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Differential Effects of Alcohol on Excitatory and Inhibitory Puberty-Related Peptides in the Basal Hypothalamus of the Female Rat

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Abstract

Background—An increase in development of excitatory inputs, along with a decline in inhibitory inputs ultimately govern the timely increased secretion of hypothalamic luteinizing hormone-releasing hormone (LHRH) at the time of puberty. Since chronic alcohol (ALC) exposure acts at the hypothalamic level to suppress LHRH secretion and delay puberty, we assessed the its ability to differentially affect the expression of key puberty-related proteins.

Methods—ALC was administered to female rats from day 27 to day 33, at which time animals were killed and tissues collected for protein expression. In the medial basal hypothalamus (MBH), we assessed kisspeptin-10 (Kp), an excitatory peptide critical for prepubertal LHRH secretion, and Lin28b, a peptide with an inhibitory influence on puberty. Since a direct mechanism action of Lin28b was not known, we determined whether its central administration could induce dynorphin (DYN), a peptide that is inhibitory on LHRH secretion. Also, ALCs effect on DYN protein expression was assessed, as well as its effect on DYN release *in vitro*.

Results—ALC markedly suppressed ($p<0.01$) the expression of the excitatory KP protein, while at the same time increased ($p<0.001$) the expression of inhibitory Lin28b protein. Subsequently, we showed for the first time that the central administration of Lin28b stimulated ($p<0.01$) the synthesis of DYN. Finally, ALC also induced ($p<0.01$) the protein expression and stimulated ($p<0.01$) the *in vitro* release of DYN from the MBH.

Conclusion—These results indicate that ALC can simultaneously and differentially alter both excitatory and inhibitory influences governing pubertal development, show for the first time a mechanism of action by which Lin28b exerts its prepubertal inhibitory tone, and further demonstrate the negative influences of ALC on the pubertal process.

Keywords

puberty; alcohol; kisspeptin; Lin28b; dynorphin

INTRODUCTION

The onset of puberty results from a complex cascade of neuronal and glial interactive events within the hypothalamus that are governed by metabolic signals, as well as genetic and environmental influences that culminate in the increased secretion of luteinizing hormone-releasing hormone (LHRH). Ultimately, these integrated events result in an enhanced developmental responsiveness to excitatory inputs to LHRH producing neurons and their nerve terminals, such as provided by insulin like growth factor -1 (IGF-1; Hiney et al., 1996; Wilson, 1998), excitatory amino acids (Gay and Plant, 1987; Urbanski and Ojeda, 1987), and kisspeptin (Kp; Navarro et al., 2004a; 2004b; Shahab et al., 2005), as well as to a concomitant and gradual removal of prepubertal inhibitory inputs, such as provided by gamma aminobutyric acid and opioid peptides (Terasawa and Hernandez, 2001). Following activation of the LHRH releasing system there is an increase in the serum levels of gonadotropins and estradiol (E₂), resulting in first ovulation and reproductive maturity. Importantly, any endogenous or exogenous substance that can stimulate or inhibit the prepubertal release of LHRH could impact one or more of the hypothalamic circuits involved in the activation of the peptide and thus, have the potential to alter the pubertal process.

Alcohol (ALC) is known to diminish the secretion of LHRH and delay signs associated with the onset of female puberty in rodents (Dees and Skelley, 1990) and primates (Dees et al., 2000; Dissen et al., 2004), including humans (Peck et al., 2011; Richards and Oinonen, 2011). Specifically, these studies show that in rodents ALC delays vaginal opening (VO) markedly beyond the time when controls show VO, in primates, it significantly delays the development of regular monthly pattern of menstruation, and in human, it causes delayed breast development and menarche. Because the increased level of LHRH secretion at puberty is critical and due to both excitatory and inhibitory influences, it is important to discern the effects of ALC on hypothalamic proteins that have these properties and are associated with the timing of puberty in order to determine the mechanisms by which ALC may alter their roles and respective contributions to the progression of the pubertal process. Thus, the present research focused on assessing the actions of ALC on Kp as an excitatory influence on puberty, and on Lin28b as a proposed inhibitory influence on the process.

The kisspeptins are a family of protein products of the KiSS-1 gene and are ligands of the G-protein-coupled receptor 54 (GPR54; Navarro et al., 2004b; Tena-Sempere, 2006). The importance of the Kp system to reproduction was revealed when a mutation of GPR54 in human (de Roux et al., 2003) and its deletion in mice (Seminara et al., 2003) resulted in hypogonadotropic hypogonadism and delayed pubertal development. Gene expressions of both KiSS-1 and GPR54 increase at puberty (Navarro et al., 2004a), and the KiSS-1 products, kisspeptin 54 and kisspeptin 10, act at the hypothalamic level to stimulate luteinizing hormone (LH) secretion in rats and rhesus monkeys (Navarro et al., 2004a; Shahab et al., 2005; Thompson et al., 2004), and advance vaginal opening in rats (Navarro et al., 2004b). ALC has been shown to suppress hypothalamic KiSS-1 gene expression (Srivastava et al., 2009), but the assessment of the Kp protein has not been evaluated. An ALC-induced decrease in the expression of this excitatory regulator of LHRH would be

consistent with the ability of ALC to decrease the secretion of this peptide and delay puberty.

The Lin28b gene is a human homolog of Lin28 in the nematode and associated with regulating the processing of micro-RNAs controlling major developmental events in that species. Genome wide association studies have revealed that Lin28b is strongly associated with a later age at menarche in humans (He et al., 2009; Elks et al., 2010; Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009). This association suggests it may be involved in the inhibitory tone that persists prior to the onset of puberty and thus, may be associated with keeping the prepubertal brake on mammalian pubertal development. In support of this, it has recently been shown that the gene expression of Lin28b decreases in male and female rats and female rhesus monkeys as puberty approaches (Sangiao-Alvarellos et al., 2013). Thus, the collective evidence to date suggests that the pre-decline expression of Lin28b may contribute to the inhibitory tone prior to puberty and that its decline may contribute to or signals the progression of the pubertal process. The fact that ALC can delay puberty suggests that it may be able to enhance the prepubertal expression of Lin28b protein in the hypothalamus. Thus, an ALC-induced increase in the expression of Lin28b would be consistent with the ability of ALC to delay puberty and would therefore, further suggest an inhibitory role for this peptide on the pubertal process.

While the stimulatory role of Kp on LHRH secretion at puberty is now well established, the physiological mechanism by which Lin28b may contribute to the inhibitory tone prior to the onset of puberty has not been investigated. One possible avenue for Lin28b to exert the putative inhibitory influence might be to stimulate opioid peptides, which are known to inhibit LHRH secretion (Faletti et al., 1999; Sirinathsinghji et al., 1985). Interestingly, ALC is known to induce opioid peptides (Boyadjieva and Sarkar, 1997; Gianoulakis, 1990; Oswald and Wand, 2004). Taken together, this raises important questions as to whether Lin28b can up-regulate opioid peptides, and whether it can be induced by ALC. Dynorphin (DYN) is an opioid localized within the MBH that is of particular importance. DYN gene expression in the hypothalamus is increased by ALC (Chang et al., 2007), and the DYN peptide has been shown to play an inhibitory role on Kp and LHRH secretion through its actions as a component of the LHRH pulse generator (Lehman et al., 2010; Navarro et al., 2009). Thus, determining the interrelationships between Lin28, DYN and ALC may serve to help discern the role of Lin28 at puberty and whether it is a target for the actions of ALC.

The present study was therefore conducted to: a) determine whether short-term ALC administration can concomitantly suppress the prepubertal expression of Kp and increase the expression of Lin28b proteins, and b) assess the mechanism by which Lin28b exerts its prepubertal inhibition. Taken together, these studies assess whether ALC can differentially affect both excitatory and inhibitory influences that contribute to the timing of puberty.

METHODS

Animals

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 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. Surgical anesthesia was an intraperitoneal injection of 2.5% Tribromoethanol (0.5ml/60g body weight; Sigma Aldrich, St. Louis, MO).

Chronic ALC Experimental Procedure

At twenty-three days of age, female rats were 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. When 26 days old, animals were weighed and divided into three groups. Group 1 consisted of animals that received a 5% ALC liquid-diet, and group 2 consisted of animals that received the companion isocaloric control liquid diet (Bioserve, Inc., Frenchtown, NJ). Group 3 served as an additional set of controls that were cannulated and maintained on rat chow and water, *ad libitum*, throughout the experiment. Each group received their respective diet by an established regimen (Dees and Skelley, 1990) with minor modifications (Srivastava et al. 2009). Briefly, on day 27, the liquid-diets were administered such that the animals received 4 intragastric injections (1.5 ml each) during the lights-on period through the gastric cannula, then 30 mls of the respective diet was provided *ad libitum* during the lights-off period by bottle. On days 28, 30 and 32, the amount of diet per injection was increased by 0.5 ml per injection, and the amount provided by bottle each evening was increased to 35, 40, and 50 ml, respectively. This procedure enabled the liquid-diet and chow-fed control groups to grow at the same rate (Dees and Skelley, 1990). Also, all of the liquid diet-fed animals received approximately the same daily number of calories, and all of the ALC-treated animals received the same daily amount of ALC.

Tissue Collection

On the morning of day 33, all animals were weighed and killed by decapitation 1.5 hours after their last gastric infusion of liquid diet. The animals were confirmed to be in the late juvenile stage of pubertal development (Dees and Skelley, 1990). Briefly, these immature, anestrus rats showed small uterus of less than 95 mg, no intraluminal fluid and closed vaginae. All of the ALC-treated rats fall into this category as a result of their markedly delayed pubertal onset. The controls however, continue to mature and thus show increased amounts of uterine fluid, with almost all of them revealing VO and first ovulation between days 34 and 37 (Dees and Skelley, 1990). Thus, in the present experiment, all of the animals in the three groups were killed on day 33 before controls began reaching maturity. Three controls showed fluid distended uteri, indicating the animals were in first proestrus, thus their tissues were not used in the experiment. Only the late juvenile (still immature) controls were used for comparisons with the immature ALC-treated animals. The brains were removed and the medial basal hypothalamic (MBH) region was dissected out. Tissues were frozen on dry ice at -80 C until analyzed by Western blot analysis.

Immunoblotting

Brain tissues were homogenized in 20mM Tris-Cl, pH (8.0), 1% Igepal CA-630, 2mM EDTA, 10% glycerol, 10mM sodium pyruvate, 10mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.25% protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO) at 4 C. The homogenates were incubated on ice for 30 minutes and centrifuged at 12,000Xg for 15 min. The quantitation of total protein was done by the Pierce 660 nm protein assay reagent (Pierce Biotechnology, Rockford, IL).

Immunoblot analysis was performed as described previously (Srivastava et al., 2014) onto SDS-PAGE under reducing conditions and the separated proteins were electrophoretically transblotted onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dried milk/0.1% Tween 20 in PBS (pH 7.4) for 3 hr and subsequently incubated at 4 C overnight with antibodies specific for rat Kisspeptin (5µg/ml; Phoenix Pharmaceuticals, Inc., Burlingame, CA), Dynorphin (1:250; Santa Cruz Biotechnology, Santa Cruz, CA), and Lin28b (1:250; Cell signaling Technology, Inc., Danvers, MA). Following incubation, membranes were washed in PBS buffer containing 0.05% Tween-20 and then incubated with horseradish peroxidase-labeled secondary antibodies (1: 50,000; Santa Cruz Biotechnology, Inc.) specific for primary antibodies. After washing, the specific protein signals were detected with the enhanced chemiluminescence method (Western Lightning Plus-ECL, PerkinElmer Inc, MA) and quantified with NIH Image J software version 1.43 (National Institute of Health, Bethesda, 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/0.1% Tween-20 in PBS (pH 7.4) for 2 hours. After blocking, membranes were reprobed with an antibody to the β -actin and horseradish peroxidase-labeled secondary antibody, to normalize for the amount of sample loaded. Following washing, the detection and quantitation of β -actin protein was carried out as described above by NIH Image J software.

Effect of Lin 28b on the synthesis of Dynorphin and Kisspeptin protein in the MBH

Twenty-four day old female rats were stereotactically implanted with a stainless steel cannula (23 gauge) in the third ventricle (3V) of the brain. After moving 1.5 mm caudally from bregma, the cannula was lowered 8.2 mm ventrally into the 3V and secured with dental cement (Dees et al. 2005). After four days of recovery, the twenty-eight day old rats were administered either sterile saline, or 500 ng Lin28b/4µl saline (Recombinant human Lin 28b, Prospeco Protein Specialists, Ness Ziona, Israel). The peptide was delivered during a 1 min period of time into the 3V at 0900 h. The animals were killed by decapitation 6 hrs post Lin28b or saline at 1500 h. Brains were removed, placement of the third ventricular cannulae verified, and the animals were confirmed to be in the juvenile stage of pubertal development (Dees and Skelley, 1990). A block of tissue containing the MBH was dissected as described previously (Hiney et al., 2009). All tissues were stored frozen until assayed for DYN and Kp protein expression by Western blot analysis.

Effect of ALC on Dynorphin release from the MBH

Thirty-two day old female rats were killed by decapitation, brains removed, and the MBH was dissected under a stereomicroscope and incubated in a vial containing 350 µl 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 20 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 replaced with medium containing 50 mM ALC for 90 minutes, an incubation period we have used in previous acute ALC studies (Hiney et al. 2003; Hiney et al. 2014). This concentration of ALC is equivalent to a blood ALC level of approximately 230 mg/dL. Following completion of the experiment, medium was frozen until assessment of DYN release by radioimmunoassay and MBH fragments were weighed to nearest 0.01 mg. Only animals in the late juvenile stage of pubertal development were used in this experiment.

DYN Radioimmunoassay

DYN radioimmunoassay was purchased as a kit from Phoenix Pharmaceuticals Inc. (Burlingame, CA). The sensitivity of the assay is 10pg/ml and the intraassay variation was less than 5%.

Statistical Analysis

Differences between the three diet groups were initially analyzed by using ANOVA, with post hoc assessments using the student-Newman-Keuls multiple range test. Because no significant differences were detected between the two control groups in any of the parameters measured, their data were combined for presentation in order to simplify comparative descriptions. The final comparison between the control and ALC-treated groups were analyzed using Student's t-test. Both methods of analysis revealed comparative results. These statistical tests were conducted with INSTAT software (GraphPad Software, San Diego, CA). Probability values less than 0.05 were considered significantly different.

Blood ALC Concentrations

Blood alcohol concentrations (BACs) were assessed from the trunk blood collected at the end of the experiment. Serum was transferred to microcentrifuge tubes for the enzymatic analysis of serum ALC levels (Dees et al., 2005) using a diagnostic kit purchased from Sekisui Diagnostics (Lexington, MA).

RESULTS

The chow-fed and liquid diet-fed control animals showed no differences with regard to body weights or any of the protein measurements during this 6 day study. This is in agreement with previous reports using this feeding regimen and showing similar animal weights and hormone levels, as well as gene and protein expressions between these two control groups of prepubertal, growing animals (Dees and Skelley, 1990; Srivastava et al., 1995; 2009). As a result of this, the controls were combined and are presented in the following figures as a single group in order to simplify comparative descriptions. On the final day of the experiment, the mean±SEM body weights of the animals treated with ALC were less ($p<0.01$) than those of control animals (CON: 92.1±2.9 vs ALC: 72.4±3.1), an effect associated with the known actions of ALC to suppress the serum levels of insulin like

growth factor-1 and growth hormone (Dees et al., 1990; Hiney et al. 1996; Srivastava et al. 2009). The mean $\pm$ SEM BAC was 179 $\pm$ 16 mg/dl when the animals were killed 1.5 hours after their last infusion of the ALC liquid diet on the morning of day 6.

Short-term ALC exposure caused a marked decrease ($p<0.01$) in Kp protein expression in the MBH (figure 1A and B), along with a concomitant increase ($p<0.001$) in Lin28b protein expression (figure 1C and D). This shows for the first time that ALC can stimulate Lin28b, which is associated with the prepubertal inhibitory tone. Since a key mechanism of action of Lin28b directly related to the generation of its apparent inhibitory action was not known, we assessed its potential for inducing DYN, a known inhibitor of LHRH in the MBH. In this regard, we have shown that the central administration of Lin 28b stimulated ($p<0.01$) DYN protein expression (figure 2A and B), but did not alter Kp protein expression (figure 2C and D). As a result of this, the effect ALC on DYN synthesis and release was then assessed. The same MBH tissue samples that were used above (figure 1) for assessing Kp and Lin28b also reveal increased ($p<0.01$) DYN protein levels in ALC-treated animals when compared to the control animals (figure 3). Finally, figure 4 demonstrates that acute *in vitro* exposure to 50mM dose of ALC caused increased ($p<0.01$) DYN release from the prepubertal MBH.

DISCUSSION

The initiation of puberty is the result of an enhanced secretion of hypothalamic LHRH to drive the pubertal process. It is now well accepted that developmental changes in upstream excitatory and inhibitory regulators of LHRH ultimately time the increased pubertal release of the peptide. Because of the excitatory role of Kp on LHRH secretion at puberty (Navarro et al. 2004a; Shahab et al., 2005; Thompson et al., 2004), the association of Lin28b with delayed menarche (He et al., 2009; Elks et al., 2010; Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009), and because of the actions of ALC to depress LHRH/LH release and delay puberty (Dees and Skelley, 1990; Dees et al., 2000) we considered that ALC administration may be a candidate for being able to suppress the synthesis of Kp and stimulate the synthesis of Lin28b; hence, negatively affecting the timing of pubertal development.

In the present study we demonstrated for the first time that short-term ALC administration caused suppressed Kp protein expression in the MBH. This extends our earlier report showing that ALC caused a decrease in KiSS-1 gene expression, which was also associated with decreased circulating levels of LH and E₂ (Srivastava et al., 2009). The suppressed circulating levels of these two important puberty-related hormones following ALC administration has been well-documented in primate (Dees et al., 2000; Dissen et al., 2004) and rodent studies (Dees and Skelley, 1990; Dees et al., 1990, Srivastava et al., 2009) and is further supported by the present results showing suppressed Kp protein available to stimulate LHRH secretion. Thus, our results regarding suppressed Kp synthesis are consistent with the well-known effects of ALC to suppress LHRH and delay puberty. Conversely, we observed an ALC-induced increase in the prepubertal expression of Lin28b protein. Because genome wide association studies mentioned above strongly associated Lin28b with a later age at menarche in humans, and because its gene expression was shown to decrease in animals as they approached puberty (Sangiao-Alvarellos et al., 2013), our

results suggest that ALC enhances Lin28b protein expression and prolongs the elevated prepubertal levels and availability of this peptide; hence, preventing its normal decline at puberty and further indicating that Lin28b may play a physiological role as a prepubertal brake. This would also be consistent with the overall effect of ALC to delay puberty. Interestingly, this delay in puberty associated with elevated Lin28b protein is in agreement with a study showing that rats exposed earlier to elevated levels of sex steroids exhibited enhanced Lin28b gene expression and delayed puberty (Sangiao-Alavrellos et al., 2013). This is also in line with another study showing delayed puberty in transgenic mice overexpressing Lin28a (Zhu et al., 2010). Importantly, the differential response of Kp and Lin28b we described using the same animal tissues reveals a dual action of ALC to affect both excitatory and inhibitory pubertal influences at the same time.

Evidence presented above suggests that Lin28b may normally act as a brake on the pubertal process and that its prolonged expression induced by ALC may contribute to the ALC-related delay in puberty; however, critical information as to the specific actions exerted by Lin28b has not been investigated. Therefore, it became important for us to begin discerning what its downstream targets are as they may relate not only to the normal prepubertal actions of Lin28b, but also to the negative prepubertal consequences of ALC. Opioid peptides have been known for years to suppress LHRH secretion ((Faletti et al., 1999; Sirinathsinghji et al., 1985; Terasawa and Hernandez, 2001) so we therefore considered Lin28b to be a prime candidate for opioid induction, thereby keeping prepubertal LHRH secretion in check. DYN is an opioid peptide that is a component, along with Kp, of the LHRH pulse generator within the MBH (Goodman et al., 2013; Lehman et al., 2010; Navarro et al., 2009). The pulse generator maintains the pulsatile nature of LHRH/LH secretion, and an increase in the amplitude and frequency of this pattern of secretion is observed as puberty approaches (Ojeda and Urbanski, 1994; Plant, 1988). Importantly, Kp acts to increase the frequency of LHRH pulses, whereas DYN acts to suppress the frequency of the LHRH pulses (Goodman et al., 2013). This action of DYN is thought to occur through its action to inhibit Kp producing neurons within the MBH and to suppress LHRH release from the nerve terminals in the median eminence (Goodman et al., 2013). Our results are the first to demonstrate the novel observation that the central administration of Lin28b induced DYN protein expression in the prepubertal MBH; therefore, supporting the concept that Lin28b is an upstream stimulator of DYN, which, as stated above, is a known inhibitor of LHRH release. Interestingly, our results did not show a concomitant decrease in Kp protein content; however, this does not rule out the possibility that any Lin28b induced increase in DYN release could have a fast acting, acute effect to suppress Kp release and thus suppress the pulse of LHRH secretion. Therefore, although more work is needed, we suggest that Lin28b may cause suppression of Kp through its action to stimulate DYN.

Our observations that Lin28b can stimulate DYN protein expression and that ALC can induce the Lin28b peptide are important and support not only a Lin28b-DYN pathway to suppress LHRH secretion prior to the normal onset of puberty, but also a pathway of action by which ALC can delay or prolong the pubertal process. Additional evidence that ALC acts on this pathway is the increased DYN protein expression shown in prepubertal animals. This increase is in agreement with a previous report showing that ALC-induced the gene expression of DYN in adult animals (Chang et al., 2007). Furthermore, we have now

demonstrated that ALC stimulated DYN protein expression *in vivo*, and induced DYN release from MBHs incubated *in vitro*. Taken together, we have shown that the Lin28b-DYN pathway is vulnerable to the effects of ALC and suggest that, at least in part, this is a path by which ALC can suppress LHRH release and delay the normal timing of puberty.

CONCLUSION

It has been accepted for years that the onset of puberty occurs when there is an increase in responsiveness to excitatory inputs to LHRH secretion, with a concomitant decrease in the prepubertal inhibitory tone. In this study we have shown that short-term ALC administration caused suppressed Kp protein expression in the MBH by prepubertal day 33, a time when this excitatory peptide has previously been shown to increase as puberty approaches. Conversely, the ALC caused an increase in Lin28b protein expression in the same tissues, at a time when this proposed inhibitory influence should be decreasing. Importantly, the central administration of the Lin28b peptide stimulates DYN protein expression, a peptide that inhibits Kp and LHRH secretion; thus, indicating for the first time a downstream target that can mediate the action of Lin28b as an inhibitory brake on the pubertal process. Additionally, we have demonstrated that ALC stimulates the synthesis and the release of the DYN peptide. The schematic shown in Fig. 5 illustrates the hypothesized actions and interactions between ALC, Kp, Lin28b and DYN with regard to prepubertal LHRH secretion. While additional studies will be required to more directly assess specific cause and effect relationships as they relate to LHRH secretion, our present results indicate that ALC can simultaneously and differentially alter both excitatory and inhibitory influences on pubertal development. Furthermore, these results suggest ALC can induce and thus prolong the prepubertal elevation in Lin28b, and that Lin28b stimulates DYN which can contribute to the prepubertal inhibitory tone.

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Figure 1.

Effect of chronic ALC exposure on basal Kisspeptin (Kp) and Lin28b protein expression in MBH of prepubertal female rats. (A) Representative Western immunoblot of Kp and β-actin proteins in the MBH isolated from control (lanes 1–3) and ALC-treated (lanes 4–6) animals. (B) Densitometric quantitation of all of the bands from two blots assessing Kp expression in the MBH. These data were normalized to the internal control β-actin protein, and the densitometric units represent the Kp/β-actin ratio. Note that Kp synthesis was decreased in the MBH of the ALC-treated animals. The respective bars illustrate the mean (±SEM) of an N of 6 in each group. **p<0.01 versus control.

(C) Representative Western immunoblot of Lin28b and β-actin proteins in the MBH isolated from control (lanes 1–3) and ALC-treated (lanes 4–6) animals. (D) Densitometric quantitation of all of the bands from two blots assessing Lin28b protein expression in the MBH. These data were normalized to the internal control β-actin protein, and the densitometric units represent the Lin28b/β-actin ratio. Note that Lin28b protein expression was increased in the MBH of the ALC-treated animals. The respective bars illustrate the mean (±SEM) of an N of 6 in each group. ***p<0.001 versus control.

Figure 2.

Effect of central administration of Lin28b on basal DYN and Kp protein expression in the MBH of prepubertal female rats. (A) Representative Western immunoblot of DYN and β -actin proteins in the MBH isolated from control (lanes 1–3) and Lin28b-treated (lanes 4–6) animals. (B) Densitometric quantitation of all of the bands from two blots assessing DYN protein expression in the MBH. These data were normalized to the internal control β -actin protein, and the densitometric units represent the DYN/ β -actin ratio. Note that DYN protein expression was increased in the MBH of the Lin28b-treated animals. The respective bars illustrate the mean ($\pm$ SEM) of an N of 6 in each group. **p<0.01 versus control. (C) Representative Western immunoblot of Kp and β -actin proteins in the MBH isolated from control (lanes 1–3) and Lin28b-treated (4–6) animals. (D) Densitometric quantitation of all of the bands from two blots assessing Kp protein expression in the MBH. These data were normalized to the internal control β -actin protein, and the densitometric units represent the Kp/ β -actin ratio. Note that Lin28b did not induce Kp protein increase in the same MBH tissue samples.

Figure 3. Effect of chronic ALC exposure on basal DYN protein expression in the MBH of prepubertal female rats. (A) Representative Western immunoblot of DYN and β -actin proteins in the MBH isolated from control (lanes 1–3) and ALC-treated (lanes 4–6) animals. (B) Densitometric quantitation of all of the bands from two blots assessing DYN protein expression in the MBH. These data were normalized to the internal control β -actin protein, and the densitometric units represent the DYN/ β -actin ratio. Note that DYN protein expression, increased in the MBH of the ALC-treated animals. The respective bars illustrate the mean ($\pm$ SEM) of an N of 6 in each group. ** $p < 0.01$ versus control.

Figure 4. Effect of ALC on the *in vitro* release of DYN from the MBH of prepubertal female rats
 The MBH of prepubertal female rats were removed and equilibrated in medium only for 20 minutes. This medium was discarded and replaced with medium to establish basal release of DYN (open bar). After basal incubation, the medium was saved then incubated in medium plus 50mM ALC (solid bar). Note that exposure to 50 mM ALC increased the release of DYN over basal secretion. The respective bars illustrate the mean ($\pm$ SEM) of an N of 9 animals ** $p < 0.01$ versus basal.

Figure 5. Actions and interactions between ALC, Kp, Lin28b, and DYN in the MBH

A: It is known that Kp is normally stimulatory on pulsatile luteinizing hormone-releasing hormone (LHRH) secretion (blue solid line) and that once activated DYN can inhibit Kp (red dotted line), which then suppresses the LHRH pulses (red solid line). Our results demonstrate for the first time a mechanism by which Lin28b, through stimulating DYN (blue dotted line) exerts its prepubertal inhibitory tone.

B: This panel depicts for the first time that ALC is capable of inducing Lin28b (blue solid line); thus suggesting that the well-known action of ALC to suppress LHRH secretion is due, at least in part, to its ability to stimulate the Lin28b-DYN pathway (blue dotted line) causing inhibition of Kp (red dotted line). (+) indicates stimulation; (-) indicates inhibition; ALC-alcohol; AP-anterior pituitary; DYN-dynorphin; Kp-kisspeptin; MBH-medial basal hypothalamus; ME-median eminence.