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Regulation of Kisspeptin Synthesis and Release in the Preoptic/ Anterior Hypothalamic Region of Prepubertal Female Rats: Actions of IGF-1 and Alcohol

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Abstract

Background—Alcohol (ALC) causes suppressed secretion of prepubertal luteinizing hormone-releasing hormone (LHRH). Insulin-like growth factor-1 (IGF-1) and kisspeptin (Kp) are major regulators of LHRH and are critical for puberty. IGF-1 may be an upstream mediator of Kp in the preoptic area and rostral hypothalamic area (POA/RHA) of the rat brain, a region containing both Kp and LHRH neurons. We investigated the ability of IGF-1 to stimulate prepubertal Kp synthesis and release in POA/RHA, and the potential inhibitory effects of ALC.

Methods—Immature female rats were administered either ALC (3g/Kg) or water via gastric gavage at 0730 h. At 0900 h both groups were subdivided where half received either saline or IGF-1 into the brain third ventricle. A second dose of ALC (2g/kg) or water was administered at 1130 h. Rats were killed 6 h after injection and POA/RHA region collected.

Results—IGF-1 stimulated Kp, an action blocked by ALC. Upstream to Kp, IGF-1 receptor (IGF-1R) activation, as demonstrated by the increase in insulin receptor substrate 1, resulted in activation of Akt, tuberous sclerosis 2, ras homologue enriched in brain and mammalian target of rapamycin (mTOR). ALC blocked the central action of IGF-1 to induce their respective phosphorylation. IGF-1 and ALC specificity for the Akt activated mTOR pathway were demonstrated by the absence of effects on PRAS40. Furthermore, IGF-1 stimulated Kp release from POA/RHA incubated *in vitro*.

Conclusion—IGF-1 stimulates prepubertal Kp synthesis and release following activation of a mTOR signaling pathway and ALC blocks this pathway at the level of IGF-1R.

Keywords

kisspeptin; puberty; alcohol; IGF-1; mTOR

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Introduction

The onset of puberty is the result of an increase in the synthesis and secretion of luteinizing hormone-releasing hormone (LHRH). This is due to a series of complex interactions within the preoptic/hypothalamic region of the brain in which there is the gradual removal of prepubertal inhibitory influences (Terasawa, 1999; Terasawa and Fernandez, 2001), along with the development of enhanced excitatory influences (Sarkar et al., 1981; Ojeda et al., 1990; Urbanski and Ojeda, 1990; Hiney et al. 1991, 1996; Claypool et al., 2000; Dearth et al., 2000; Navarro et al. 2004a; Thompson et al. 2004). Among the excitatory influences, both insulin like growth factor -1 (IGF-1) and the kisspeptins (Kp), the latter being peptide products of the *KiSS-1* gene, are considered pivotal in the upstream control of LHRH synthesis and secretion at puberty (Hiney et al. 1996; Wilson ME, 1998; Navarro et al. 2004 a, 2004 b, Shahab et al., 2005; Keen et al., 2008). Thus, gaining a better understanding of the actions and potential interactions between these two peptides with regard to the control of LHRH could provide important information about critical events leading to the onset of puberty. In this regard, the IGF-1 peptide has been shown to upregulate prepubertal *KiSS-1* gene expression in the preoptic area and rostral hypothalamic area (POA/RHA) of the rat brain; a region that includes the anteroventral periventricular (AVPV) nucleus (Hiney et al., 2009). More recently, research has shown that Kp synthesis is mediated by an Akt-mammalian target of rapamycin (mTOR) pathway (Srivastava et al., 2016). It is noteworthy that IGF-1 normally increases as puberty approaches and that Akt is a transduction signal that is involved in mediating some of the effects of IGF-1 (Cardona-Gomez et al., 2002). Furthermore, Kp producing neurons in the AVPV nucleus project to LHRH synthesizing neurons in the preoptic area and rostral hypothalamic area (POA/RHA) that express Kp receptors (Parhar et al, 2004; Han et al 2005; Yeo et al. 2011). Taken together, this information provides evidence suggesting important interactions between IGF-1, Kp and LHRH.

Defining the interactions between IGF-1 and Kp is not only important for discerning normal pubertal events, but also provides avenues for determining mechanisms by which neuroendocrine disrupting substances, such as alcohol (ALC), can alter LHRH and the pubertal process. Prepubertal ALC exposure has been shown to act at the hypothalamic level to suppress the release of LHRH (Hiney and Dees 1991; Dissen et al., 2004) resulting in delayed pubertal events in rats (Bo et al., 1982; Dees and Skelley, 1990; Emanuelle et al., 2002), rhesus monkeys (Dees et al., 2000), and adolescent girls (Peck et al., 2011; Richards et al., 2011). In recent years, we have investigated the influences of ALC on both IGF-1 and the *KiSS-1*/Kp system with regard to LHRH synthesis and release at puberty. In this regard, it is well documented that prepubertal ALC administration causes suppressed circulating levels of IGF-1 in female rats (Srivastava et al., 1995; Emanuelle et al., 2002) and rhesus monkeys (Dees et al., 2000); thus, suppressing the amount of the peptide available to the hypothalamus. Importantly, ALC acts within the hypothalamus to inhibit IGF-1 induced LHRH/luteinizing hormone (LH) release (Hiney et al., 1998). Also, both chronic (Srivastava et al., 2009) and acute (Hiney et al., 2010) ALC administration causes suppressed basal *KiSS-1* gene expression in the prepubertal female hypothalamus. Thus, while progress has been made, more attention is needed to define the sites and mechanisms of the actions and

interactions between IGF-1 and ALC regarding physiological control of the prepubertal *Kiss-1/Kp* system, a critical regulator of LHRH synthesis and secretion at the time of puberty.

Materials and Methods

Animals for in vivo study

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 d of age and housed three per cage (Allentown Nexgen individually ventilated caging system) under controlled conditions of light (lights on, 0600h; lights off, 1800h) and temperature (23 °C), with *ad libitum* access to food (Teklad Global 16% Protein rodent diet, Envigo, Houston, TX) 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).

Assessment of Kp protein synthesis

Twenty-two-day-old female rats were implanted with third ventricular (3V) cannulae as described previously (Hiney et al. 2009). After four days of recovery, half of the rats were administered water (control group) and the other half received a dose of ALC (3g/Kg; 1.5 ml 25% ethanol /100 g rat) by gastric gavage at 0730 h. This dose of ALC was chosen because a single intragastric injection to immature female rats yields a moderate blood ALC level and is capable of consistently suppressing LH release (Hiney, et al., 1998; 2003). The animals were undisturbed for 90 min. to allow time for ethanol absorption. The ALC and control groups were then subdivided such that half of the rats in each group were injected with IGF-1 (rat IGF-1, Prospec, Israel, 200ng/3µl) and the other half received an equal volume of saline into the 3V at 0900 h. A second dose of ALC (2g/kg) or water was administered gastrically at 1130 h (4 hr after the first dose) in order to maintain a moderately elevated serum ethanol level over the course of the 6 h after the IGF-1 injection. This overall protocol was repeated several times to complete the study, and each replica experiment always contained control and IGF-1 only groups, and the ALC dose with and without IGF-1. All animals were killed at 1500 h, 6 h after the 3V injection of IGF-1 or saline. The IGF-1 dose and 6 h time point were chosen based on our recent dose response study demonstrating IGF-1 induction of *Kiss-1* gene transcription (Hiney et al., 2009). The brains were removed, 3V placement verified and blocks of tissue containing the POA/RHA were dissected from the brain as described previously (Hiney et al., 2009). This brain region was chosen since, in the rat, populations of both Kp and LHRH producing neurons are present, most of the LHRH neurons express the Kp receptors (Parhar et al., 2004, Han et al., 2005, Messenger et al., 2005) and because IGF-1 has been shown to stimulate *Kiss-1* gene expression in this area (Hiney et al., 2009). The medial basal hypothalamus was not assessed because IGF-1 does not stimulate the *Kiss-1* gene in this region (Hiney et al., 2009) and because this area does not contain the LHRH neurons in the rat (Kozłowski and Dees, 1984), only the nerve terminals. After tissue collection, the samples were frozen on dry ice for protein assessments

by Western blot analysis. Trunk blood was collected at that time for subsequent measurement of blood ALC concentrations by an enzymatic method using a diagnostic kit purchase from BioAssay Systems, Hayward, CA.

Western blot analysis

Brain 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, 0.25% protease inhibitor cocktail (Sigma Aldrich) at 4 C. The homogenates were incubated on ice for 30 min and centrifuged at 12,000×g for 15 min. The concentration of total protein in the resulting supernatant was determined by the Pierce 660nm Protein assay kit (Thermo Scientific, Rockford, IL) using bovine serum albumin as standard. Immunoblot analysis was performed by solubilizing the proteins (100 µg) in a sample buffer containing 62.5 mM Tris-Cl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue and electrophoresed through 4-20% SDS-PAGE for insulin receptor substrate 1 (IRS-1), Proline-rich Akt substrate of 40kDa (PRAS40), Akt, tuberous sclerosis 2 (TSC2), ras homologue enriched in brain (Rheb), mTOR and KP under reducing conditions. The separated proteins were electrophoretically transblotted onto polyvinylidene difluoride membranes. Following transfer, 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 rabbit anti-total-IRS-1 (1:500; Cohesion Biosciences Ltd, London, UK) or anti-p-IRS-1 (Ser307; 1:500; Assay Biotechnology Company, Inc, Sunnyvale, CA), rabbit anti-total (1:1000) or anti-p-Akt (Ser473, 1:3000; Cell Signaling Tech., Danvers, MA), rabbit anti-total (1:2000) or anti-p-TSC2 (Thr1462, 1:250; Cell Signaling Tech., Danvers, MA), rabbit anti-Rheb (1µg/ml; Abcam Inc., Cambridge, MA), rabbit anti-total or anti-p-mTOR (Ser2448, 1:1000; Cell Signaling Tech., Danvers, MA) and rabbit anti-Kisspeptin (3µg/ml; Novus Biologicals, Littleton, CO). The specificity of each primary antibody was checked by preabsorbing with an excess (4-5 fold) of the specific protein/peptide being tested and incubating at 23 C for 3 hr. After the incubation, membranes were washed in PBS/0.1% Tween-20 and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1: 50,000; Santa Cruz Biotech., Santa Cruz, CA) for 2 hour at room temperature. Following washing, the specific signals were detected with the enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer, Shelton, CT) and quantified with NIH Image J software version 1.43 (National Institutes of Health, MD). Subsequently, all membranes were also stripped using Re-Blot Plus kit (EMD Millipore, Temecula, CA) and reprobed with mouse monoclonal antibody to β-actin and goat anti-mouse secondary antibody, to normalize for the amount of sample loading when appropriate. Following washing, the detection and quantitation of β-actin was conducted as described above.

Animals for assessing IGF-1 induced Kp release in vitro

POA/RHA tissues were collected from animals killed either during late juvenile development or during first proestrus. Tissues from the juvenile animals were collected from 28-32 day-old rats prior to the animals entering the peripubertal phase of development. In order to obtain sufficient numbers of proestrus tissues at one time, we administered pregnant mare serum gonadotropin (PMSG; 15 IU/.1 ml saline, Bioworld, Dublin, OH) to 32 day-old

rats to stimulate estradiol (E₂) secretion by the ovaries and induce first proestrus. The tissues were collected from these animals 48 hours later. The rats were confirmed to be in these phases of puberty by criteria we have previously described (Dees and Skelley, 1990). Briefly, juvenile animals all had small uteri (less than 150 mg) with no intraluminal fluid content and closed vaginae. Animals in the late proestrous phase of the peripubertal period were selected and had large uteri (over 300 mg) with a substantial amount of intraluminal fluid and usually closed vaginae. Since some rats show an open vagina at the time of first proestrus, vaginal smears were performed on those animals and the presence of a typical proestrus cytology was confirmed.

Effect of ALC on IGF-1-induced kisspeptin release in vitro

Prepubertal female rats were decapitated, the POA/RHA fragments, which include the AVPV nucleus, were dissected under a stereomicroscope as previously described (Hiney et al., 2009). Briefly, each tissue block was incubated in 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, pH 7.4) inside a Dubnoff shaker (95 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 with fresh medium for 30 min to establish basal release. Subsequently, these media were saved in microcentrifuge tubes then replaced with medium containing IGF-1 (200ng/ml). After a 30 min incubation, the samples were collected and saved for subsequent assessment of kisspeptin using a radioimmunoassay kit purchased from Phoenix Pharmaceuticals (Burlingame, CA). To verify that ALC blocks the IGF-1 induced Kp release, POA/RHA fragments were incubated as above with the following changes. Tissues were incubated for 60 minutes after the basal incubation in medium plus 50 mM ALC, and then collected and incubated an additional thirty minutes in medium with 50 mM ALC plus IGF-1. The 50 mM dose has an initial ALC content approximately equivalent to 220- 230 mg/dL in blood and was chosen because it is the minimal effective dose of ALC that blocks IGF-1 induced LHRH release in vitro (Hiney et al. 1998, 2003). Following completion of the all the experiments, POA/RHA fragments were weighed to nearest 0.1mg.

Statistical Analysis

Student's t test analyzed the differences between basal Kp secretion and IGF-1 stimulated Kp release in the *in vitro* experiments. Multiple comparisons were performed using ANOVA with *post hoc* testing using the Student-Newman-Keuls multiple comparison test between the four treatment groups analyzed by quantitation of the Western blots. These statistical tests were conducted with INSTAT software (GraphPad Software, San Diego, CA). Probability values less than 0.05 were considered significantly different.

Results

IGF-1 and ALC effects on Kp synthesis

The mean ($\pm$ SEM) blood ALC concentration two hours after the second ALC dose was 165 $\pm$ 10.2 mg/dL, similar to moderate levels attained in previous studies (Dees et al., 2005; Hiney et al., 2010). Figure 1 shows that the central administration of IGF-1 to control animals induced an increase in Kp protein expression in the POA/RHA. The ALC-treated

animals that received the 3V injection of saline did not reveal altered basal Kp expression, but the increase in Kp expression induced by the central delivery of IGF-1 was blocked by the presence of ALC. Although these novel effects of IGF-1 and ALC are important, it then became necessary to more completely delineate the signaling pathway utilized by IGF-1 within the POA/RHA, and determine the site and mechanism by which ALC altered the IGF-1 induction of Kp.

Since Akt is a transduction signal mediating some of the actions of IGF-1 (Cardona-Gomez 2002), including activation of mTOR (Srivastava et al, 2016), a regulator of Kp (Roa et al., 2009), we used the same tissues as above to assess the effects of IGF-1 and ALC on an interactive pathway from Akt to mTOR. Figure 2 demonstrates that central delivery of IGF-1 to control animals stimulated an increase in the phosphorylation of the Akt (p-Akt) protein expression in the POA/RHA compared to those control animals that received saline. Furthermore, although ALC did not alter basal Akt or p-Akt expressions after the central delivery of saline, the drug blocked the central action of IGF-1 to induce p-Akt. Downstream from Akt, we showed that the central administration of IGF-1 to control animals also stimulated the phosphorylation of TSC2 compared to the controls that received the saline (Fig. 3 A&B). This action to phosphorylate TSC2 subsequently allowed for the increased expression observed in Rheb (Fig. 3 C&D), and therefore, inducing the increased expression of mTOR protein (Fig. 4). Figures 3 and 4 also show that the basal expressions of TSC2, Rheb and mTOR proteins were not altered in the ALC-treated animals by the central delivery of saline, but that the ALC prevented the central delivery of IGF-1 from stimulating increased expressions of each of these proteins.

While the above reveals an IGF-1-induced Akt signaling pathway to mTOR, we could not rule out the possibility that an alternate pathway utilized by Akt to regulate mTOR was also activated by IGF-1. Therefore, we assessed whether IGF-1 activated Akt would alter PRAS40, the inhibitory subunit of the mTOR complex (Wang et al 2012). Our results indicate that neither the central delivery of IGF-1 to control animals ($0.53 \pm .04$), nor ALC administration either without ($0.55 \pm .03$) or with ($0.51 \pm .04$) the administration of IGF-1 altered PRAS40 expression compared to the control animals that received saline centrally ($0.48 \pm .04$).

In order to determine the site and mechanism by which ALC inhibits IGF-1 regulated mTOR and Kp synthesis, we assessed the effects of ALC upstream to Akt within the POA/RHA. The results showed that in control animals the centrally delivered IGF-1 stimulated phosphorylation of the main substrate of the IGF-1 receptor (IGF-1R), IRS-1 protein (Fig. 5), thus, confirming peptide activation of IGF-1R, and ultimately, the downstream increases in Akt, Rheb, mTOR and Kp proteins shown above. Furthermore, figure 5 also demonstrates that basal expression of IRS-1 was not altered in the ALC-treated animals by the central delivery of saline; however, the ALC prevented the IGF-1 stimulation of IRS-1, indicating the site of the inhibitory effect of ALC was at the IGF-1R/IRS-1 complex.

IGF-1 and ALC effects on Kp release

To determine the ability of IGF-1 to stimulate Kp release, POA/RHA tissues from immature rats collected during juvenile and first proestrus phases of development were incubated *in*

vitro. Results revealed that IGF-1 did not alter Kp secretion as compared to their basal levels from the tissues obtained from juvenile animals (basal: 1.32 ± 0.10 vs IGF-1: 1.08 ± 0.11 ; N=16). However, tissues obtained from animals during their first proestrus showed that IGF-1 (200ng/ml) markedly increased ($P<0.001$) the release of Kp when compared to their basal levels of secretion (Fig. 6A). As expected, since IGF-1Rs are blocked by ALC, no IGF-1 stimulation was observed from the proestrus tissues when ALC (50 mM) was present in the medium (Fig 6B).

Discussion

Prepubertal ALC consumption by young girls has been associated with delayed breast development (Peck et al., 2011) and the onset of menarche (Richards et al., 2011). Also girls using ALC have a four times greater chance of showing signs of delayed puberty than non-users (Peck et al., 2011). Animal models show very similar results regarding ALC-induced delayed pubertal development. In rats, ALC caused a delay in vaginal opening and the age of first estrus (Dees and Skelley, 1990; Emanuelle et al., 2002), and in rhesus monkeys, ALC caused a delay in the development of a regular monthly pattern of menstruation (Dees et al., 2000). Animal studies also demonstrated that altered pubertal development resulting from ALC is due to an action within the hypothalamus to suppress LHRH synthesis and secretion and therefore, its ability to stimulate the puberty-related pituitary and ovarian hormones involved in the developmental process (for review, see Dees et al., 2017). Because LHRH is the driving force behind the onset of puberty, identifying factors that control its synthesis and secretion are important not only for addressing control of normal puberty, but also for assessing mechanisms by which ALC, for example, can alter its release and hence, the progression of puberty.

Although several neuromodulators can affect LHRH secretion, IGF-1 and Kp are now considered pivotal upstream signaling components that contribute to regulation of LHRH and the timing of puberty (Hiney et al. 1991, 1996; Wilson ME, 1998; Navarro et al. 2004a, 2004b; Thompson et al., 2004; Shahab et al., 2005; Keen et al., 2008). Importantly, centrally administered IGF-1 has been shown to upregulate the *Kiss-1* gene in the POA/RHA obtained from juvenile, anestrous animals with normally low levels of E_2 and thus, suggesting an IGF-1 action and not that of E_2 (Hiney et al., 2009). Furthermore, the stimulation of endogenous IGF-1 caused an increase in Kp protein expression in the same brain region (Srivastava et al., 2016); thus, suggesting IGF-1 is an upstream regulator of Kp synthesis. The ability of ALC to block the central action of IGF-1 to induce *Kiss-1* gene expression (Hiney et al., 2010) prompted us to more closely investigate IGF-1 and ALC interactions as they relate to the control of Kp and ultimately, LHRH. Similar to the above study that showed IGF-1 induction of *Kiss-1* gene expression, we now demonstrate that the direct central delivery of IGF-1 caused increased Kp protein expression in the POA/RHA of anestrous animals, an action that was blocked by ALC. To determine the upstream site of this action we initially assessed the effect of ALC on a recently identified Akt mediated pathway used by IGF-1 to induce Kp expression (Srivastava et al, 2016). In this regard, we showed that ALC blocked the IGF-1 induced phosphorylation of Akt, an action that is in agreement with a previous study (Hiney et al., 2010). Using the same tissues, we demonstrated here that downstream from Akt the ALC suppressed phosphorylation of TSC2,

Rheb, and finally, mTOR. This suppression of IGF-1 induced mTOR by ALC is important, since mTOR is a known regulator of *Kiss-1*/Kp (Roa et al., 2009), and thus, LHRH neuronal activity.

To confirm the above pathway specificity, an alternate Akt pathway to mTOR was assessed. In this case, activation of PRAS40 would result in suppressed mTOR (Wang et al. 2012). This possibility was eliminated since neither IGF-1 stimulation of Akt, or ALC alone, affected basal PRAS40 expression. Thus, it is clear from these studies that while a specific IGF-1 induced transduction signaling pathway mediated by Akt was affected by ALC, the exact site and mechanism of this action to inhibit the IGF-1 induced Kp synthesis appeared to be upstream to Akt. Subsequent assessments upstream to Akt revealed that IGF-1 induced the phosphorylation of IRS-1 in the POA/RHA; thus, confirming IGF-1 peptide activation of the IGF-1R. Importantly, ALC blocked this action, thereby demonstrating the inhibitory effect of ALC on the IGF-1R/IRS-1 complex.

Current evidence indicates that once Kp is released it stimulates LHRH synthesis and secretion by a direct action on LHRH neuronal perikarya present in the POA/RHA. In this regard, Kp receptors are expressed on the majority of LHRH neurons localized in the POA/RHA of the rat brain (Parhar et al, 2004; Han et al., 2005; Messenger et al., 2005) and Kp nerve processes have been shown to be closely associated with these neurons (Clarkson et al., 2006; Smith et al., 2008; Decourt et al., 2008). The present results now demonstrate that while IGF-1 was not effective in inducing Kp release from POA/RHA explants removed from rats during late juvenile development, when E₂ levels are low, but was capable of stimulating the release of Kp from explants removed from rats in their first proestrus. This is important since Kp is a potent stimulator of LHRH/LH release during the proestrus phase of development (Clarkson et al., 2010; Popa et al., 2008; Clarkson et al., 2009), when E₂ levels are known to be rising toward the LHRH/LH surge. Furthermore, firing rates of Kp producing neurons in the RHA are increased during proestrus (Ducret et al., 2010), and at this time Kp can directly depolarize and induce LHRH neuronal firing rates *in vitro* (Liu et al., 2008; Zhang et al., 2008; Pielecka et al., 2008). Hence, the action we describe here with regard to IGF-1 stimulated Kp release during proestrus supports the fact Kp neurons in the RHA region of the rodent brain play a pivotal role in relaying the positive feedback effect of rising E₂ to the LHRH neurons at this time of development (Terasawa et al., 1980; Wintermantel et al., 2006). Thus, in addition to stimulating Kp synthesis, our results demonstrate a role for IGF-1 in controlling the prepubertal release of Kp and therefore, ultimately contributing to the synthesis and release of LHRH as puberty approaches. Additionally, as described above regarding synthesis, the ability of IGF-1 to stimulate Kp release was also inhibited by the action of ALC to block the IGF-1R.

The results from this study demonstrate for the first time the ability of IGF-1 to stimulate prepubertal synthesis of the Kp protein, as well as its peripubertal release within the POA/RHA. The IGF-1 influence was due to the peptide activating an IGF-1R/Akt mediated signaling pathway regulating Kp. Furthermore, we show that ALC acts at the level of the IGF-1R to block the downstream components of the pathway resulting in suppressed prepubertal Kp protein synthesis, and an inhibition in its release during first proestrus. Figure 7A represents a schematic drawing depicting the IGF-1 mediated pathway to Kp and

7B reveals the upstream site of the ALC inhibition. Overall, these data show conclusively an important prepubertal interrelationship between IGF-1 and Kp, and demonstrates the ability of ALC to alter this relationship causing suppressed Kp, a critical prepubertal regulator of LHRH. We suggest this action, at least in part, contributes to the ALC-induced suppression in LHRH and delayed puberty.

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References

- Bo WJ, Krueger WA, Rudeen PK, Symmes SK. Ethanol-induced alterations in the morphology and function of the rat ovary. *Anat Rec.* 1982; 202:255–260. [PubMed: 7199834]
- Cardona-Gomez GP, Mendez P, Garcia-Segura LM. Synergistic interaction of estradiol and insulin-like growth factor-1 in the activation of P13K/Akt signaling in the adult rat hypothalamus. *Mol Brain Research.* 2002; 107:80–88. [PubMed: 12414126]
- Clarkson J, Han SK, Liu X, Lee K, Herbison AE. Neurobiological mechanism underlying kisspeptin activation of gonadotropin-releasing hormone (GnRH) neurons at puberty. *Mol Cell Endocrinol.* 2010; 324:45–50. [PubMed: 20109523]
- Clarkson J, Herbison AE. Postnatal Development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology.* 2006; 147:5817–5825. [PubMed: 16959837]
- Clarkson J, Herbison AE. Oestrogen, kisspeptin, GPR54 and the pre-ovulatory luteinizing hormone surge. *J Neuroendocrinol.* 2009; 21:305–311. [PubMed: 19207812]
- Claypool LE, Kasuya E, Saitoh Y, Marzban F, Terasawa E. N-methyl-D,L-aspartate induces the release of LHRH in the prepubertal and pubertal female rhesus monkey as measured by in vivo push-pull perfusion in the stalk-median eminence. *Endocrinology.* 2000; 141:219–228. [PubMed: 10614642]
- Dearth RK, Hiney JK, Dees WL. Leptin acts centrally to induce the prepubertal secretion of luteinizing hormone in the female rat. *Peptides.* 2000; 21:387–392. [PubMed: 10793221]
- Decourt C, Tillet Y, Caraty A, Franceschini I, Briant C. Kisspeptin immunoreactive neurons in the equine hypothalamus interactions with GnRH neuronal system. *J Chem Neuroanat.* 2008; 36:131–137. [PubMed: 18761083]
- Dees WL, Skelley CW. The effects of ethanol during the onset of female puberty. *Neuroendocrinology.* 1990; 51:64–69. [PubMed: 2106089]
- Dees WL, Skelley CW, Hiney JK, Johnston CA. Actions of ethanol on hypothalamic and pituitary hormones in prepubertal female rats. *Alcohol.* 1990; 7:21–25. [PubMed: 1968748]
- 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, Hiney JK, Srivastava VK. Alcohol and puberty: mechanisms of delayed development. *Alcohol Research: Current Reviews, Journal of the NIAAA.* 2017; 38:277–282.
- Dees WL, Srivastava VK, Hiney JK. Alcohol alters insulin-like growth factor-1 activated Oct 2 POU domain gene expression in the immature female hypothalamus. *J Stud Alcohol.* 2005; 66:35–45. [PubMed: 15830901]
- Dissen GA, Dearth RK, Scott HM, Ojeda SR, Dees WL. Alcohol alters prepubertal luteinizing hormone secretion in immature female rhesus monkeys by a hypothalamic action. *Endocrinology.* 2004; 145:4558–4564. [PubMed: 15217984]
- Ducret E, Gaidamaka G, Herbison AE. Electrical and morphological characteristics of anteroventral periventricular nucleus kisspeptin and other neurons in the female mouse. *Endocrinology.* 2010; 151:2223–2232. [PubMed: 20211970]
- Emanuele N, Ren J, LaPaglia N, Steiner J. Ethanol disrupts female mammalian puberty: age and dependence. *Endocrine.* 2002; 18:247–254. [PubMed: 12450316]

- Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *J Neuroscience*. 2005; 25:11349–11356.
- Hiney JK, Ojeda SR, Dees WL. Insulin-like growth factor (IGF-I) stimulates LHRH release from the prepubertal female median eminence in vitro. *Neuroendocrinology*. 1991; 54:420–423. [PubMed: 1758585]
- Hiney JK, Srivastava VK, Nyberg CL, Ojeda SR, Dees WL. Insulin-like growth factor-1 (IGF-1) of peripheral origin acts centrally to accelerate the initiation of female puberty. *Endocrinology*. 1996; 137:3717–3727. [PubMed: 8756538]
- Hiney JK, Srivastava VK, Lara F, Dees WL. Ethanol blocks the central action of IGF-1 to induce luteinizing hormone secretion in the prepubertal female rat. *Life Sci*. 1998; 62:301–308. [PubMed: 9450501]
- Hiney JK, Dearth RK, Srivastava V, Rettori V, Dees WL. Actions of ethanol on epidermal growth factor receptor activated luteinizing hormone secretion. *J Stud Alcohol*. 2003; 64:809–816. [PubMed: 14743943]
- Hiney JK, Srivastava VK, Pine MD, Dees WL. Insulin-like growth factor-1 activates *Kiss-1* gene expression in the brain of the prepubertal female rat. *Endocrinology*. 2009; 150:376–384. [PubMed: 18703622]
- 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]
- Keen KL, Wegner FH, Bloom SR, Ghatei Ma, Terasawa E. An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. *Endocrinology*. 2008; 149:151–157.
- Koslowski GP, Dees WL. Immunocytochemistry for LHRH neurons in the arcuate nucleus area of the rat: fact or artifact? *J Histochem Cytochem*. 1984; 32:83–91. [PubMed: 6690601]
- Liu X, Lee K, Herbison AE. Kisspeptin excites gonadotropin-releasing hormone neurons through a phospholipase C/calcium-dependent pathway regulating multiple ion channels. *Endocrinology*. 2008; 149:4605–4614. [PubMed: 18483150]
- Messenger S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci*. 2005; 102:1761–1776. [PubMed: 15665093]
- 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*. 2004a; 145:4565–4574. [PubMed: 15242985]
- Navarro VM, Fernandez-Fernandez R, Castellano JM, Roa J, Mayen A, Barreiro ML, Gaytan F, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M. Advanced vaginal opening and precocious activation of the reproductive axis by Kiss-1 peptide, the endogenous ligand of GPR54. *J Physiol*. 2004b; 561:379–386. [PubMed: 15486019]
- 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. [PubMed: 2263621]
- Parhar IS, Ogawa S, Sakuma Y. Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. *Endocrinology*. 2004; 145:3613–3618. [PubMed: 15155576]
- Pielecka-Fortuna J, Chu Z, Moenter SM. Kisspeptin act directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. *Endocrinology*. 2008; 149:1979–1986. [PubMed: 18162521]
- Peck JD, Peck BM, Skaggs VJ, Fukushima M, Kaplan HB. Socio-environmental factors associated with pubertal development in female adolescents: The role of tobacco and alcohol use. *J Adolesc Health*. 2011; 48:241–246. [PubMed: 21338894]

- Popa SM, Clifton DK, Steiner RA. The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. *Ann Rev Physiol.* 2008; 70:213–238. [PubMed: 17988212]
- Richards MA, Oinonen KA. Age at menarche is associated with divergent alcohol use patterns in early adolescence and early adulthood. *J Adolesc.* 2011; 34:1065–1076. [PubMed: 21115194]
- Roa J, Garcia-Galiano D, Varela L, Sanchez-Garrido MA, Pineda R, Castellano JM, Ruiz-Pino F, Romero M, Aguilar E, Lopez M, Gaytan F, Dieguez C, Pinilla L, Tena-sempere M. The mammalian target of rapamycin as novel central regulator of puberty onset via modulation of hypothalamic Kiss1 system. *Endocrinology.* 2009; 150:5016–5026. [PubMed: 19734277]
- 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]
- Shahab M, Mastronardi C, Seminara S, Crowley WF, Ojeda SR, Plant TM. Increased hypothalamic GPR54 signaling: A potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci.* 2005; 102:2129–2134. [PubMed: 15684075]
- Smith JT, Collen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, Bateman K, Goodman RL, Tilbrook AJ, Ubuka T, Bentley GE, Clarke IJ, Lehman MN. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. *Endocrinology.* 2008; 149:5770–5782. [PubMed: 18617612]
- Srivastava V, Hiney JK, Nyberg CL, Dees WL. Influence of ethanol (ETOH) on the synthesis of insulin-like growth factor-1 (IGF-1) and the IGF-1 receptor (IGF-1R) during female puberty. *Alcohol Clin Exper Res.* 1995; 19:1467–1473. [PubMed: 8749812]
- Srivastava VK, Hiney JK, Dees WL. Short-term alcohol administration alters KiSS-1 gene expression in the reproductive hypothalamus of the prepubertal female rat. *Alcohol Clin Exper Res.* 2009; 33:1605–1615. [PubMed: 19519717]
- Srivastava VK, Hiney JK, Dees WL. Manganese stimulated kisspeptin is mediated by the IGF-1/akt/ mammalian target of rapamycin pathway in the prepubertal female rat. *Endocrinology.* 2016; 157:3233–3241. [PubMed: 27309941]
- 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]
- Terasawa E, Wiegand SJ, Bridson WE. A role for medial preoptic nucleus on afternoon of proestrus in female rats. *Am J Physiol.* 1980; 238:E533–E539. [PubMed: 7189967]
- Thompson EL, Patterson M, Murphy KG, Smith KL, Dhillo WS, Todd JF, Ghatei MA, Bloom SR. Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. *J Neuroendocrinology.* 2004; 16:850–858. [PubMed: 15500545]
- 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; 126:774–1776.
- Wang H, Zhang Q, Wen Q, Zheng Y, Philip L, Jiang H, Lin J, Zheng W. Proline-rich Akt substrate of 40 kda (PRAS40): A novel downstream target of PI3k/Akt signaling pathway. *Cell Signal.* 2012; 24:17–24. [PubMed: 21906675]
- Wilson ME. Premature elevation in serum insulin-like growth factor-1 advances first ovulation in monkeys. *J Endocrinology.* 1998; 158:247–257. [PubMed: 9771469]
- Wintermantel TM, Campbell RE, Porteous R, Bock D, Gröne HJ, Todman MG, Korach KS, Greiner E, Pérez CA, Schütz G, Herbison AE. Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron.* 2006; 52:271–280. [PubMed: 17046690]
- Yeo SH, Herbison AE. Proliferations of arcuate nucleus and rostral periventricular kisspeptin neurons in adult female mouse brain. *Neuroendocrinology.* 2011; 152:2387–2399.
- Zhang C, Roepke TA, Kelly MJ, Røbbejleiv OK. Kisspeptin depolarizes gonadotropin-releasing hormone neurons through activation of TRPC-like cationic channels. *J Neurosci.* 2008; 28:4423–4434. [PubMed: 18434521]

Figure 1.

Effects of IGF-1 and acute ALC exposure on Kp protein in the POA/RHA of prepubertal female rats. A) Representative Western blot of Kp and β -actin proteins from control (lanes 1-3), IGF-1 (lanes 4-6), ALC (7-9) and IGF-1 + ALC (lanes 10-12) animals. B) Densitometric quantification of all bands assessing the Kp protein. These data were normalized to the internal control β -actin protein. IGF-1 (checked bar) induced an increase in Kp over control (solid bar) animals. Note that 3g/kg exposure to ALC (open bar) did not alter basal expression of Kp protein levels, but the IGF-1 induced expression of Kp protein was blocked by acute ALC exposure (hatched bar). Each bar represents the mean $\pm$ SEM of the Kp/ β -actin ratio. The number of animals represented by each bar is 6. ** $p < 0.01$.

Figure 2.

Effects of IGF-1 and acute ALC exposure on Akt protein in the POA/RHA of prepubertal female rats. A) Representative Western blot of phosphorylated (p)-Akt and total Akt from control (lanes 1-3), IGF-1 (lanes 4-6), ALC (7-9) and IGF-1 + ALC (lanes 10-12) animals. B) Each bar represents the mean $\pm$ SEM of the densitometric quantification of all bands assessing the p-Akt normalized to total Akt protein. IGF-1 (checked bar) stimulated an increase in p-Akt ($p < 0.05$) over control (solid bar) animals. Note that 3g/kg exposure to ALC (open bar) did not affect phosphorylation of Akt but the IGF-1 induced expression of p-Akt was blocked by acute ALC exposure (hatched bar). The number of animals represented by each bar is 6. * $p < 0.05$.

Figure 3.

Effects of IGF-1 and acute ALC exposure on TSC2 and Rheb proteins in the POA/RHA of prepubertal female rats. A) Representative Western blot of phosphorylated (p)- and total-TSC2 proteins from control (lanes 1-3), IGF-1 (lanes 4-6), ALC (7-9) and IGF-1 + ALC (lanes 10-12) animals. B) Each bar represents the mean $\pm$ SEM of the densitometric quantification of all bands assessing p-TSC2 protein normalized to total TSC2 protein. C) Representative Western blot of Rheb and β -actin proteins from control (lanes 1-3), IGF-1 (lanes 4-6), ALC (7-9) and IGF-1 + ALC (lanes 10-12) animals. D) Densitometric quantification of all bands assessing Rheb protein normalized to β -actin protein. IGF-1 (checked bars) induced an increase in p-TSC2 and Rheb over control (solid bars) animals. Note that 3g/kg exposure to ALC (open bars) did not alter basal expression of either protein, but the IGF-1-induced increase of p-TSC2 and Rheb proteins were both blocked by acute ALC exposure (hatched bars). The number of animals represented by each bar is 6.

** $p < 0.01$, * $p < 0.05$.

Figure 4.

Effects of IGF-1 and acute ALC exposure on mTOR protein in the POA/RHA of prepubertal female rats. A) Representative Western blot of phosphorylated (p)-mTOR and total mTOR from control (lanes 1-3), IGF-1 (lanes 4-6), ALC (7-9) and IGF-1 + ALC (lanes 10-12) animals. B) Each bar represents the mean $\pm$ SEM of the densitometric quantification of all bands assessing the p-mTOR normalized to total mTOR protein. IGF-1 (checked bar) stimulated an increase in p-mTOR over control (solid bar) animals. Note that 3g/kg exposure to ALC (open bar) did not affect phosphorylation of mTOR but the IGF-1 induced expression of p-mTOR was blocked by acute ALC exposure (hatched bar). The number of animals represented by each bar is 6. * $p < 0.05$.

Figure 5.

Effects of IGF-1 and acute ALC exposure on IRS-1 protein in the POA/RHA of prepubertal female rats. A) Representative Western blot of phosphorylated (p)- and total-IRS-1 proteins from control (lanes 1-3), IGF-1 (lanes 4-6), ALC (7-9) and IGF-1 + ALC (lanes 10-12) animals. B) Each bar represents the mean $\pm$ SEM of the densitometric quantification of all bands assessing p-IRS-1 protein normalized to total IRS-1 protein. IGF-1 (checked bar) induced an increase in p-IRS-1 over control (solid bar) animals. Note that 3g/kg exposure to ALC (open bar) did not alter basal expression of p-IRS-1, but that the IGF-1 induced increase of p-IRS-1 was blocked by acute ALC exposure (hatched bar). The number of animals represented by each bar is 6. ** $p < 0.01$.

Figure 6.

IGF-1 induced Kp secretion from POA/RHA of female rats in first proestrus and the effect of ALC. A) Solid bar represents the mean ($\pm$ SEM) basal secretion of Kp from POA/RHA tissues in medium only. Following the basal incubation, tissues were stimulated with IGF-1 (200 ng/ml) and the checked bar represents the mean ($\pm$ SEM) of the IGF-1 induced Kp release. B) Solid bar represents the mean ($\pm$ SEM) basal secretion of Kp. Following the basal incubation, tissues were incubated in the presence of 50 mM ALC and IGF-1. Note, as expected, ALC blocked the IGF-1 induced Kp release (hatched bar). ** $p < 0.01$. N=16 for IGF-1 animals and N-14 for IGF-1 + ALC animals.

Figure 7.

Schematic drawing showing the effects of IGF-1 and ALC on prepubertal Kp synthesis and release. A) IGF-1 activates the IGF-1R/IRS-1 complex, which subsequently initiates critical phosphorylations within the Akt mediated pathway to mTOR; hence resulting in increased Kp synthesis and release. B) ALC blocks the activation of IGF-1R/IRS-1 complex by inhibiting the phosphorylation of IRS-1 resulting in the inhibition of key downstream phosphorylations within the Akt pathway to mTOR, thus suppressing Kp synthesis and release. IGF-1, Insulin-like growth factor 1; IGF-1R; insulin-like growth factor receptor 1; IRS-1, insulin receptor substrate 1; Kp, kisspeptin; mTOR, mammalian target of rapamycin; phosphorylated site, p; ras homologue enriched in brain, Rheb; TSC1, tuberous sclerosis complex 2.