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Prepubertal ethanol exposure alters hypothalamic transforming growth factor- α and erbB1 receptor signaling in the female rat

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Abstract

Glial derived transforming growth factor- α (TGF α) activates the erbB1/erbB2 receptor complex on adjacent glial cells in the medial basal hypothalamus (MBH). This receptor activation stimulates the synthesis and release of prostaglandin-E₂ (PGE₂) from the glial cells, which then induces the release of prepubertal luteinizing hormone releasing hormone (LHRH) secretion from nearby nerve terminals; thus, showing the importance of glial-neuronal communications at the time of puberty. Ethanol (EtOH) is known to cause depressed prepubertal LHRH secretion and delayed pubertal development. In this study, we assessed whether short-term EtOH exposure could alter the hypothalamic glial to glial signaling components involved in prepubertal PGE₂ secretion. Immature female rats began receiving control or EtOH diets beginning when 27 days old. The animals were killed by decapitation after 4 and 6 days of treatment and confirmed to be in the late juvenile stage of development. Blood and brain tissues were collected for gene, protein and hormonal assessments. Real-time PCR analysis demonstrated that EtOH did not affect basal levels of erbB1 gene expression in the MBH. Expression of total erbB1 protein was also unaffected; however, the EtOH caused suppressed phosphorylation of erbB1 protein in the MBH at both 4 and 6 days ($p < 0.01$) as revealed by Western blotting. Phosphorylation and total protein levels of erbB2 receptor were not affected by EtOH exposure. Because this receptor is critical for PGE₂ synthesis/release, which mediates the secretion of LHRH, we assessed whether *in vivo* EtOH exposure could affect the release of PGE₂. EtOH exposure for 6 days suppressed ($p < 0.01$) basal levels of PGE₂ released into the medium. The effects of 4 and 6 day EtOH exposure on gene and protein expressions of TGF α , an upstream component in the activation of erbB1/erbB2, were also studied. The levels of TGF α mRNA were increased markedly at 4 days ($p < 0.001$), but declined to near basal levels by 6 days in the EtOH-treated animals. The EtOH caused increases in TGF α protein expression at both 4 ($p < 0.001$) and 6 ($p < 0.01$) days; hence, suggesting that the EtOH inhibited release of the peptide. We confirmed this inhibition by showing decreased ($p < 0.01$) TGF α released from MBHs incubated *in vitro* following 6 days of EtOH exposure *in vivo*. Thus, these results demonstrate that EtOH is capable of interfering with hypothalamic glial to glial signaling processes involved in prepubertal PGE₂ secretion.

Keywords

Ethanol; Hypothalamus; TGF α ; erbB1; Puberty

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INTRODUCTION

The onset of puberty relies on complex interactions within the hypothalamus that lead to increased secretion of luteinizing hormone-releasing hormone (LHRH). This increased level of secretion at puberty is due to a decrease in inhibitory neurotransmitters such as gamma aminobutyric acid and opioid peptides (Terasawa, 1999; Terasawa and Fernandez, 2001), as well as the enhanced developmental responsiveness to excitatory neurotransmitters such as excitatory amino acids (Claypool et al., 2000; Urbanski and Ojeda, 1990), norepinephrine (Sarkar, et al., 1981), transforming growth factor alpha (TGF α ; Ojeda et al., 1990), leptin (Dearth et al., 2000), insulin like growth factor -1 (Hiney et al., 1991,1996), and the kisspeptins (Navarro et al., 2004). Actions of these neurotransmitters at puberty are dependent upon the maturation and interactive participation of neuronal circuits and glial cells. The glial cells within the medial basal hypothalamus (MBH) facilitate prepubertal LHRH release by producing cell adhesion molecules and growth factors. Growth factors of glial origin are now recognized as key components in the functional control of mammalian puberty via hypothalamic glial-neuronal communication (Cardona-Gomez et al., 2000; Ma et al., 1997; Ojeda et al., 2000, 2008). TGF α and epidermal growth factor (EGF) are glial derived peptides that signal through a specific tyrosine kinase receptor known as erbB1. While both peptides can stimulate LHRH release (Ojeda et al., 1990), TGF α has been shown to play the more pivotal role in the glial-neuronal signaling process by which glial cells, through their close association with LHRH nerve terminals in the MBH, regulate LHRH neuronal function during puberty in rodents (Ma et al., 1994a, Ojeda et al., 2008) and primates (Ma et al., 1994b). In this regard, it is now recognized that once this growth factor is released, it binds to and activates the erbB1 receptor on adjacent glial cells (Ma et al., 1994c). Binding of the ligand (TGF α or EGF) to the erbB1 receptor results in dimerization with the co-receptor, erbB2, with activates the signaling cascade that results in the synthesis and release of prostaglandin-E₂ (PGE₂). PGE₂ released from the glial cells then binds to its receptor on nearby LHRH nerve terminals (Rage et al., 1997) and ultimately, stimulates release of the LHRH peptide (Ma et al., 1997; 1999; Ojeda et al., 1990). Therefore, glial-neuronal communications play an important role with regard to LHRH secretion at puberty (Ma et al., 1997; Cardona-Gomez et al., 2000; Ojeda et al., 2000; 2008).

Previous studies have shown that chronic ethanol (EtOH) exposure to females alters puberty-related hormones (for review see Dees et al., 1995; 2009) and delays sexual maturation in both rodents (Bo et al., 1982; Dees and Skelley, 1990, Emanuele et al., 2002) and primates (Dees et al., 2000). These effects in both species are due to the hypothalamic action of EtOH to diminish LHRH secretion (Dees et al., 2005; Hiney and Dees, 1991; Hiney et al., 2003; Dissen et al., 2004; Ogilvie and Rivier, 1997). Understanding the mechanism by which EtOH alters prepubertal LHRH secretion is important. Because PGE₂ plays such a well known and critical role in controlling LHRH secretion, investigators have used prepubertal female hypothalami incubated *in vitro* to assess the effects of EtOH on this prostanoid. In this regard, acute EtOH exposure has been shown to block PGE₂ release induced by several substances (Hiney and Dees, 1991; Hiney et al., 1998; Lomniczi et al., 2000), including EGF (Hiney et al., 2003). The effects of chronic EtOH exposure on hypothalamic TGF α /erbB1-erbB2 signaling in prepubertal animals have not yet been studied. Since this signaling pathway is important within the MBH for glial-mediated PGE₂ synthesis and release at the time of puberty, we investigated whether chronic EtOH exposure to prepubertal female rats alters the synthesis and secretion of TGF α , and whether this drug affects the signaling mechanisms of the erbB1-erbB2 receptor complex.

METHODS

Animals and Surgery

Eighteen-day pregnant female rats of the Sprague-Dawley line were purchased from Charles River (Boston, MA) and allowed to deliver pups normally in the Texas A&M University lab animal facility. Female pups were weaned at twenty-one days of age and housed three per cage under controlled conditions of light (lights on, 0600h; lights off, 1800h) and temperature (23 C), with ad libitum access to food (Harland Teklad Diet, Madison, WI) and water. All procedures performed on the animals were approved by the University Animal Care and Use Committee and in accordance with the NAS-NRC Guidelines for the Care and Use of Laboratory Animals. Each animal was anesthetized at 23 days of age with 2.5% tribromoethanol (0.5ml/60g body weight) and was surgically implanted with a permanent intragastric cannula by a procedure that has been described previously (Dees et al., 1984). All animals were allowed to recover from surgery for 4 days prior to the beginning of the experiments.

Short-term EtOH Exposure Regimen

When the rats were 26 days old, they were weighed and divided into three groups. Group 1 received a 5% EtOH-liquid diet, and group 2 received the companion isocaloric control liquid diet (Bioserve, Inc., Frenchtown, NJ). Group 3 served as an additional set of controls that consisted of animals that were cannulated and maintained on lab chow and water, ad libitum, throughout the experiment. On day 27, the animals began receiving their respective diet regimens as described previously (Dees and Skelley, 1990; Srivastava et al., 1995) and modified only slightly (Srivastava et al., 2009). Briefly, the liquid-diets were administered in such a manner that 6 ml of the respective diet was injected via the intragastric cannula (four injections of 1.5 ml each) equally dispersed at approximately three hour intervals over the lights-on period, and then 30 mls of diet was available ad libitum (bottle) during the lights-off period. To provide an adequate food supply for these immature growing animals, beginning on day 28 and again on days 30 and 32, the amount of diet injected via the cannula was increased by 0.5 ml per injection. Also, at these same times, the amount of diet made available each night in the bottle was increased to 35, 40, and 50 ml, respectively. Thus, by day 33, each animal was receiving the maximum of four intragastric injections (3.0 ml each) of diet during the lights-on period and 50 ml of diet, ad libitum, during the lights-off period (Srivastava et al., 2009). Half of the animals received their respective diets for four days (days 27–31), and the other half of the animals for six days (day 27–33). We have reported previously that this feeding regimen enabled those animals in the liquid-diet control group to grow at exactly the same rate as the animals in the chow-fed control group (Dees and Skelley, 1990; Srivastava et al., 2002; 2009). Additionally, this method ensures not only that all liquid diet-fed animals received approximately the same number of calories per day, but also that all of the EtOH-treated animals received the same amount of EtOH per day.

Tissue Collection

On the morning of day 31 (4 day EtOH exposure) or 33 (6 day EtOH exposure), all animals were weighed and killed by decapitation 1.5 hours after their last gastric infusion. The animals were confirmed to be in the late juvenile stage of pubertal development as assessed by criteria described previously (Dees and Skelley, 1990; Srivastava et al., 1995). These animals all had closed vaginae, small uteri, and no intraluminal fluid. Since none of the EtOH-treated rats had entered the peripubertal period, it was important that only late juvenile controls be used for comparisons with the EtOH-treated animals; hence, the few control animals that had entered the peripubertal period by day 33 were not utilized. Serum was stored at –80 C until assayed for blood alcohol concentrations (BAC). The brains were removed and the MBH block of tissue was dissected by vertical cuts along the posterior side

of the optic chiasm and the anterior edge of the mammillary bodies and laterally at the tuberoinfundibular sulci of the hypothalamus. Tissues were frozen on dry ice at -80°C until analyzed by Real-time PCR or Western blot analysis. The experiment above was repeated three times in order to achieve the number of animals in each of the three groups.

Isolation of Total RNA

Total RNA was initially isolated from the brain tissues by homogenizing in TRIzol Reagent (Invitrogen, CA). The homogenates were further extracted for RNA using QIAGEN RNeasy kit and treated with RNase-free DNase I according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). Total RNA was quantitated spectrophotometrically by absorbance at 260 nm in a model Smartspec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The integrity of the RNA was checked by the visualization of the ethidium bromide-stained 28S and 18S ribosomal RNA bands.

Reverse Transcription and Real-time Quantitative PCR

Reverse transcription was performed with 1 μg of total RNA from each sample according to the instruction manual using oligo (dT) and SuperScript III First-strand Synthesis System (Invitrogen Life Tech., CA). Real-time PCR was done on an ABI PRISM 7500 sequence detection system as described previously (Hiney et al., 2009). Briefly, PCR reactions were performed in 25 μl reactions containing 2 μl cDNA, 500nM primer pairs and 1X SYBR green PCR master mix in 96-well plates. Primers for each gene were designed according to the guidelines of Applied Biosystems with the help of Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Each primer was checked for the absence of cross-reactivity by BLAST search. Primers, specific for the house keeping gene, β -actin, were also included in all reactions separately under the same experimental conditions to normalize for the amount of RNA in the initial reverse transcription reaction. A reaction without reverse transcriptase was also carried out to ensure the specificity of the expected amplicons. The primers for the PCR reactions are as follows: rat TGF α [GeneBank accession M31076], forward, 5'-TTCTCCATGGGCCGTCTAAC-3', reverse, 5'-TGTTTCCCCTTGTCATTTTGTA-3' (product size 69 bp); rat erbB1 [GeneBank accession NM_031507], forward, 5'-CCTCCTGGGCATCTGTCTGA-3', reverse, 5'-GGCAACCATAGGGCATGAGT-3' (product size 65 bp); rat β -actin [GenBank accession NM_031144], forward, 5'-ATGCCCCGAGGCTCTCTT-3', reverse, 5'-TGGATGCCACAGGATTCCA-3' (product size 57 bp). The PCR cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. PCR product purity was confirmed by dissociation curve analysis for each gene at the end of the PCR reaction. In this regard, each amplicon yielded a single peak and did not show any peak when the template was not included in the PCR reaction. Additionally, to check the specificity of the primers, each PCR-generated DNA product was electrophoresed onto 2% agarose gel containing ethidium bromide, which showed a single band of the desired size. This ruled out the possibility of any unexpected formation of primer-dimers. The relative levels of expression for each gene were determined from the raw data as described by Hettinger et al., (2001) using delta-delta C_T method.

Immunoblotting

Brain tissues were homogenized in a buffer containing 1X PBS, 1% Igepal CA 630, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM sodium orthovanadate at 4°C . The homogenates were incubated on ice for 30 minutes and centrifuged at 12,000 $\times g$ for 15 min. The concentration of total protein in the supernatants was then determined by the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standard. Immunoblot analysis was performed by solubilizing an equal amount of protein (100 μg) in sample buffer containing

25 mM Tris Cl, pH 6.8, 1% SDS, 5% β -mercaptoethanol, 1mM EDTA, 4% glycerol, and 0.01% bromophenol blue and electrophoresed through a 12% SDS-PAGE for TGF α and 8% SDS-PAGE for erbB1 and erbB2 under reducing conditions. The separated proteins were electrophoretically transblotted onto polyvinylidene difluoride membranes. Membranes were blocked at room temperature in the presence of 5% nonfat dried milk/0.05% Tween 20 in PBS (pH 7.4) for 3 hr and subsequently incubated at 4°C overnight with rabbit polyclonal antibody specific for TGF α (1:500; Abcam Inc, Cambridge, MA) or rabbit polyclonal antibody specific for either phospho-erbB1 (Tyr1068, 1:1000; Cell Signaling Technology, Inc. Danvers, MA) or phospho-erbB2 (Tyr1248, 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or nonphosphorylated erbB1 (1:1000; Cell Signaling Technology, Inc.) or nonphosphorylated erbB2 (1:500; Santa Cruz Biotechnology, Inc.). Following incubation, membranes were washed in PBS buffer containing 0.05% Tween-20 and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:10000; Abcam Inc, Cambridge, MA) for 2 hr at room temperature. The specific proteins were detected with the enhanced chemiluminescence method (Western Lightning Plus-ECL, Perkin Elmer, Shelton, CT) and quantified with NIH Image J software version 1.42 (National Institute of Health, Maryland). Subsequently, membranes were stripped with 100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris HCl, pH 6.7 and reprobed with a goat anti-human polyclonal antibody to the β -actin and anti-goat horseradish peroxidase –conjugated secondary antibody, to normalize for the amount of sample loading. Following washing, the visualization and quantitation of β -actin protein was done as described above.

Effects of EtOH Exposure on TGF α and PGE₂ Secretion in vitro

Animals were treated from days 27 to 33 (6 day EtOH exposure) as described above and on day 33, brains were removed from animals in all three groups. The MBH was dissected under a stereomicroscope as described above and incubated as described previously (Lee et al., 2007) with minor modifications. Briefly, each MBH was incubated in a vial containing 150 μ l of Locke's Buffer (2mM Hepes, 154 mM NaCl, 5.6 mM KCl, 1 mM MgCl₂, 6 mM NaHCO₃, 10 mM glucose, 1.25 mM CaCl₂, and 1 mg ml⁻¹ BSA, pH 7.4) inside a Dubnoff shaker (80 cycles/min) at 37 °C in an atmosphere of 95% O₂ and 5% CO₂. After a 60 min equilibration period, the medium was discarded and replaced with fresh medium for 60 minutes to determine basal release. Subsequently, these media were removed and saved in two aliquots for assessment of TGF α and PGE₂ release. Following completion of the experiment, MBH fragments were weighed. The TGF α release was measured in 15 μ g total protein of the media by Western immunoblotting as described above. PGE₂ release was measured by enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked Immunosorbent Assay (ELISA)

PGE₂ levels were measured from the media using an ELISA kit (Caymen Chemical Inc., Ann Arbor, MI) according to the manufacturer's instructions. The sensitivity of the assay was 5 pg/ml. Values are expressed as pg PGE₂/mg tissue/60 minutes.

Serum Alcohol Analysis

Blood alcohol concentration (BAC) was assessed from samples collected on days 31 and 33 from the trunk blood collected at the end of the experiment. Serum was transferred to microcentrifuge tubes for analysis as described previously (Dees et al., 2005) by an enzymatic method using a diagnostic kit purchased from Genzyme (Oxford, CT).

Statistical Analysis

Differences between groups in the experiment were analyzed by using ANOVA with post hoc testing, with the student-Newman-Keuls multiple range test. Because no significant

differences were detected in any of the assessments measured in the two control groups, their data were combined for presentation in figures to simplify comparative descriptions. The differences between the combined control groups and the ALC-treated group were then analyzed using Student's t-test. These statistical tests were conducted with INSTAT software (GraphPad Software, San Diego, CA). Probability values less than 0.05 were considered significantly different.

RESULTS

As in our previous studies (Dees and Skelley, 1990; Dees et al., 1990; Srivastava et al., 1995; 2002; 2009) differences were not detected between the chow-fed and liquid diet-fed control animals with regard to body weight gain, gene or hormonal measurements; thus, values from these two groups were combined and presented as a single control group in the following figures to simplify comparative descriptions. BACs in the animals exposed to EtOH for 4 days were 188 ± 8 mg/dl, and those exposed for 6 days were 210 ± 6 mg/dl.

Since the activation of the erbB1/erbB2 receptor complex is critical for a cascade of events leading to the release of PGE₂, we first assessed the effect of EtOH on the gene and protein expression of erbB1. Real-time PCR analysis revealed that EtOH exposure did not affect basal levels of erbB1 gene expression in the MBH at either 4 or 6 days (Fig. 1A&B). Also, the protein expressions of total erbB1 were not changed in the MBH following EtOH exposure for 4 and 6 days (not shown); however, EtOH caused a marked suppression ($p < 0.01$) in the phosphorylation of erbB1 protein in the MBH at both 4 (Fig. 2A&B) and 6 days (Fig. 2C & D). Since dimerization of erbB1 and erbB2 is necessary for the ligand to activate the signaling pathway, we assessed whether or not EtOH exposure affected the phosphorylation of erbB2 and detected no changes in total and phosphorylated forms of erbB2 (data not shown). Because of the suppressed phosphorylation of erbB1 following short-term EtOH exposure and because this receptor is critical for PGE₂ synthesis/release, we investigated whether *in vivo* EtOH exposure for 6 days would result in suppressed PGE₂ released from MBHs incubated *in vitro*. In this regard, we observed suppressed ($p < 0.01$) basal levels of PGE₂ released into the medium (Fig. 3).

To determine effects upstream to the erbB1 receptor, we assessed the effects of 4 and 6 day EtOH exposure on gene and protein levels of TGF α , the principal ligand for erbB1. Real-time PCR analysis of MBH tissue demonstrated that the levels of basal TGF α mRNA were increased ($p < 0.001$) by 4 days in the EtOH-treated animals compared with control animals (Fig. 4A). After 6 days of EtOH exposure (Fig. 4B) the level of TGF α gene expression was still modestly elevated ($p < 0.05$) over the control animals; however, the expression levels had declined markedly compared to those observed after 4 days of exposure. The levels of TGF α protein were increased after both 4 ($p < 0.01$) and 6 ($p < 0.001$) days of EtOH exposure (Fig. 5A–D). To assess whether the increased hypothalamic content of TGF α protein was due to an inhibition in its release, we assessed the basal *in vitro* release of the peptide from MBH explants following *in vivo* exposure to EtOH. In this regard, Western blot analysis of the incubation medium revealed that animals exposed to EtOH exhibited a suppression ($p < 0.01$) in the release of TGF α (Fig. 6).

DISCUSSION

It is now well established that EtOH acts within the hypothalamus to cause suppressed release of LHRH in both prepubertal and adult rats (Ching and Negro-Villar, 1988; Dees et al., 1983; 1990; 1995; 2005; 2009; Hiney and Dees, 1991; Ogilvie and Rivier, 1997) and primates (Dissen et al., 2004). Studies to discern the mechanism of this EtOH action are important for understanding how this drug disrupts puberty and reproductive function. For

years it has been known that PGE₂ plays a major role in the LHRH secretory process in prepubertal animals (Ojeda et al., 1986; 1988). We showed previously that EtOH is capable of inhibiting PGE₂ secretion and thereby suppressing LHRH release (Hiney and Dees, 1991); however, until now there has been a void in our knowledge regarding the mechanism by which EtOH affects PGE₂ in the prepubertal hypothalamus. The present results are the first to reveal upstream inhibitory effects of EtOH on glial cell proteins that control the production and secretion of PGE₂ within the MBH.

In recent years it has become clear that glial cells in the MBH regulate prepubertal LHRH neuronal secretion indirectly (Ojeda et al., 2003). TGF α is a glial derived peptide that binds to erbB1 receptors on adjacent glial cells, resulting in dimerization of this receptor with the erbB2 receptor and initiating intracellular transduction/signaling pathways (Stern, 2008) leading to the secretion of glial derived PGE₂ (Prevot et al., 2003). Once PGE₂ is released it then binds to its receptor on LHRH nerve terminals in the median eminence, causing the release of this peptide that controls the timing of puberty and subsequent reproductive function. Thus, we considered that this TGF α /erbB1-erbB2/PGE₂ glial pathway could be a target of EtOH's actions within the MBH.

We initially showed that ALC affects erbB1 expression in the MBH of prepubertal female rats. Specifically, our study demonstrated that while EtOH exposure for 4 and 6 days did not alter erbB1 gene expression or the synthesis of total, non-phosphorylated erbB1 protein, it did cause a marked decrease in the synthesis of the phosphorylated form of the receptor at both 4 days and 6 days. While this is the first report of EtOH altering phosphorylated erbB1 in the prepubertal female hypothalamus, it has previously been reported that chronic EtOH exposure inhibits erbB1 phosphorylation in liver (O'Rourke et al., 1997; Tuma et al., 1998) and stomach (Wang et al., 1997). It is important to note that while we have demonstrated that short-term EtOH exposure did not affect the hypothalamic synthesis of erbB1, as reflected by unaltered gene and total protein expressions, it markedly altered its activity, as reflected by decreased basal phosphorylated erbB1 protein. Additionally, our observation that EtOH did not affect the synthesis of total and phosphorylated erbB2 in this study is also important because it further demonstrates a specific effect on the erbB1 receptor. Since suppressed erbB1 phosphorylation would be expected to result in decreased PGE₂ release, we exposed animals to EtOH then assessed the release of PGE₂ from hypothalami incubated *in vitro*, but without EtOH in the incubation medium. In this regard, we observed a marked decrease in PGE₂ secreted into the medium from the hypothalami obtained from EtOH-treated animals compared to controls. This is in agreement with previous studies showing that acute, *in vitro* only EtOH exposure resulted in decreased stimulation of PGE₂ release (Dees and Hiney, 1991; Hiney et al., 1998, 2003; Lomniczi et al., 2000).

Whether the suppressed PGE₂ secretion which we observed following short-term EtOH exposure was the result of a principal action of the drug on erbB1, or whether it reflects an upstream effect of the drug to alter the synthesis or secretion of glial derived TGF α , the most important ligand for this receptor, remained an important question that needed to be addressed. In this regard, we have shown that short-term EtOH administration caused a marked increase in TGF α gene expression in the MBH at 4 days, then declined to near basal levels of expression at 6 days. Importantly, the hypothalamic content of TGF α protein was increased on both days, an effect coinciding with our earlier observations of suppressed erbB1 phosphorylation and decreased PGE₂ release. Thus, we hypothesized that EtOH may have caused the diminished secretion of TGF α ; hence, resulting in the noted increased accumulation of the peptide in the MBH, and also suggesting that the decrease gene expression observed between 4 and 6 days was due to down regulation of the gene. Therefore, it would be expected that the same tissues that were incubated *in vitro* (without EtOH in the incubation medium) following *in vivo* EtOH exposure and showing a decrease

in the amount of PGE₂ released into the medium, would also show a decreased content of TGF α released into the medium. Our results indicated that this was indeed the case and thus, allowed us to conclude that EtOH is capable of interfering with the hypothalamic release of TGF α .

Since TGF α binding initiates the autophosphorylation of the erbB1 receptor (Carpenter and Wahl, 1990; Ebner and Derynck, 1991), it is therefore likely that the EtOH-induced impairment of TGF α release is, at least in part, a major contributing factor responsible for decreased erbB1 phosphorylation observed in EtOH-treated animals. However, we cannot rule out the possibility that EtOH may also have a direct effect on the erbB1 autophosphorylation process independent of its effect to suppress TGF α secretion. In this regard, several studies have demonstrated that chronic EtOH exposure influences the phosphorylation of erbB1 by altering the receptor affinity and/or tyrosine kinase activity in rats (Tuma et al., 1998; Wang et al., 1997).

The results of the present study are important since they address mechanisms of EtOH action on processes involved with the onset of puberty. Our results showing that EtOH can suppress the release of TGF α and alter erbB1 signaling is important, since it is known that once this receptor is altered, puberty is delayed (Ojeda and Ma, 1998; Ma et al., 1999; Apostolakis et al., 2000), and furthermore, that EtOH is a drug known to cause delayed puberty in both rodents (Bo et al., 1982; Dees and Skelley, 1990; Emanuele et al., 2002) and primates (Dees et al., 2000). Thus, our results demonstrate that EtOH is capable of interfering with glial to glial interactions that are important for prepubertal PGE₂ secretion.

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Figure 1. Effect of EtOH exposure on basal erbB1 gene expression in the MBH of prepubertal female rats. (A & B) Real-time PCR analysis of erbB1 gene expression after 4 and 6 days of exposure to EtOH, respectively. Note that EtOH did not alter erbB1 gene expression on either day compared with control animals. The respective bars illustrate the mean ($\pm$ SEM) of an N of 14 per group at 4 days and an N of 8 per group at 6 days.

Figure 2. Effect of EtOH exposure for 4 and 6 days on phosphorylated erbB1 protein expressions in the MBH of prepubertal female rats. (A) Representative Western blot of phosphorylated erbB1 and total, nonphosphorylated erbB1 proteins in the MBH from control (lanes 1–3) and animals exposed to EtOH for 4 days (lanes 4–6). (B) Densitometric quantitation of all bands from 2 blots assessing phosphorylated erbB1 protein in the MBH. (C) Representative Western blot of phosphorylated erbB1 and total, nonphosphorylated erbB1 proteins in the MBH from control (lanes 1–3) and animals exposed to EtOH for 6 days (lanes 4–6). (D) Densitometric quantitation of all of the bands from 2 blots assessing phosphorylated erbB1 protein in the MBH. Note that phosphorylated erbB1 protein expression decreased markedly in both the 4 and 6 day of EtOH - treated animals compared with controls. These data were normalized to the total, nonphosphorylated erbB1 protein, and the densitometric units represent the phosphorylated erbB1/total, non-phosphorylated erbB1 ratio. The respective bars illustrate the mean ($\pm$ SEM) of an N of 8 per group at both 4 and 6 days. ** $p < 0.01$ versus control.

Figure 3.

Effect of *in vivo* EtOH exposure for 6 days on prostaglandin-E₂ (PGE₂) release *in vitro* from the MBH of prepubertal rats. The content of PGE₂ released into the medium was determined by ELISA. Note that EtOH-treated animals showed a marked decrease in the basal PGE₂ release compared with control animals. The respective bars illustrate the mean ($\pm$ SEM) of an N of 14 for control and an N of 9 for EtOH. ** $p < 0.01$ versus control.

Figure 4. Effect of EtOH exposure for 4 (panel A) and 6 (panel B) days on basal TGFα gene expression in the MBH of prepubertal female rats as determined by real-time PCR. Note that EtOH caused an increase in the expression of the TGFα gene compared with control animals at 4 days, but while still slightly elevated ($p < 0.05$) at 6 days they were markedly suppressed compared to the levels at 4 days. The respective bars illustrate the mean ($\pm$ SEM) of an N of 12–14 per group (panel A) and an N of 21 per group (panel B). * $p < 0.05$; *** $p < 0.001$ versus control.

Figure 5. Effect of EtOH exposure for 4 (panels A&B) and 6 days (panels C&D) on TGFα protein expression in the MBH of prepubertal female rats. (A & C) Representative Western immunoblot of TGFα and β-actin proteins in the MBH isolated from control (lanes 1–3) and ALC-treated (lanes 4–6) animals. (B & D) Densitometric quantitation of all the bands from 2 blots assessing TGFα protein expression in the MBH. These data were normalized to the internal control β-actin protein, and the densitometric units represent the TGFα/β-actin ratio. Note that EtOH–treated animals showed increased TGFα protein expression on day 4 (panel B) and day 6 (panel D) compared with control animals. The respective bars illustrate the mean (±SEM) of an N of 7– 8 per group. **p<0.01;***p<0.001 versus control.

Figure 6.

Effect of *in vivo* EtOH exposure for 6 days on TGFα protein released *in vitro* from the MBH of prepubertal female rats. The content of TGFα released into the medium was determined by Western immunoblotting. Note that EtOH-treated animals showed a marked decrease in basal TGFα release compared with control animals. These data were normalized to the internal control β-actin protein, and the densitometric units represent the TGFα/β-actin ratio. The respective bars illustrate the mean (±SEM) of an N of 14 for control and an N of 9 for EtOH. **p<0.01 versus control.