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Actions and Interactions of Alcohol and Transforming Growth Factor β 1 on Prepubertal Hypothalamic Gonadotropin-Releasing Hormone

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Abstract

Background—Alcohol (ALC) diminishes gonadotropin-releasing hormone (GnRH) secretion and delays puberty. Glial transforming growth factor β 1 (TGF β 1) plays a role in glial-neuronal communications facilitating prepubertal GnRH secretion. We assessed the effects of acute ALC administration on TGF β 1-induced GnRH gene expression in the brain preoptic area (POA), and release of the peptide from the medial basal hypothalamus (MBH). Furthermore, we assessed actions and interactions of TGF β 1 and ALC on an adhesion/signaling gene family involved in glial-neuronal communications.

Methods—Prepubertal female rats were administered ALC or water via gastric gavage at 0730 h. At 0900 h saline or TGF β 1 (100ng/3 μ l) was administered into the third ventricle. At 1500 h the POA was removed and frozen for gene expression analysis and repeated for protein assessments. In another experiment, the MBH was removed from ALC-free rats. After equilibration, tissues were incubated in Locke's medium only or medium containing TGF β 1 with or without 50 mM ALC for measurement of GnRH peptide released *in vitro*.

Results—TGF β 1 induced GnRH gene expression in the POA and this effect was blocked by ALC. We also described the presence and responsiveness of the TGF β 1 receptor in the POA and showed that acute ALC exposure not only altered the TGF β 1 induced increase in TGF β -R1 protein expression but also the activation of receptor associated proteins, Smad2 and Smad3, key downstream components of the TGF β 1 signaling pathway. Assessment of an adhesion/signaling family consisting of glial RPTP β and neuronal Caspr1 and contactin showed that the neuronal components were induced by TGF β 1 and that ALC blocked these effects. Finally, TGF β 1 was shown to induce release of the GnRH peptide *in vitro*, an action that was blocked by ALC.

Conclusion—We have demonstrated glial-derived TGF β 1 induces GnRH gene expression in the POA, and stimulates release of the peptide from the MBH; actions necessary for driving the pubertal process. Importantly, ALC acted at both brain regions to block stimulatory effects of TGF β 1. Furthermore, ALC altered neuronal components of an adhesion/signaling family previously shown to be expressed on GnRH neurons and implicated in glial-GnRH neuronal communications. These results further demonstrate detrimental effects of ALC at puberty.

Keywords

Transforming growth factor β 1; alcohol; puberty; Caspr1; contactin

INTRODUCTION

The onset of mammalian puberty is the result of a culmination of synchronized events within the preoptic-hypothalamic region of the brain, which leads to the activation and increased secretion of gonadotropin-releasing hormone (GnRH) and the subsequent increased secretion of gonadotropins from the pituitary gland. Initial studies showed that prepubertal alcohol (ALC) exposure resulted in delayed pubertal development (Anderson et al., 1981; Bo et al., 1982; Ramaley, 1982). Subsequently, it was revealed that this delayed development in rats, as well as the delayed development of a normal menstruation pattern in rhesus monkeys, was due to an ALC-induced suppression in luteinizing hormone (LH) secretion, suggesting that the drug was indeed acting within the region of the preoptic area (POA) and hypothalamus (Dees and Skelley, 1990; Dees et al., 2000; Dissen et al., 2004). In the rat, the GnRH peptide is mainly synthesized in neuronal cell bodies located within the POA, but is secreted from the median eminence (ME) region at the base of the hypothalamus. Prepubertal GnRH secretion is regulated by a decrease in the release of inhibitory neurotransmitters (Terasawa, 1999; Terasawa and Fernandez, 2001), as well as by an increase in numerous excitatory inputs (Dearth et al., 2000; Hiney et al., 1991b, 1996, 2009; Navarro et al., 2004; Ojeda et al., 1990; Sarkar et al., 1981; Urbanski and Ojeda, 1987; 1990). Importantly, it is now well documented that prepubertal ALC exposure acts at the hypothalamic level, at least in part, to suppress the stimulatory actions of excitatory neurotransmitters on GnRH secretion (Dissen et al., 2004; Hiney and Dees, 1991a, Hiney et al., 2003, 2010; Nyberg et al., 1993, 1995). In addition to these excitatory influences regulating GnRH, there are glial-glial and glial-neuronal influences that contribute to GnRH release that may also be affected by ALC.

Glial cells in the hypothalamus produce and release various growth factors which play important roles in glial-GnRH neuronal communications facilitating release of the GnRH peptide (Ma et al., 1997; Melcangi et al., 1995; Ojeda et al., 2008). There is a close association between glia and GnRH nerve terminals in the ME (Ojeda et al., 2008), as well as an intimate association between astrocytes and GnRH neuronal parakarya in POA (Witkin and Silverman, 1985; Witkin et al., 1991). TGF β 1 is produced by hypothalamic astrocytes in cell culture (Buchanan et al., 2000; Galbiati et al., 1996; Melcangi et al., 1995) and its gene expression has been shown in both the POA and medial basal hypothalamic (MBH) tissues (Bouret et al., 2004; Galbiati et al., 2001). Additionally, GnRH neurons in the POA express both TGF β -receptor-1 (TGF β -R1, Prevot et al., 2000) and TGF β -receptor-2 (TGF β -R2, Bouret et al., 2004). Studies using a GnRH secreting cell line have shown that TGF β 1 was capable of stimulating GnRH release (Buchanan et al., 2000; Galbiati et al., 1996; Melcangi et al., 1995); however, when only the ME tissue fragment was incubated *in vitro* the release of GnRH from the terminals was not observed (Ojeda et al., 1990). Although these studies have collectively demonstrated some evidence for TGF β 1 actions at the level of the GnRH cell body, they were not able to address the issue of release

of the peptide from the terminals in the basal hypothalamus. The present study has used additional methods to more closely assess the issues related to sites and mechanisms of TGF β 1 actions on the GnRH system, as well as to determine the effects of ALC.

Also relevant to glial-neuronal communications and GnRH secretion at puberty is the identification of genes which synthesize adhesion/signaling proteins involved in bi-directional communications between the two cell types. In this regard, a three member gene family has been identified which consists of neuronal contactin associated protein-1 (Caspr1), a transmembrane protein that binds to contactin on the same neuronal membrane. The contactin portion of this Caspr1/contactin complex is bound by receptor protein tyrosine phosphatase beta (RPTP β), the glial transmembrane protein; therefore, forming an assembly capable of contributing to glial-neuronal adhesiveness (Peles et al., 1995; 1998). Because this family, when bound, has cell signaling capabilities (Peles et al., 1997), it has been suggested that it may not only contribute to glial cell adhesion with GnRH neuron terminals, but also may regulate intracellular processes (Lomniczi and Ojeda, 2009). In support of this, it has been shown that GnRH neurons express contactin and Caspr1 (Mungenast and Ojeda, 2005), the neuronal connections required for glial RPTP β recognition (Peles et al., 1997). Regarding the RPTP β , we previously observed a decrease in its basal expression in the MBH following chronic ALC exposure and also, that it was induced by IGF-1, an action that was blocked by acute ALC exposure (Srivastava et al., 2011). The present study has assessed the effects of TGF β 1 on this three member family and determined the actions of ALC.

Assessing the importance of glial-GnRH communication networks and analyzing substances that alter them is important. The influences of ALC on glial-derived substances and on cell signaling systems have received little attention; thus, because TGF β 1 can influence GnRH secretion and because of the described central effects of ALC to alter GnRH and the pubertal process, we have assessed the actions and interactions between ALC and TGF β 1 as they relate to prepubertal GnRH secretion.

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 four 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 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. Surgical anesthesia was an intraperitoneal injection of 2.5% Tribromoethanol (0.5ml/60g body weight).

Actions of TGF β 1 and ALC on GnRH Gene Expression in the POA

Twenty-four day old female rats were implanted with third ventricular cannulae as described previously (Hiney et al., 2009). After 4 days of recovery, the rats were divided into three groups. At 0730 hours groups 1 and 2 were administered water, and group 3 received ALC (3g/kg; 1.5 ml 25% ALC /100 g rat) by gastric gavage. This dose of ALC was chosen because a single intragastric injection to immature female rats yields a moderate ALC level and is capable of consistently suppressing LH release (Hiney et al., 2003). The animals were left undisturbed for 90 minutes to allow time for ALC absorption. At 0900 hours groups 2 and 3 received a third ventricular injection of TGF β 1 (Abcam, Cambridge, MA) at a dose of 100 ng/3 μ l saline. Group 1 (control) was injected with an equal volume of saline. The injections were delivered into the ventricle over a 1 minute period of time. A 2g/kg dose of ALC or water was administered gastrically at 1130 hours (4 hours after the first dose) to maintain moderately elevated serum levels of ALC over the course of the day (Hiney et al., 2010). All animals were killed by decapitation at 1500 hours, 6 hours after receiving the TGF β 1 or saline. Trunk blood was collected at that time for subsequent assessment of blood ALC concentrations by an enzymatic method using a diagnostic kit purchased from Sekisui Diagnostics P.E.I. Inc. (Prince Edwards Island, Canada). The brains were removed, placement of the third ventricular cannula verified, and the animals were confirmed to be in the late juvenile stage of pubertal development (Dees and Skelley, 1990). A block of tissue containing the POA was dissected as described previously (Hiney et al., 2009). All tissues were stored frozen as described above until analyzed by Real-time polymerase chain reaction (PCR) for GnRH gene expression. A total of 27 rats were used.

TGF β -R1 and Signal Gene Expression in the POA

This experiment was conducted as above except without ALC in order to first determine whether the gene for TGF β -R1 is expressed in the prepubertal female POA and if so, to assess its responsiveness to the TGF β 1 peptide. Additionally, we tested the responsiveness of a family of signaling genes consisting of RPTP β , Caspr1 and contactin to the peptide as well. A total of 15 rats were used.

Isolation of total RNA

Total RNA was initially isolated from the POA tissues by homogenizing in TRIzol Reagent (Invitrogen, Life Tech., Grand Island, NY). 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). The integrity of the RNA was checked by the visualization of the ethidium bromide-stained 28S and 18S ribosomal RNA bands under UV light. Total RNA was quantitated spectrophotometrically by measuring its absorbance at 260 nm.

Reverse Transcription and Real-time Quantitative PCR

Total RNA (1 μ g) from each sample was reverse transcribed into cDNA according to the instruction manual using oligo (dT) and SuperScript III First-strand Synthesis System (Invitrogen Life Tech., Grand Island, NY). Real-time PCR was performed on an ABI PRISM 7500 sequence detection system as described previously (Srivastava et al., 2011).

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 GnRH [GenBank accession NM_012767], forward, 5'-GGGCAAGGAGGAGGATCAAA-3', reverse, 5'-GGCCAGTGGACAGTGCATT-3' (product size 60 bp); rat TGF β -R1 [GenBank accession NM_0127752], forward, 5'-CACCGCGTACCAAATGAAGA-3', reverse, 5'-TGGTGCCCTCTGAAATGAAAG-3' (product size 61 bp); rat Caspr1 [GenBank accession NM_032061], forward, 5'-AACGCGACCTTCTTCGGTAA-3', reverse, 5'-GCGAGCCGTAAAATGGTAGTG-3' (product size 75 bp); rat contactin [GenBank accession NM_057118], forward, 5'-AGAGCCCAGCATACCCTCAA-3', reverse, 5'-TACGTCTGAGGGAGCCACATT-3' (product size 70 bp); rat RPTP β [GenBank accession U09357], forward, 5'-GAACGGGCACATACATTGTACTAG-3', reverse, 5'-TGCTCCTCTGTTTGCACCAA-3' (product size 127 bp); rat β -actin [GenBank accession NM_031144], forward, 5'-ATGCCCCGAGGCTCTCTT-3', reverse, 5'-TGGATGCCACAGGATTCCA-3' (product size 57 bp). Thermal cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Specificity of PCR product was confirmed by melting curve analysis for each gene at the end of the PCR reaction. Each PCR product was also electrophoresed onto 2% agarose gel containing ethidium bromide, which showed a single band of the desired size. The relative levels of expression for each gene were determined using comparative C_T (threshold cycle) method as described previously (Srivastava et al., 2011).

Effect of ALC on TGF β 1-induced TGF β -R1 and Signaling Gene Proteins in the POA

This experiment was conducted exactly as the first experiment described above except brain tissue was collected for assessment of protein expression for TGF β -R1, RPTP β , Caspr1, and contactin. These proteins were analyzed by Western Blotting. A total of 39 rats were used.

Immunoblotting

Tissues were homogenized in 1% Igepal CA-630, 20 mM Tris-Cl, pH (8.0), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 10 mM sodium pyruvate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.25% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) at 4°C. The homogenates were incubated on ice for 30 minutes and centrifuged at 12,000Xg for 15 min. The concentration of total protein in the resulting supernatant was determined by the RC DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard. Immunoblot analysis was performed by solubilizing equal amounts of protein (100µg) in a 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 electrophores through 10% SDS-PAGE for TGF β -R1, phospho (p)-Smad2(Ser465/467), phospho (p)-Smad3(Ser423/425), contactin and

8% SDS-PAGE for Caspr1 and RPTP β under reducing conditions. The separated proteins were electrophoretically transblotted onto polyvinylidene difluoride (PVDF) membranes. Following transfer, 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 β -R1 (1:500; Millipore corporation, Temecula, CA), Caspr1 (1:750; Santa Cruz Biotechnology, Inc., CA) rabbit monoclonal antibody specific for p-Smad2 or Smad3 (1:250; Cell Signaling Technology, Inc., Danvers MA) or contactin (1:250; Santa Cruz Biotechnology, Inc., CA) and with mouse monoclonal antibody specific for RPTP β (1:250; BD Transduction Laboratories, Franklin Lakes, NJ, USA). Following incubation, membranes were washed in PBS buffer containing 0.05% Tween-20 and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology, Inc., CA) specific for TGF β -R1, p-Smad2, p-Smad3, Caspr1 or contactin and with goat anti-mouse IgG (1:10,000; Santa Cruz Biotechnology, Inc., CA) specific for RPTP β for 2 hr at room temperature. Following washing, the specific proteins signals were detected with the enhanced chemiluminescence method (Western Lightning Plus-ECL, Perkin Elmer, Shelton, CT) and quantified with NIH Image J software version 1.43 (National Institute of Health, MD). Subsequently, membranes were stripped using Re-Blot Plus kit (EMD Millipore, Temecula, CA), washed and blocked at room temperature in the presence of 5% nonfat dried milk/0.05% Tween 20 in PBS (pH 7.4) for 2 hr. After blocking, membranes were reprobed with a mouse monoclonal antibody to the β -actin and goat anti-mouse secondary antibody, to normalize for the amount of sample loading. Following washing, the detection and quantitation of β -actin protein was done as described above by NIH Image J software.

Effect of ALC on TGF β 1-induced GnRH Peptide Release from the MBH

Twenty-eight day old female rats were killed by decapitation, the brains removed, and the MBH consisting of the arcuate nucleus and median eminence was dissected under a stereomicroscope as described previously (Lee et al., 2007). Briefly, each MBH was incubated in a vial containing 300 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 30 min equilibration period, the medium was discarded and replaced medium alone, medium containing TGF β 1 (100ng/300 μ l), or medium containing ALC (50mM) and TGF β 1 for a 90 minute incubation period. These media were removed and saved for assessment of GnRH release by radioimmunoassay. Following completion of the experiment, MBH fragments were weighed. The samples were boiled for 10 min. to break down proteases and then frozen at -80 C. A total of 27 rats were used.

Hormone Analysis

GnRH was measured as previously described (Nyberg et al., 1993) using Antiserum R11B73 kindly provided by Dr. V.D. Ramirez. Synthetic LHRH used for the standards and iodinations was purchased from Sigma Chemical Co. (St. Louis, MO). The sensitivity of the assay was 0.2 pg tube⁻¹, and the intra and interassay coefficients of variation were < 10%.

Statistical Analysis

All values are expressed as the mean ($\pm$ SEM). Differences between treatment groups were analyzed by Kruskal-Wallis nonparametric analysis of variance (ANOVA) followed by post hoc testing using Student-Neuman-Keuls multiple range test. *P*-values less than 0.05 were considered significant. The IBM PC programs INSTAT 3.0 and PRISM 5.0 (GraphPad, San Diego, CA) were used to calculate and graph the results.

RESULTS

The experiments in which ALC was administered for one day and tissues were collected at 1500 hours for either gene or protein assessments were conducted in exactly the same manner. The blood ALC concentrations for these acute *in vivo* experiments were averaged. The BACs showed a mean ($\pm$ SEM) of 131 ± 10.2 mg/dL at the time the tissues were collected.

Actions of TGF β 1 and ALC on GnRH and TGF β -R1 expressions in the POA

The action of glial-derived TGF β 1 on GnRH gene expression and the effect of ALC on this action were assessed in the POA, the principal site of GnRH synthesizing neurons in the rat. Figure 1 demonstrates that the central delivery of TGF β 1 caused an increase ($p < 0.05$) in GnRH gene expression by 6 hours post-injection over that expressed by the controls. Furthermore, this action of TGF β 1 within the POA was blocked ($p < 0.05$) by acute ALC administration. Because of the ability of TGF β 1 to induce the GnRH gene, we then determined whether the TGF β -R1 was expressed in the POA and its ability to respond to the peptide at this early stage of development. Figure 2 demonstrates the mean ($\pm$ SEM) basal level of TGF β -R1 gene expression, as well as an increase ($p < 0.01$) in the level of TGF β -R1 expression following the central administration of TGF β 1. Since these results demonstrate for the first time both the presence and responsiveness of the TGF β -R1 gene in the POA of prepubertal female rats, we subsequently assessed the effect of ALC on TGF β -R1 protein expression. Figure 3A shows the representative Western blot of the TGF β -R1 protein. The composite of all animals shown in figure 3B demonstrates the ability of the TGF β 1 to markedly up-regulate ($p < 0.001$) the expression of TGF β -R1 protein expression by 6 hours post-injection, and furthermore, that this action was blocked in the ALC-treated animals. Smad2 and Smad3 proteins were assessed as downstream markers of receptor activation and the maximum phosphorylation activity of these two proteins was determined using a time course experiment. Table 1A and B shows a time course of protein expression of p-Smad2 (A) and p-Smad(3) 4-6 hours post TGF β 1. At 4 and 5 hours after TGF β 1 administration, both p-Smad2 (A) and p-Smad3 (B) protein expressions showed a non-significant increase over controls. However, TGF β 1 induced a marked increase 6 hours ($p < .05$) following administration confirming the activation of TGF β -R1. Importantly, the activation of both Smad2 and Smad3 ($p < 0.05$) by TGF β 1 was blocked by ALC.

Actions of TGF β 1 and ALC on Adhesion/Signaling Molecules within the POA

An experiment was conducted to first determine if the central administration of TGF β 1 could induce the gene expressions of RPTP β , Caspr1 and contactin in the POA of prepubertal female rats. Real-time PCR results indicated that the peptide did not affect the

expression of the glial RPTP β gene, but elicited an increase ($p<0.001$) in the mean ($\pm$ SEM) expression of neuronal Caspr1 (saline-treated: 1.2 ± 0.04 ; TGF β 1-treated $2.2\pm.18$; N=9/group), as well as an increase ($p<0.05$) in the expression of neuronal contactin (saline-treated: 2.23 ± 0.21 ; TGF β 1-treated: 2.94 ± 0.16 ; N=9/group). Once the effects of TGF β 1 on the expression of these genes was determined then the actions and interactions of TGF β 1 and ALC on the expression of their protein was assessed. In this regard, figure 4A shows the representative Western blot and figure 4B shows the composite of all animals demonstrating that neither TGF β 1 nor ALC affected the protein expression of the glial component, RPTP β . Conversely, figures 4C and D, as well as 4E and F, respectively, demonstrate that the central administration of TGF β 1 elicited increases in the protein expressions ($p<0.05$) of both of the neuronal components, Caspr1 and contactin. Furthermore, these TGF β 1-stimulated protein expressions were blocked by ALC; hence, indicating that ALC can interfere with the neuronal component of this glial-neuronal signaling system in the POA.

Actions of TGF β 1 and ALC on in vitro GnRH release from the MBH

Figure 5 shows the mean ($\pm$ SEM) basal secretion of the GnRH peptide from the MBH when incubated in medium only. Tissues incubated in medium plus TGF β 1 showed a marked ($p<0.01$) increase in the release of GnRH into the medium. This TGF β 1-induced increase in peptide release was blocked when tissues were incubated in the presence of 50 mM ALC.

DISCUSSION

ALC exposure causes suppressed serum levels of LH and delayed pubertal development in both rats and rhesus monkeys (Dees and Skelley, 1990; Dees et al., 2000). This is due to the hypothalamic action of ALC to suppress the secretion of GnRH by blocking the influences of several excitatory neuronal regulators (Dissen et al., 2004; Hiney and Dees, 1991a; Hiney et al., 2010; Nyberg et al., 1993). In addition to neuronal influences, glial-derived factors can also influence GnRH secretion (Hiney et al., 2003; Ma et al., 1997; Mahesh et al., 2006; Ojeda and Skinner, 2006). Some of these glial substances, such as epidermal growth factor and transforming growth factor α , bind to erbB1 receptors and have been shown to be altered by ALC (Hiney et al., 2003). The present study has assessed the actions and interactions of ALC and glial-derived TGF β 1 with regard to their potential to influence hypothalamic glial-glial and glial-neuronal communications.

The close anatomical relationship between glial cells and GnRH cell bodies in the POA (Witkin and Silverman, 1985; Witkin et al., 1991) and between glia and GnRH nerve terminals in the MBH/ME region (Ojeda et al., 2008), suggests a functional interaction between these cell types in both brain regions. This is also supported by the fact the POA of adult rats express TGF β receptors (Bouret et al., 2004; Prevot et al., 2000), with some adult GnRH neurons showing positive receptor staining (Prevot et al., 2000). The present study first addressed whether TGF β 1 can act within the POA to induce GnRH gene expression. We showed that the peptide can induce the prepubertal GnRH gene six hours after being injected into the brain third ventricle. Furthermore, this action of TGF β 1 was blocked by ALC. Previous studies showed that medium containing TGF β 1 secreted from astrocytes maintained in culture can up-regulate GnRH gene expression in GT1 neurons, a GnRH

secreting immortalized cell line (Galbiati et al., 1996). The present study shows for the first time that the TGF β -R1 gene is expressed in the POA of prepubertal female rats, and that the TGF β 1 peptide up-regulated both gene and protein expression of the receptor, as well as that of Smad 2 and Smad 3, downstream markers demonstrating receptor activation and confirming the involvement of this signaling pathway (Massague and Wotton, 2000). Furthermore, this action to induce the receptor protein and downstream activation was blocked by ALC. Thus, our results provide the first evidence *in vivo* supporting the notion that TGF β 1 can act directly on GnRH neurons and further demonstrates the *in vivo* detrimental effects of ALC within the hypothalamus to alter the prepubertal GnRH system.

In recent years, attention has been given to other cell-cell signaling molecules that contribute to the structural organization and bi-directional glial-neuronal communications. In this regard, a three member gene family consisting of glial RPTP β and the neuronal Caspr1/contactin complex has been shown to play a role in glial-neuronal adhesiveness and cellular communications (Peles et al., 1997, 1998; Pierre et al., 1998). Once these three molecules are bound together, this adhesion/signaling family can contribute to hypothalamic neuroendocrine functions (Pierre et al., 1998). Importantly, an immortalized GnRH secreting cell line has been shown to express both neuronal contactin and Caspr1 (Mungenast and Ojeda, 2005). Because of this and the intimate relationship discussed between glial cells and GnRH cell bodies in the POA, we assessed in this brain region whether TGF β 1 administered *in vivo* could up-regulate components of this gene family. Although glial RPTP β was not altered, marked increases were observed in both gene and protein expressions of neuronal Caspr1 and contactin after a third ventricular injection of TGF β 1. Furthermore, we revealed that ALC blocked this action of TGF β 1 to induce both of these neuronal components of this adhesion/signaling family. The fact that ALC can alter the synthesis of the neuronal Caspr1/contactin complex, which is required for binding to glial RPTP β , indicates its potential to disrupt the glial-neuronal adhesiveness function associated with the binding together of this three member family. While additional research is needed to more closely assess the precise contribution of Caspr1-contactin-RPTP β adhesion and signaling interactions in the POA, the present results provide new evidence *in vivo* indicating that TGF β 1 may act directly on GnRH neurons through this adhesion/signaling family and suggests that the detrimental action of ALC to alter this cell-cell communication may contribute to this drugs ability to suppress GnRH neuronal function.

In this study we have also addressed more closely whether TGF β 1 can influence release of GnRH from the basal hypothalamus. Previous studies have suggested that TGF β 1 caused GnRH release from GT1 cells in culture (Melcangi et al., 1995), but not from a ME explant that was incubated alone *in vitro* (Ojeda et al., 1990). In the present study, we showed that TGF β 1 was capable of inducing GnRH release *in vitro* when the entire MBH was present. The difference between our study and Ojeda et al., 1990 is that the MBH contains the arcuate nucleus (AN) as well as the ME, and not the ME alone. Thus, our results indicate that the TGF β 1 action within the MBH to stimulate GnRH release is indirect since GnRH neurons are not localized within the AN of the rat (Kozlowski and Dees, 1984) and because TGF β 1 receptors are not localized on the GnRH nerve terminals in the ME (Bouret et al., 2004). Therefore, in this brain region, TGF β 1 must first be stimulating another

neurotransmitter synthesized by neurons within the AN, and that once secreted, it is capable of stimulating the GnRH peptide from the nerve terminals in the ME; hence, mediating the TGF β 1 effect. This is supported by the fact that TGF β 1 receptors are present in the AN (Bouret et al., 2004; Prevot et al., 2000) and because it is well known that several neurotransmitters/peptides synthesized by neurons in the AN can stimulate GnRH release directly from the nerve terminals in the ME. More research is needed to determine which neuron(s) in the AN respond to TGF β 1 and thus, mediate its action with regard to GnRH secretion. Regardless of the exact neuromodulator involved, we have shown here for the first time that TGF β 1 can stimulate GnRH release from the MBH, and that this action was blocked by ALC. Importantly, while GnRH neuronal parakarya are not present in the arcuate nucleus of the rat, they are in the human; thus, suggesting both direct and indirect means by which TGF β 1 can contribute to GnRH release.

In addition to the TGF β 1 action within the AN, this peptide can also act through glial-glial actions within the ME itself to further facilitate GnRH release. Tanycytes are specialized glia within the ME that line the ventricular wall and are known to express the TGF β -R1 gene (Prevot et al, 2000). A close anatomical relationship exists between GnRH nerve terminals and tanycyte processes within the ME (Kozłowski and Coates, 1985), and there is a change in the dynamics of tanycyte plasticity depending upon the steroid milieu (Kozłowski and Coates, 1985; King and Letourneau, 1994). At times of increased GnRH secretion, the tanycytes retract their end feet from around portal vessels, thus, facilitating the GnRH release process by allowing the peptide, once released from the nerve terminals, access to the blood vasculature (King and Letourneau, 1994). Importantly, tanycytes retract in the presence of TGF β 1 (Prevot et al., 2003); hence, supporting the concept that this peptide can facilitate GnRH release from within the ME. Interestingly, other substances such as IGF-1, which is known to be produced by adjacent glial cells and also crosses the blood brain barrier at the ME in increased amounts as puberty approaches, is capable of stimulating TGF β 1 protein expression in the MBH and inducing release of the protein from hypothalamic astrocytes in culture (Hiney et al., unpublished observation). It is not known what effects ALC may have on this glial-glial process.

In summary, the present study demonstrates for the first time that glial-derived TGF β 1 is capable of inducing GnRH gene expression in the POA, as well as stimulating GnRH release from the MBH; actions important at puberty when increased synthesis and release of the peptide are necessary to drive the pubertal process. Importantly, ALC blocked the stimulatory effects of TGF β 1 on both GnRH synthesis and its release. Furthermore, we have provided additional evidence supporting the existence of glial-GnRH neuronal interactions by demonstrating that TGF β 1 can induce gene and protein expressions of neuronal Caspr1 and contactin, both of which are expressed on GnRH cell bodies. We have also shown that ALC is capable of altering prepubertal glial-neuronal communications by blocking the stimulatory effect of TGF β 1 on the neuronal Caspr1/contactin complex. Taken together, these results further demonstrate the mechanisms by which ALC detrimentally affects the GnRH system at a critical time of development.

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References

- Anderson RA, Willis BR, Oswald C, Gupta A, Zaneveld L. Delayed male sexual maturation induced by chronic ethanol ingestion. *Fed Proc.* 1981; 40:825–829.
- Bo WJ, Krueger WA, Rudeen PK, Symees SK. Ethanol-induced alterations in the morphology and function of the rat ovary. *Anat Rec.* 1982; 202:255–260. [PubMed: 7199834]
- Bouret S, De Seranno S, Beauvillian JC, Prevot V. Transforming growth factor β 1 may directly influence gonadotropin releasing hormone gene expression in the rat hypothalamus. *Endocrinology.* 2004; 145:1794–1801. [PubMed: 14670985]
- Buchanan CD, Mahesh VB, Brann DW. Estrogen-astrocyte-luteinizing hormone-releasing hormone signaling: a role for transforming growth factor β 1. *Biol Reprod.* 2000; 62:1710–1721. [PubMed: 10819775]
- Dearth RK, Hiney JK, Dees WL. Leptin acts centrally to induce prepubertal secretion of luteinizing hormone in the female rat. *Peptides.* 2000; 21:387–392. [PubMed: 10793221]
- Dees WL, Dissen GA, Hiney JK, Lara F, Ojeda SR. Alcohol ingestion inhibits the increased secretion of puberty-related hormones in the developing female rhesus monkey. *Endocrinology.* 2000; 141:1325–1331. [PubMed: 10746635]
- Dees WL, Skelley CW. The effects of ethanol during the onset of puberty. *Neuroendocrinology.* 1990; 51:64–69. [PubMed: 2106089]
- Dissen GA, Ojeda SR, Dearth RK, Scott H, Dees WL. Alcohol alters prepubertal luteinizing hormone (LH) secretion in female rhesus monkeys by a hypothalamic action. *Endocrinology.* 2004; 145:4558–4564. [PubMed: 15217984]
- Galbiati M, Magnaghi V, Martini L, Melcang RC. Hypothalamic transforming growth factor β 1 and basic fibroblast growth factor mRNA expression is modified during the rat estrous cycle. *J Neuroendocrinol.* 2001; 13:483–489. [PubMed: 11412334]
- Galbiati M, Zanisi M, Messi E, Cavarretta I, Martini L, Melcangi RC. Transforming growth factor β and astrocyte conditioned medium influence luteinizing hormone-releasing hormone gene expression in the hypothalamic cell line GT1. *Endocrinology.* 1996; 137:5605–5609. [PubMed: 8940390]
- Hiney JK, Dearth RK, Srivastava VK, Rettori V, Dees WL. Actions of alcohol on epidermal growth factor-receptor activated luteinizing hormone secretion. *J Studies Alcohol.* 2003; 64:809–816.
- Hiney JK, Dees WL. Ethanol inhibits luteinizing hormone-releasing hormone from the median eminence of prepubertal female rats in vitro: Investigation of its actions on norepinephrine and prostaglandin E_2 . *Endocrinology.* 1991a; 128:1404–1408. [PubMed: 1999162]
- Hiney JK, Ojeda SR, Dees WL. Insulin-like growth factor-1: A possible metabolic signal involved in the regulation of female puberty. *Neuroendocrinology.* 1991b; 54:420–423. [PubMed: 1758585]
- Hiney JK, Srivastava VK, Dees WL. Insulin-like growth factor-1 stimulates hypothalamic KiSS-1 gene expression by activating Akt: Effect of alcohol. *Neuroscience.* 2010; 166:625–632. [PubMed: 20034543]
- Hiney JK, Srivastava VK, Nyberg CL, Ojeda SR, Dees WL. Insulin-like growth factor -1 of peripheral origin acts centrally to accelerate the initiation of female puberty. *Endocrinology.* 1996; 137:3717–3728. [PubMed: 8756538]
- Hiney JK, Srivastava VK, Pine MD, Dees WL. IGF-1 activates KiSS-1 gene expression in the brain of the prepubertal female rat. *Endocrinology.* 2009; 150:376–384. [PubMed: 18703622]
- King JC, Letourneau RL. Luteinizing hormone releasing hormone terminals in the median eminence of rats undergo dynamic changes after gonadectomy, as revealed by electron microscopic image analysis. *Endocrinology.* 1994; 134:1340–1351. [PubMed: 8119174]
- Kozłowski GP, Coats PW. Ependymoneuronal specializations between LHRH fibers and cells of the cerebroventricular system. *Cell Tissue Res.* 1985; 242:301–311. [PubMed: 3902246]

- Lomniczi, A.; Ojeda, SR. A role for glial cells of the neuroendocrine brain in the central control of female sexual development. In: Purpura, V.; Haydon, P., editors. *Astrocytes in Pathophysiology of the Nervous System*. Springer; New York: 2009. p. 487-511.
- Lee B, Hiney JK, Pine MD, Srivastava VK, Dees WL. Manganese stimulates luteinizing hormone-releasing hormone secretion in prepubertal female rats: Hypothalamic site and mechanism of action. *J Physiol*. 2007; 578:765–772. [PubMed: 17110411]
- Ma YJ, Berg-von der Emde K, Rage F, Wetsel WC, Ojeda SR. Hypothalamic astrocytes respond to transforming growth factor alpha with secretion of neuroactive substances that stimulate the release of luteinizing hormone-releasing hormone. *Endocrinology*. 1997; 138:19–35. [PubMed: 8977380]
- Mahesh VB, Dhandapani KM, Brann DW. Role of astrocytes in reproduction and neuroprotection. *Mol Cell Endocrinology*. 2006; 246:1–9.
- Massague J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J*. 2000; 19:1745–1754. [PubMed: 10775259]
- Melcangi RC, Galbiati M, Messi E, Piva F, Martini L, Matta M. Type 1 astrocytes influence luteinizing hormone releasing hormone release from the hypothalamic cell line GT1: Is transforming growth factor β the principal involved? *Endocrinology*. 1995; 136:679–686. [PubMed: 7835301]
- Mungenast AE, Ojeda SR. Expression of three gene families encoding cell-cell communication molecules in the prepubertal nonhuman primate hypothalamus. *J Neuroendocrinology*. 2005; 17:208–219. [PubMed: 15842232]
- Navarro VM, Castellano JM, Fernandez-Fernandez R, Barriero ML, Roa J, Sanchez-Criado JE, Agilar E, Dieguez C, Pinilla L, Tena-Sempere M. Developmental and hormonally regulated mRNA expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent LH-releasing activity of KiSS-1 peptide. *Endocrinology*. 2004; 145:4565–4574. [PubMed: 15242985]
- Nyberg CL, Hiney JK, Minks JB, Dees WL. Ethanol alters N-methyl-DL-aspartic acid induced secretion of luteinizing hormone releasing hormone and the onset of puberty in the female rat. *Neuroendocrinology*. 1993; 57:863–868. [PubMed: 8413822]
- Nyberg CL, Srivastava V, Hiney JK, Lara F, Dees WL. N-methyl-D-Aspartic acid-receptor (NMDA-R) synthesis and luteinizing hormone release in immature female rats: Effects of stage of pubertal development and exposure to ethanol. *Endocrinology*. 1995; 136:2874–2880. [PubMed: 7789312]
- Ojeda SR, Lomniczi A, Sandau US. Glial-gonadotropin hormone (GnRH) neurone interactions in the median eminence and the control of GnRH secretion. *J Neuroendocrinology*. 2008; 20:732–742. [PubMed: 18601696]
- Ojeda, SR.; Skinner, MK. Puberty in the rat. In: Neill, JD., editor. *The Physiology of Reproduction*. 3. Academic Press/Elsevier; California: 2006. p. 2061-2126.
- Ojeda SR, Urbanski HF, Costa ME, Hill DF, Moholt-Siebert M. Involvement of transforming growth factor alpha in the release of luteinizing hormone releasing hormone from the developing female hypothalamus. *Proc Natl Acad Sci*. 1990; 87:9698–9702.
- Peles E, Nativ M, Campbell PL, Sakuria T, Martinez R, Lev S, Clary DO, Schilling J, Barnea G, Plowman GD, Grumet M, Schlessinger J. The carbonic anhydrase domain of receptor tyrosine phosphatase β is a functional ligand for the axonal cell recognition molecule contactin. *Cell*. 1995; 82:251–260. [PubMed: 7628014]
- Peles E, Nativ M, Lustig M, Grumet M, Schilling J, Martinez R, Plowman GD, Schlessinger J. Identification of a novel contactin-associated transmembrane receptor with multiple domains implicated in protein-protein interactions. *EMBO J*. 1997; 16:978–988. [PubMed: 9118959]
- Peles E, Schlessinger J, Grumet M. Multi-ligand interactions with receptor-like protein tyrosine phosphatase β : implications for intercellular signaling. *Trends Biochem Sci*. 1998; 23:121–124. [PubMed: 9584610]
- Pierre K, Rougon G, Allard M, Bonhomme R, Gennarini G, Poulain DA, Theodosis DT. Regulated expression of the cell adhesion glucoprotein F3 in adult hypothalamic magnocellular neurons. *J Neuroscience*. 1998; 18:5333–5343.
- Prevot V, Bouret S, Croix D, Takumi T, Jennes L, Mitchell V, Beauvillain JC. Evidence that members of the TGF β family superfamily play a role in regulation of the GnRH neuroendocrine axis:

- expression of type 1 serine-threonine kinase receptor for TGF β and activin in GnRH neurons and hypothalamic areas of the female rat. *J Neuroendocrinol.* 2000; 12:665–670. [PubMed: 10849211]
- Prevot C, Cornea A, Mungenast A, Smiley G, Ojeda SR. Activation of erbB-1 signaling in tanycytes of the median eminence stimulates transforming growth factor β 1 release via prostaglandin E₂ production and induces cell plasticity. *J Neurosci.* 2003; 23:10622–10632. [PubMed: 14627647]
- Ramaley JA. The regulation of gonadotropin secretion in immature ethanol-treated male rats. *J Androl.* 1982; 3:248–252.
- Sarkar DK, Smith GC, Fink G. Effect of manipulating central catecholamines on puberty and the surge of luteinizing hormone and gonadotropin releasing hormone induced by pregnant mare serum gonadotropin in female rats. *Brain Res.* 1981; 213:335–349. [PubMed: 6113874]
- Srivastava VK, Hiney JK, Dees WL. Hypothalamic actions and interactions of alcohol and IGF-1 on the expression of glial receptor protein tyrosine phosphatase- β during female pubertal development. *Alc Clin Exper Res.* 2011; 35:1812–1821.
- Terasawa E. Hypothalamic control of the onset of puberty. *Curr Opin Endocrinol Diabetes.* 1999; 6:44–49.
- Terasawa E, Fernandez DL. Neurobiological-mechanisms of the onset of puberty in primates. *Endocr Rev.* 2001; 22:111–151. [PubMed: 11159818]
- Urbanski HF, Ojeda SR. A role for N-methyl -D-Aspartate (NMDA) receptors in the control of LH secretion and initiation of female puberty. *Endocrinology.* 1990; 126:1774–1776. [PubMed: 1968384]
- Urbanski HF, Ojeda SR. Activation of lutenizing hormone-releasing hormone release advances the onset of female puberty. *Neuroendocrinology.* 1987; 46:273–276. [PubMed: 3309706]
- Witkin JW, Ferin M, Popilskis SJ, Silverman AJ. Effects of gonadal steroids on the ultrastructure of GnRH neurons in the rhesus monkey: synaptic input and glial apposition. *Endocrinology.* 1991; 129:183–1092.
- Witkin JW, Silverman AJ. Synaptology of lutenizing hormone-releasing hormone neurons in the rat preoptic area. *Peptides.* 1985; 6:263–271. [PubMed: 3898038]

Figure 1.

The effects of transforming growth factor β 1 (TGF β 1) and alcohol (ALC) on gonadotropin-releasing hormone (GnRH) gene expression in the preoptic area (POA) of prepubertal female rats. The central administration of TGF β 1 (100ng) induced the expression of the GnRH gene at 6 hours post-injection in animals that did not receive ALC (solid bar) when compared to control animals (open bar). Note that this TGF β 1-induced GnRH gene expression was blocked in the ALC-treated animals (lined bar). The bars illustrate the mean ($\pm$ SEM) of an N of 9 per group. * p <0.05 versus control and TGF β 1+ALC.

Figure 2.

Transforming growth factor β1 (TGFβ1) stimulation of TGFβ-R1 gene expression in the preoptic area (POA) of the prepubertal female rat. Central administration of TGFβ1 (100ng/3μl) stimulated an increase in the gene expression of TGFβ-R1 (solid bar) at 6 hours post-injection compared to control animals that received saline (open bar) in the third ventricle. The bars illustrate the mean (±SEM) of an N of 5 per group. **p<0.01 versus control.

Figure 3.

The effect of ALC on TGFβ1-induced TGFβ-R1 protein expression in the POA of prepubertal female rats. (A) Representative Western immunoblot of TGFβ-R1 and β-actin proteins in the POA isolated from control (lanes 1-3), TGFβ1-treated (lanes 4-6) and TGFβ1+ALC-treated (lanes 7-9) animals. (B) Densitometric quantitation of all the bands from two immunoblots corresponding to the TGFβ-R1 and β-actin proteins. The central administration of TGFβ1 (100ng) stimulated an increase in TGFβ-R1 protein expression 6 hours post-injection in animals that did not receive ALC (solid bar) when compared to control animals (open bar). Note that this TGFβ1-induced increase of TGFβ-R1 protein expression was blocked in the ALC-treated animals (lined bar). These data were normalized to the internal control, β-actin protein. The bars illustrate the mean (±SEM) of an N of 8 per group. ***p<0.001 versus control and TGFβ1+ALC.

Figure 4.

Effects of TGFβ1 and ALC on glial RPTPβ and neuronal Caspr1 and contactin protein expressions in the POA of prepubertal rats. (A) Representative Western immunoblot of RPTPβ and β-actin proteins isolated from control (lanes 1 -3), TGFβ1-treated (lanes 4-6) and TGFβ1+ALC-treated (lanes 7-10) animals. (B) Densitometric quantitation of all the bands from two immunoblots corresponding to the RPTPβ protein. (C) Representative Western immunoblot of Caspr1 and β-actin proteins isolated from control (lanes 1 -3), TGFβ1-treated (lanes 4-6) and TGFβ1+ALC-treated (lanes 7-10) animals. (D) Densitometric quantitation of all the bands from two immunoblots corresponding to the caspr1 protein. (E) Representative Western immunoblot of contactin and β-actin proteins isolated from control (lanes 1 -3), TGFβ1-treated (lanes 4-6) and TGFβ1+ALC-treated (lanes 7-10) animals. (F) Densitometric quantitation of all the bands from two immunoblots corresponding to the contactin protein. These data were normalized to the internal control β-actin protein. Neither the central administration of TGFβ1 nor acute ALC exposure altered the protein expression of RPTPβ. The TGFβ1 significantly increased expression levels of both neuronal Caspr1 and contactin proteins 6 hours post-injection in the animals that did not receive ALC. Note that the TGFβ1 stimulated protein expressions of both Caspr1 and contactin were blocked in the ALC-treated animals. Open bar represents control animals, solid bar represents TGFβ1-treated animals and lined bar represents TGFβ1+ALC-treated

animals. The respective bars illustrate the mean ($\pm$ SEM) of an N of 5 per group. * $p < 0.05$ versus control and TGF β 1+ALC.

Figure 5.

Effect of ALC on TGFβ-1-induced GnRH release from the MBH of prepubertal female rats incubated *in vitro*. Open bar indicates basal secretion of GnRH in medium only. Solid bar represents GnRH release in medium containing TGFβ1 and lined bar represents GnRH release in the presence of medium containing TGFβ1 plus 50 mM ALC. Note that TGFβ1-induced the secretion of GnRH compared to the medium only group and that the presence of ALC in the medium blocked the TGFβ1-induced release of the peptide. The bars illustrate the mean ($\pm$ SEM) of an N of 9 per group. ** $p < 0.01$ versus medium only and medium + TGFβ1 + ALC.

Table 1

Effect of acute alcohol (ALC) exposure on transforming growth factor β 1 (TGF β 1)-induced phosphorylation of Smad2 and 3 protein expressions at 4, 5, and 6 hrs in the preoptic area of prepubertal female rats. Densitometric values were determined by Western blot analysis and normalized to the internal control β -action protein. At 4 and 5 hrs, central administration of TGF β 1 showed a slight elevation in the phosphorylation of both Smad2 and 3 protein expressions, but at 6 hrs phosphorylation of both Smad2 (A) and Smad3 (B) were significantly elevated over saline-treated controls. Note that ALC inhibited the TGF β 1-induced increase in both p-Smad2 and p-Smad3 at 6 hrs. The values illustrate the mean ($\pm$ SEM) of an N of 4 for 4 hrs, N of 6 for 5 hrs and an N of 5 for 6 hrs in each group.

A. p-Smad2	Control	TGFβ1	TGFβ1+ALC
4hr	0.71 $\pm$.12	0.92 $\pm$.12	0.88 $\pm$.12
5hr	0.75 $\pm$.08	0.93 $\pm$.04	0.87 $\pm$.04
6hr	1.17 $\pm$.11	1.81 $\pm$.23 *	1.23 $\pm$.14 ⁺
B. p-Smad3	Control	TGFβ1	TGFβ1+ALC
4hr	0.56 $\pm$.15	0.65 $\pm$.06	0.53 $\pm$.08
5hr	0.42 $\pm$.11	0.62 $\pm$.10	0.48 $\pm$.07
6hr	0.54 $\pm$.10	1.11 $\pm$.17 *	0.64 $\pm$.05 ⁺

* p<0.05 versus control and

⁺ p<0.05 vs TGF β 1 + ALC