropriate primary antibody.

**Oxidative Products and Enzymatic Activities**

Lipid peroxides were measured as described by Ohkawa et al. (46) in the same extraction medium as that for the antioxidant enzyme assays. Tissue lysate (500 µL) was mixed with 500 µL thiobarbituric acid (1% in 50 mM NaOH) and 500 µL of 25% HCl. The samples were then heated in a boiling water bath for 10 min and, after cooling, were extracted with 1.5 mL of n-butanol-pyridine (1/15, vol/vol). The mixture was centrifuged at 12,000 g for 10 min and the absorbance of the supernatant was determined at 532 nm. Thiobarbituric acid reacts with products of lipid peroxidation, mainly malondialdehyde, producing a colored compound.

Protein carbonyls were determined by the method of Levine (37). Proteins were precipitated with 20% trichloroacetic acid and, after centrifugation, the precipitate was resuspended in 2,4-dinitrophenylhydrazine (10 mM). After incubation for 60 min in the dark, 0.5 mL of 20 % trichloroacetic acid was added and samples were centrifuged for 3 min. Pellets were washed twice in ethanol:ethyl acetate with incubation for 10 min each
time. The final precipitates were resuspended in 6 M guanidine solution, centrifuged for 3 min and insoluble debris removed. The maximum absorbance (360–390 nm) of the supernatants was read against appropriate blanks (water, 2 M HCl) and the carbonyl content was calculated using the molar absorption coefficient of 22,000 M⁻¹ cm⁻¹.

Total glutathione (GSH) was measured by the method of Anderson (3) by following the formation of 5-thio-2-nitrobenzoic acid from 5,5'-dithiobis-2-nitrobenzoic acid at 412 nm in the presence of glutathione reductase (GSH-R) and NADPH. Glutathione peroxidase (GPx) activity was determined by following the rate of NADPH oxidation at 340 nm in the presence of GSH-R, tert-butyl hydroperoxide and reduced GSH (26). One unit of GPx activity is equal to moles NADPH oxidized per minute per mg protein. GSH-R activity was determined by following the NADPH-dependent reduction of oxidized GSH at 340 nm (9). One unit of GSH-R activity is equal to moles NADPH oxidized per minute per mg protein. Catalase activity was determined by measuring the decomposition of hydrogen peroxide into H₂O at 230 nm (1). A standard curve was established using purified catalase. One unit of catalase activity equals moles hydrogen peroxide degraded per minute per mg protein.

**Polymerase Chain Reactions**

RT-PCR assays were performed as described (13, 16). Total RNA was isolated by tissue homogenization in Trizol reagent, and the first-strand cDNAs were synthesized using SuperScript reverse transcriptase and oligo(deoxythymidine) primers. The reverse transcription products were amplified by PCR using Taq DNA polymerase and specific primers (Table 1). Each reaction also included two oligonucleotide primers to amplify
GAPDH as an internal control. The PCR procedure consisted of denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Amplified cDNAs were separated by 1% agarose gel electrophoresis, and PCR products were stained with ethidium bromide. The conditions were such that the product amplification was in the exponential phase and the assay was linear with respect to the amount of input RNA. Photography of the gel was performed in a Kodak DC 120 Zoom Digital Camera. The bands were compared by using the Kodak 1D Image Analysis Software.

Statistics

Statistical analyses were performed with SPSS software (version 11.0 for Windows, SPSS Inc., Chicago, IL). Data were log-transformed when required, to achieve normality before analysis. Differences in the means of 3 groups were tested by one-way ANOVA with Tukey’s-b post hoc test. Two-tailed unpaired t test was used to compare means of 2 groups. Values are expressed as means ± SE. P<0.05 was considered significant.

RESULTS

Animal characteristics

The characteristics of the animals used in this study are shown in Table 2. The rat dams had similar intergroup weights before and at the end of gestation. Prenatal EtOH exposure resulted in IUGR as reflected by decreased birth weight in EtOH offspring compared with GAV offspring [group effect: F(2,44)=3.24, P<0.05], but there was no significant difference in birth weight between EtOH and PF or between PF and GAV groups. At PD7, body weight was still lower in EtOH offspring compared with the other
2 groups [group effect: F(2,38)=6.76, P<0.005]. At 3 months of age, however, EtOH offspring were heavier than the other 2 groups, whose weights remained similar [group effect: F(2,26)=6.42, P<0.005]. NORM rats used to control for the effect of gavage had similar body weight (455±12 g, n=7) as GAV rats. Because the purpose of pair-feeding was to control for possible weight loss in EtOH dams, which did not occur in this study and also because there was no metabolic difference between PF and GAV groups (see below), pair-feeding studies were limited to PD7 rats only.

**Oxidative stress in rats exposed to EtOH in utero.**

The excess weight gain of adult EtOH compared with GAV offspring led us to investigate the presence of hypothalamic dysregulation by oxidative stress in these rats (Table 3). We found that GSH levels in EtOH offspring were ~2-fold lower than in GAV and ~2.8 fold than in NORM rats [group effect: F(2,12)=4.72, P<0.05]. The levels of lipid peroxides [group effect: F(2,14)=24.3, P<0.0001] and protein carbonyls [group effect: F(2,9)=11.39, P<0.005] were elevated in EtOH offspring compared with both GAV and NORM rats. The activity of the anti-oxidant enzyme GPx was decreased [group effect: F(2,13)=4.67, P<0.05], whereas GSH-R and catalase activities were normal, in EtOH offspring (Table 3). None of these oxidative markers was significantly different between GAV and NORM rats. We found a dissociation in the expression of dismutases in that MnSOD expression (Fig.1) was increased in EtOH rats [group effect: F(2,7)=4.94, P<0.05], whereas Cu/ZnSOD expression was unaffected (not shown). We next investigated whether oxidative stress was present earlier in the life of EtOH offspring. Because no difference was seen between GAV and NORM in adult rats,
however, no NORM group was used in PD7 rats. GSH concentrations in EtOH PD7 rats were ~2-fold lower than in GAV and ~1.5-fold lower than in PF [group effect: F(2,15)=5.07, P<0.05, Table 3], but the levels of lipid peroxides and protein carbonyls were similar between the 3 groups. Among the anti-oxidant enzymes, GPx activity was increased [group effect: F(2,15)=3.67, P<0.05], catalase activity was normal, whereas GSH-R activity was decreased [group effect: F(2,15)=4.01, P<0.05] in EtOH compared with GAV rats (Table 3). The expression of both MnSOD and Cu/ZnSOD was normal in PD7 EtOH offspring. All of the markers of oxidative stress were similar between PF and GAV PD7 groups.

**Hypothalamic Neuroendocrine Alterations**

We investigated whether hypothalamic neuropeptides involved in appetite and body weight regulation are altered after prenatal EtOH exposure. In adult rat offspring, the levels of hypothalamic NPY mRNA (arbitrary units) were similar between EtOH (0.96 ± 0.19, n=3) and GAV (0.88 ± 0.25, n=3, p=NS). POMC mRNA levels were also similar between EtOH (1.71 ± 0.51, n=5) and GAV (2.09 ± 0.63, n=4, p=NS) offspring. Similar results were found in PD7 rats (not shown). Because markers of lipid and protein oxidation indicated macromolecular damage in adult rats only, further assessment of hypothalamic peptides was carried out in this age group. POMC protein levels were significantly decreased in adult EtOH rats compared with NORM and GAV offspring [group effect: F(2,14)=5.49, P<0.05, Fig.2]. Because POMC mRNA levels were normal, we hypothesized that the decrease of POMC protein was likely due to a post-translational modification caused by oxidative damage. We found a slight increase of HNE-adducts of
POMC in EtOH rats, but this was not statistically significant (Fig.2). The expression of MC3R and MC4R was not different between groups (not shown).

**DISCUSSION**

In this study, we demonstrate the presence of oxidative stress in the hypothalamus of rat offspring after prenatal exposure to EtOH. We found changes in various molecules involved in cellular redox balance, and there were differences in markers of oxidative stress between PD7 and adult rat offspring (Table 3). Whereas hypothalamic GSH levels were decreased in both PD7 and adult rat offspring compared with their respective controls, lipid and protein oxidation as determined by lipid peroxides and protein carbonyls, respectively, was increased only in adult offspring. Thus, although oxidative stress was already present in early life, tissue damage was delayed and manifested in adult rats only.

Oxidative stress results from an imbalance between the formation of reactive oxygen species (ROS) and anti-oxidant defense mechanisms. It is known that EtOH exposure induces production of ROS, which has been widely documented in liver and some brain cells. GSH is an important endogenous anti-oxidant, which reduces hydrogen peroxide and lipid hydroperoxides. The decrease in GSH levels was, therefore, expected to result in oxidative damage caused by EtOH-generated ROS or hydroperoxides in both adult and PD7 rats, or even more so in the latter because of a more recent EtOH exposure compared to the adults. Superoxides are cleared by the oxygen free radical scavengers SODs, which convert superoxides into hydrogen peroxide, which is then degraded by catalase and GPx. MnSOD is predominantly mitochondrial, whereas Cu/ZnSOD is predominantly
cytoplasmic. Both groups of rats had normal expression of Cu/ZnSOD, but increased expression of MnSOD, suggesting that mitochondrial superoxides were probably adequately cleared and were not the direct cause of the increased lipid peroxidation found in the adult rats. The activities of GPx and GSH-R, which are coupled in the recycling of oxidized and reduced forms of GSH, were differentially regulated in the rat age groups. After prenatal EtOH exposure, GSH-R activity was decreased in PD7, but normal in adult rats, whereas GPx activity was increased in PD7 rats, but decreased in adult rats.

Since GSH-R and GPx are coupled in the recycling of GSH, the decreased GPx activity in the adult rat offspring indicates an increase in the levels of hydroperoxides, which may arise from the reaction of superoxide with SODs, the activity of several enzymes, or the oxidation of endogenous substances. The decreased GPx activity can be interpreted as a lack of compensatory response to restore depleted levels of GSH which would remove hydroperoxides, and therefore as an additional indication for increased oxidative pressure. This inadequate anti-oxidant compensation appeared to inadequately protect the adult offspring against oxidative damage. Hydrogen peroxide clearance is a critical step for the removal of ROS in neurons, and exposure to hydrogen peroxide results in oxidative stress in these tissues. Since GPx transforms hydroperoxides into water, the difference in GPx activity between the 2 age groups suggests that peroxides were adequately cleared in PD7, but not in adult rat offspring. Hydroperoxides accumulating due to this GPx insufficiency may have caused damage in the adult rats. Furthermore, the duration of the oxidative insult resulting from a longstanding GSH insufficiency was likely instrumental in tissue damage in adult rats.
It is unclear why GPx and GSH-R activities were differently regulated between the rat age groups. GSH functions as a substrate for GPx and GSH-R, and decreased GSH levels may result in a decrease of GPx or an increase of GSH-R activity. It has been suggested that chronic EtOH feeding reduces the entry of cytosolic GSH into mitochondria and that the decreased mitochondrial pool size of GSH results in reduced GPx activity (25). Perinatal EtOH is known to increase systemic oxidative stress in developing organs, particularly the liver and the brain (18, 32, 33, 41, 55). There have been reports of EtOH-associated oxidative stress in the hippocampus and other brain regions, where postnatal EtOH decreased GSH content and increased lipid peroxides and protein carbonyls, with brain region and age dependent differences in EtOH sensitivity and the response of antioxidant enzymes (29, 32, 33, 41, 55). For example, Heaton et al (32) reported that 7 days-old rats postnatally exposed to EtOH had delayed SOD and catalase responses compared with 21 days-old rats and concluded that older brains have protective mechanisms against EtOH. Our study does not reflect direct EtOH effects, because the results were obtained several days and months after intrauterine EtOH exposure. Persistence of oxidative stress several weeks after EtOH exposure has been reported in postpartum rat dams (54). Thus, in the presence of oxidative stress, two mitochondrial anti-oxidant enzymes (MnSOD and GPx) increased in PD7 rats, whereas only MnSOD increased in adult rats. These differences could explain the difference in lipid and protein peroxidation, but the exact reason for this age-dependent difference in oxidative damage is unknown. Thus far, we know of no data on prenatal EtOH-associated oxidative stress in the hypothalamus, and no brain oxidative stress has been reported before in adult offspring prenatally exposed to EtOH. We speculate that EtOH-induced oxidative stress undergoes cycles of self-
reinforcement and perpetuation through activation of cytokines (35, 36). In addition, the progressive development of hyperglycemia and hyperlipidemia (14) may add to the reinforcement cycle, which causes oxidative tissue damage.

Another important finding is that of decreased POMC levels in the hypothalamus of EtOH exposed adult rats. Recent studies indicate that the hypothalamic melanocortin system is important in the regulation of energy balance and insulin action. In rodents, genetic or pharmacologic manipulations causing impairment of melanocortin signaling lead to hyperphagia, obesity, insulin resistance and various degrees of glucose intolerance, while stimulation of melanocortin signaling results in increased weight loss and insulin sensitivity (24, 42, 44). In humans, polymorphic changes in POMC or MC4R genes have been described that predispose to obesity (22, 39, 64). Whereas POMC mRNA levels were normal in rats exposed to EtOH, POMC protein levels were decreased. The levels of NPY and the melanocortin receptors MC3R and MC4R were normal. To our knowledge, this is the first in vivo demonstration of the hypothalamic melanocortin system down-regulation in adult rats by prenatal EtOH exposure. However, prenatal EtOH has been reported to decrease POMC mRNA levels in the anterior pituitary of 7-21 days old male rat offspring (2). POMC expression has also been shown in primary culture of hypothalamic neurons to be increased by acute, but suppressed by chronic EtOH exposure (21). The length of EtOH exposure may explain why a direct EtOH exposure decreased hypothalamic POMC mRNA in some studies, while increasing it or having no effect in others (4, 52, 56, 59, 70). Our results, which are a consequence of chronic gestational EtOH exposure, are in agreement with these reports. Our results are also in line with reports in adult rats showing that a 3-week EtOH diet reduced α-MSH levels
immunoreactivity in the hypothalamus and the pituitary (51). These results further suggest that POMC down-regulation by prenatal EtOH involves post-translational mechanisms. We found an HNE-modification of POMC, which, although not statistically significant, could be an indication that this protein may be modified by oxidative stress. Such modification has not been described before in adult rats exposed to EtOH in utero and needs to be confirmed by further studies.

Because of reports of detrimental effects of the gavage procedure during pregnancy or early development on offspring brain function (60, 62), a group of normal age-matched male rats were used as non-gavaged controls. We found no effect of gavage on the parameters studied, in agreement with most (38, 41, 61, 62), but not all (60, 62), previous reports. Torres and Zimmerberg (60) reported that gavage treatment of rat dams during pregnancy affected neuromotor development in the offspring. In a study by Tran and Kelly (62) where pregnant rats and their pups were gavaged daily, the results were mixed in that gavage reduced offspring serotonin level in the hypothalamus, but had no effect on norepinephrine concentration in the hippocampus (62). In other studies by the same authors, gavage treatment of rat pups had no effect on hippocampal oxidative stress (41) or cerebellar function (61). Others found no effect of gavage on pups weight gain (38). The reasons for these discrepancies remain unclear, but may be related to the gavage vehicle, dose, volume and duration or to the overall experience with the procedure (8, 45, 62). Thus, the evidence that maternal gavage causes hypothalamic oxidative stress in offspring is still lacking.

We conclude that prenatal EtOH exposure induces oxidative damage in the hypothalamus and reduces POMC levels, which by reducing melanocortin signaling
could explain previously documented alterations of food intake, body weight and insulin sensitivity in rats exposed to EtOH in utero (12-15, 68).

ACKNOWLEDGEMENTS

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<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Size (bp)</th>
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</thead>
<tbody>
<tr>
<td>Cu/Zn-SOD S</td>
<td>TTCGAGCAGAAGGCAAGCGGTGAA</td>
<td>396</td>
</tr>
<tr>
<td>Cu/Zn-SOD AS</td>
<td>AATCCAATCACACCACAAGCCAA</td>
<td></td>
</tr>
<tr>
<td>Mn-SOD S</td>
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<tr>
<td>Mn-SOD AS</td>
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<tr>
<td>POMC S</td>
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<td>POMC AS</td>
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<tr>
<td>GAPDH AS</td>
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</table>

S, sense primer; AS, antisense primer; bp, base pairs.
Table 2: Body weights

<table>
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<tr>
<th></th>
<th>Pregnant dams</th>
<th></th>
<th>Offspring</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 21</td>
<td>PD1</td>
<td>PD7</td>
<td>Adult</td>
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<tr>
<td>GAV</td>
<td>271 ± 7</td>
<td>405 ± 9</td>
<td>6.3 ± 0.2</td>
<td>14.9 ± 0.4</td>
<td>450 ± 4</td>
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<tr>
<td></td>
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<td>(n=8)</td>
<td>(n=12)</td>
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<tr>
<td>PF</td>
<td>297 ± 12</td>
<td>425 ± 14</td>
<td>5.9 ± 0.2</td>
<td>15.4 ± 0.5</td>
<td>463 ± 6</td>
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<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=17)</td>
<td>(n=14)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>EtOH</td>
<td>296 ± 17</td>
<td>410 ± 16</td>
<td>5.7 ± 0.1*</td>
<td>13.1 ± 0.5*</td>
<td>490 ± 11*</td>
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<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=18)</td>
<td>(n=15)</td>
<td>(n=11)</td>
</tr>
</tbody>
</table>

Data is shown as means±SE. The number of rats is shown in parenthesis. *P<0.05 vs. other groups.
Table 3: Markers of oxidative damage in the hypothalamus of rat offspring

<table>
<thead>
<tr>
<th></th>
<th>GSH (nmol/mg tissue)</th>
<th>Lipid peroxides (nmol/mg tissue)</th>
<th>Protein carbonyls (nmol/mg protein)</th>
<th>GPx (mU/min/mg protein)</th>
<th>GSH-R (mU/min/mg protein)</th>
<th>Catalase (µmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PD7 rats</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>GAV</td>
<td>92.5 ± 16.4</td>
<td>0.051 ± 0.003</td>
<td>7.2 ± 0.5</td>
<td>61.2 ± 10.0</td>
<td>37.2 ± 6.4</td>
<td>5.79 ± 1.00</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(8)</td>
<td>(4)</td>
<td>(6)</td>
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<td>(3)</td>
</tr>
<tr>
<td>PF</td>
<td>69.7 ± 10.0</td>
<td>0.054 ± 0.002</td>
<td>7.1 ± 1.3</td>
<td>85.2 ± 13.0</td>
<td>40.7 ± 5.8</td>
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</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(2)</td>
<td>(5)</td>
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<tr>
<td>EtOH</td>
<td>41.9 ± 7.3*</td>
<td>0.053 ± 0.002</td>
<td>7.2 ± 0.3</td>
<td>88.5 ± 6.0*</td>
<td>23.5 ± 3.0*</td>
<td>3.87 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(8)</td>
<td>(4)</td>
<td>(7)</td>
<td>(7)</td>
<td>(4)</td>
</tr>
<tr>
<td><strong>Adult rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NORM</td>
<td>1.33 ± 0.24</td>
<td>0.61 ± 0.17</td>
<td>4.5 ± 1.4</td>
<td>80.4 ± 9.5</td>
<td>2.66 ± 1.15</td>
<td>0.74 ± 0.32</td>
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<tr>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>GAV</td>
<td>1.04 ± 0.18</td>
<td>0.56 ± 0.01</td>
<td>9.6 ± 1.1</td>
<td>125.8 ± 19.3</td>
<td>1.04 ± 0.14</td>
<td>0.94 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(6)</td>
<td>(4)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.48 ± 0.20*</td>
<td>1.35 ± 0.04*</td>
<td>19.4 ± 4.3*</td>
<td>68.5 ± 11.8#</td>
<td>1.56 ± 0.14</td>
<td>0.60 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(6)</td>
<td>(3)</td>
<td>(6)</td>
<td>(6)</td>
<td>(8)</td>
</tr>
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</table>

Data is shown as means±SE. The number of rats is shown in parenthesis. #P=0.05 vs. GAV, * P<0.05 vs. other groups
Table 4: Directional changes in oxidative markers in the hypothalamus of prenatally EtOH-exposed rats

<table>
<thead>
<tr>
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<th>PD7</th>
<th>Adult</th>
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<tr>
<td>Lipid peroxides</td>
<td>≈</td>
<td>↑</td>
</tr>
<tr>
<td>Protein carbonyls</td>
<td>≈</td>
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</tr>
<tr>
<td>Glutathione</td>
<td>↓</td>
<td>↓</td>
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<tr>
<td>GSH-R</td>
<td>↓</td>
<td>≈</td>
</tr>
<tr>
<td>GPx</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Catalase</td>
<td>≈</td>
<td>≈</td>
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<tr>
<td>MnSOD</td>
<td>≈</td>
<td>↑</td>
</tr>
<tr>
<td>Cu/ZnSOD</td>
<td>≈</td>
<td>≈</td>
</tr>
</tbody>
</table>

↓, decreased; ↑, increased; ≈, unchanged vs. controls.
Figures Legend

Figure 1. Western blots of MnSOD and Cu/ZnSOD in the hypothalamus of rat offspring. This figure shows representative immunoblots and densitometric analyses of the blots. Each age and treatment group included 3-4 rats. *P<0.05 vs. other 2 groups.

Figure 2. POMC protein and its HNE-modification detected by western blots in the hypothalamus of rat offspring, n=6 rats/group. The figure shows representative immunoblots and densitometric analyses of the blots. Immunoprecipitation with anti-HNE was followed by Western blot with anti-POMC. *P<0.05 vs. other 2 groups.


54. Ren J, Roughhead ZK, Wold LE, Norby FL, Rakoczy S, Mabey RL and Brown-Borg HM. Increases in insulin-like growth factor-1 level and


66. **Vickers MH, Breier BH, Cutfield WS, Hofman PL and Gluckman PD.** Fetal origins of hyperphagia, obesity, and hypertension and postnatal


Figure 1
Figure 2