The Mammalian Target of Rapamycin as Novel Central Regulator of Puberty Onset via Modulation of Hypothalamic Kiss1 System

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The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that operates as sensor of cellular energy status and effector for its coupling to cell growth and proliferation. At the hypothalamic arcuate nucleus, mTOR signaling has been recently proposed as transducer for leptin effects on energy homeostasis and food intake. However, whether central mTOR also participates in metabolic regulation of fertility remains unexplored. We provide herein evidence for the involvement of mTOR in the control of puberty onset and LH secretion, likely via modulation of hypothalamic expression of Kiss1. Acute activation of mTOR by L-leucine stimulated LH secretion in pubertal female rats, whereas chronic L-leucine infusion partially rescued the state of hypogonadotropism induced by food restriction. Conversely, blockade of central mTOR signaling by rapamycin caused inhibition of the gonadotropic axis at puberty, with significantly delayed vaginal opening, decreased LH and estradiol levels, and ovarian and uterine atrophy. Inactivation of mTOR also blunted the positive effects of leptin on puberty onset in food-restricted females. Yet the GnRH/LH system retained their ability to respond to ovariectomy and kisspeptin-10 after sustained blockade of mTOR, ruling out the possibility of unspecific disruption of GnRH function by rapamycin. Finally, mTOR inactivation evoked a significant decrease of Kiss1 expression at the hypothalamus, with dramatic suppression of Kiss1 mRNA levels at the arcuate nucleus. Altogether our results unveil the role of central mTOR signaling in the control of puberty onset and gonadotropin secretion, a phenomenon that involves the regulation of Kiss1 and may contribute to the functional coupling between energy balance and gonadal activation and function. **(*Endocrinology* 150: 5016–5026, 2009)**

Maturation and function of the reproductive system are metabolically demanding phenomena gated by a network of nutritional and hormonal cues, responsible for the coupling between the fuel reserves of the organism and its capacity to reproduce (1–3). Most of the metabolic regulators of reproductive axis integrate at the central nervous system (CNS), and specifically at discrete hypothalamic areas, in which they impinge onto the neuronal circuitries governing GnRH secretion (2, 3). As a prototypic example, the adipose hormone, leptin, has been demonstrated to conduct permissive/stimulatory actions on the gonadotropic axis by (indirectly) modulating GnRH neu-

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Abbreviations: AMPK, AMP-activated protein kinase; ARC, arcuate nucleus; AVPV, anteroventral paraventricular nucleus; CNS, central nervous system; Crtc1, cAMP response element-binding protein-regulated transcriptional coactivator-1; DMSO, dimethylsulfoxide; E2, 17β-estradiol; i.c.v., intracerebroventricular; Kp-10, kisspeptin-10; L-Leu, L-leucine; mTOR, mammalian target of rapamycin; OVX, ovariectomy; p, phosphorylated; S6K1, S6 kinase1; semi-Q, semiquantitative; VO, vaginal opening.

rons (1). Likewise, other metabolic hormones modulate reproductive maturation and/or function (2, 4). Yet whereas the nature of many of those regulators has been elucidated recently, the molecular mechanisms for their integration and action at the CNS remain ill defined.

The mammalian target of rapamycin (mTOR) is a ubiquitous serine/threonine kinase regulated by different stressors, growth factors, nutrients, and hormones, which participates in the control of key cellular functions, including cell proliferation, growth, and metabolism (5–9). This kinase system exists as two complexes: the rapamycin-sensitive, mTORC1, responsible for the integration of nutritional and metabolic cues as well as different stressors, mostly by targeting S6 kinase1 (S6K1) and 4E-binding protein 1 (5–8, 10); and mTORC2, which operates via activation of Akt/protein kinase B to modulate cytoskeletal reorganization and is largely rapamycin insensitive (5–7, 10). In addition to its role as sensor of intracellular energy status and key environmental cues, recent evidence suggests that mTOR signaling at discrete neuronal populations within the hypothalamic arcuate nucleus plays a key function in the control of energy homeostasis by linking the state of body fuel reserves and food intake (11). Notably, hypothalamic mTOR has been proposed as mediator for the anorectic effects of leptin because leptin enhanced mTOR activity (11), and its effects on food intake appeared to require preserved mTOR signaling (11). Conceivably, other fuel-sensitive kinases, such as AMP-activated protein kinase (AMPK) (12), are likely to cooperate with mTOR in the central regulation of food intake and energy balance (11). Further interest on the mTOR pathway has been fostered recently by the proposal that it may play a major role as link between obesity and carcinogenesis (13).

Our understanding of the neuroendocrine mechanisms responsible for the control of reproductive axis, and its modulation by metabolic cues, has been revolutionized by the identification of kisspeptins, encoded by the *Kiss1* gene, and their receptor, G protein-coupled receptor-54 (also termed Kiss1R) (14, 15). Indeed, the hypothalamic Kiss1 system has been proposed as critical transmitter of metabolic information onto the centers governing the reproductive axis (16). Thus, conditions of negative energy balance and metabolic stress inhibit hypothalamic expression of Kiss1 (17–20), whereas kisspeptin treatment was sufficient to ameliorate gonadotropin levels in those conditions (18). The metabolic regulation of Kiss1 seems to involve leptin because leptin receptors have been described in kisspeptin neurons in ob/ob mice (21), and leptin has been shown to rescue Kiss1 mRNA levels in rodent models of leptin deficiency (18, 21). However, the molecular mechanisms for the transmission of leptin effects onto *Kiss1* gene transcription, and its potential integration with other regulators of Kiss1 expression, are yet to be elucidated.

Despite the proposed role of mTOR signaling as hypothalamic gauge for the whole-body control of energy homeostasis (22), its potential function in the control of other metabolically gated neurohormonal systems, such as the reproductive axis, remains to date virtually unexplored. In this context, assessment of the contribution of central mTOR to metabolic regulation of fertility is posed of not only physiological relevance but also potential physiopathological implications; mTOR inhibitors are being widely used in cancer patients and organ transplantation, and chronic treatment with these drugs has been reported to impair gonadal function through as-yet-ill-defined mechanisms (23). Using pharmacological manipulations coupled to functional tests and expression analyses, we provide herein compelling evidence for the involvement of central mTOR signaling in the control of puberty onset and gonadotropin secretion in the female, a phenomenon that seems to involve the modulation of Kiss1 expression at the hypothalamus.

Materials and Methods

Wistar female rats were used. Experimental procedures were approved by Córdoba University Ethical Committee and in accordance with European Union normative. L-Leucine (L-Leu) was purchased from Scharlau Chemie (Barcelona, Spain). Rapamycin was obtained from LC Laboratories (Woburn, MA) and Eton Bioscience Inc. (San Diego, CA). Kisspeptin (110–119)-NH_2 (termed kisspeptin-10 or Kp-10) was purchased from Phoenix Pharmaceuticals Ltd. (Belmont, CA). 17β-Estradiol (E2) was obtained from Sigma Chemical Co. (St. Louis, MO), and recombinant leptin was supplied by ProSpec-Tany Techno-Gene Ltd. (Rehovot, Israel).

Experimental designs

All experiments were conducted in peripubertal rats and subjected to pharmacological manipulation of brain mTOR signaling. Drugs and dosages for activation or inactivation of brain mTOR signaling were taken from previous references, with minor modifications (11). Standard procedures of cannulation of the lateral cerebral ventricle and acute or repeated intracerebroventricular (i.c.v.) injection/infusion of compounds were implemented, as described elsewhere (24, 25).

In the first set of studies, the effects of acute or chronic activation of mTOR were monitored. In experiment 1, groups of pubertal (35 d old) female rats (n = 10–12) were subjected to i.c.v. administration of a single bolus of L-Leu (10 nmol; equivalent to 1.3 μg) or vehicle. Blood samples for hormone assays were obtained by jugular venipuncture at 15 and 60 min after i.c.v. injections. In experiment 2, the ability of continuous infusion of L-Leu to advance puberty onset in the female was evaluated. Groups of female rats (n = 12), fed *ad libitum*, were implanted with osmotic minipumps (1 μl/h delivery rate; Alzet

model 2001; DURECT Corp., Cupertino, CA) containing vehicle or L-Leu, at a final concentration 1 μg per 1 μl. The osmotic pumps were connected to i.c.v. cannulae. Chronic infusion of L-Leu was conducted between d 26 and d 32, following previously described procedures (25). Body weights and vaginal opening were daily monitored and trunk blood collected at the end of experiment. In addition, in experiment 3, the effects of continuous infusion of L-Leu were studied in a model of negative energy balance at puberty. Groups of female rats (n = 12) were submitted to a protocol of 30% restriction in daily food intake, as previously described (17); food restriction was initiated on d 23. Chronic infusion of L-Leu was conducted, as described above, between d 30 and d 36. Body weights and vaginal opening were daily monitored and trunk blood collected at the end of experiment.

In the second set of studies, the effects of chronic inactivation of mTOR were assessed. In experiment 4, groups of female rats (n = 20–22) were subjected to repeated i.c.v. injections of rapamycin (50 μg per 5 μl; twice daily) or vehicle [dimethylsulfoxide (DMSO)], between d 30 and d 36, following previously published protocols (17). Body weights and vaginal opening were daily monitored. On d 32, a subset of females (n = 10/group) were killed and weights from uteri and ovaries recorded. The experiment was terminated on d 36, when trunk blood was collected 60 min after rapamycin injection, uteri and ovaries weighed, and hypothalami excised for phosphor-protein determinations. A subset of ovaries (n = 5) from control and rapamycin-treated groups were fixed in Bouin solution and processed for paraffin embedding, following standard procedures. Histological analyses of these samples were conducted as previously described (26), using serial 7-μm-thick sections stained with hematoxylin and eosin. The above protocols were implemented in duplicate, using rapamycin from two different commercial sources. Because initial tests demonstrated a consistent decrease in food intake and body weight gain in rapamycin-treated females (mean reduction ~15–20% *vs.* controls), a group (n = 20) of age-matched females, pair fed to rapamycin-injected animals, was also included in our experiments. For comparative purposes, in experiment 5, a similar protocol of repeated rapamycin injections was carried out in pubertal male rats. Groups of male rats (n = 10–12) were i.c.v. injected with rapamycin (50 μg per 5 μl; twice daily) or vehicle (DMSO), between d 40 and d 47, as described above. Body weights and balano-preputial separation were daily monitored. At the end of the experiment, testes, epididymis, seminal vesicles, and prostate from each animal were dissected and weighed.

In experiment 6, the impact of central mTOR inactivation on the positive effects of leptin on puberty onset was assayed. Given the permissive role of leptin (27, 28), these studies were conducted in pubertal female rats under a moderate state of negative energy balance, imposed by 20% restriction in daily food intake, starting at d 23. Food-restricted animals were submitted to repeated i.c.v. injections of vehicle, leptin (3 μg per 10 μl; twice daily), or leptin+rapamycin (50 μg per 5 μl; twice daily) between d 30 and d 36 (n = 8–9). Body weights and vaginal opening were daily monitored. At the end of the experiment, trunk blood was collected and uterine and ovarian weights recorded. Histological analyses were conducted in ovarian samples, as described in experiment 3. Semiquantitative (semi-Q) estimation of the degree of ovarian maturation was provided by considering the most advanced stage of the follicle-corpus luteum continuum, as previously described (26). For scoring, only healthy follicles were considered.

In the third set of studies, the profiles of LH secretion, and responses to potent stimuli of gonadotropin release, were evaluated after chronic mTOR inactivation. In experiment 7, groups of pubertal females (n = 10–12) were subjected to repeated i.c.v. injections of rapamycin or vehicle between d 30 and d 36, as described in experiment 3. At the end of treatment, blood samples were taken by jugular venipuncture, before (0) and 60 and 180 min after the last i.c.v. injection. In experiment 8, pubertal female rats, submitted to a similar protocol of chronic i.c.v. administration of rapamycin or vehicle were i.c.v. injected on d 36 with an effective dose of the potent gonadotropin secretagogue, Kp-10 (1 nmol/rat), 60 min after the last bolus of rapamycin. Blood samples were taken immediately before (0) and 15 and 60 min after Kp-10. In experiment 9, groups of female rats were subjected to bilateral ovariectomy (OVX) on d 29, following standard protocols. On d 30, repeated i.c.v. injections of rapamycin were initiated, as described in previous experiments. On d 36, the animals were killed at 60 min after the last bolus of rapamycin. Age-matched, intact females, i.c.v. injected with vehicle, were also included.

Given the lack of effects of rapamycin on serum LH levels in OVX animals, the role of estrogen in this phenomenon was explored. In experiment 10, groups of female rats (n = 10–12) were OVX on d 29 and subjected to estradiol replacement, as described elsewhere (29). Animals were implanted with SILASTIC brand silicon tubing (Dow Corning, Midland, MI) elastomers (12.5 mm length) containing E2 (solution of 10 μg/ml in olive oil). This dose of replacement was selected to achieve moderate levels of circulating E2. One day after surgery (d 30), repeated i.c.v. injection of rapamycin was initiated, as described above. On d 36, the animals were killed 60 min after the last bolus of rapamycin.

Hypothalamic Kiss1 mRNA levels were assayed after mTOR inactivation. To avoid the potential confounding factor of changes in circulating estrogen, these experiments were conducted in OVX+E2 females. In experiment 11, groups of female rats (n = 10) were subjected to OVX and E2 replacement, followed by repeated i.c.v. injections of rapamycin or vehicle, as described in experiment 7. Groups of intact and OVX rats without steroid replacement were also included. On d 36, the animals were killed, trunk blood was collected, and hypothalami were dissected as previously described (17). In experiment 12, groups of female rats (n = 4–5) were subjected to OVX+E2 replacement and repeated i.c.v. injections of rapamycin, as described above. An additional group of OVX+E2 animals pair fed to rapamycin-treated rats was included. On d 36, the animals were decapitated and brains were removed, frozen on dry ice, and stored at −80 C for *in situ* hybridization.

Protein analysis by Western blot

Hypothalamic total protein lysates (40 μg) were subjected to SDS-PAGE electrophoresis and transferred onto polyvinyl difluoride membranes. Membranes were probed for 16 h at 4 C in the presence of the appropriate dilution of the indicated antibodies [mTOR: 1:1000; phosphorylated (p)-mTOR: 1:1000; S6K1: 1:500; p-S6K1: 1:500; S6: 1:500; p-S6: 1:500; β-actin: 1:5000]. Protein detection was performed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagent (Amersham, Aylesbury, UK). Antibodies were obtained from Cell Signaling (Danvers, MA).

RNA measurements by real-time RT-PCR

Real-time RT-PCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Procedures for real-time RT-PCR of Kiss1 mRNA were as previously described (18). Similar protocols were used to assay GnRH mRNA levels, with the primer pair: *GnRH* forward (5′-CCGCTGT TGTTCTGTTGACTGTG-3′); *GnRH* reverse (5′-GGGGTTCTGCCATTTGATCCTC-3′). Relative expression levels of the targets was calculated based on the cycle threshold method, as previously described (18).

In situ hybridization

Coronal hypothalamic sections (20 μm) were used. A specific oligoprobe for Kiss1 mRNA detection was used (5′-GCCTCCT-GCCGTAGCGCAGGCCAAA GGAGTTCCAGTTGTA-3′) located at nt 328-367 of the cDNA sequence (GenBank NM_181692.1). This probe was 3′-end labeled with 33P-αdATP using terminal deoxynucleotidyl transferase (Amersham). Hybridizations were carried out in the presence of 1×10^6 cpm/slide of the labeled probe, as described elsewhere (30). Sections were dipped in Kodak autoradiography emulsion nitroblue tetrazolium salt (Kodak, Rochester, NY) and exposed for 6 wk at 4 C. The slides were developed in Dektol developer (Kodak). Similar anatomical regions were analyzed using the rat brain atlas of Paxinos and Watson, and specific hybridization signals were quantified by densitometry using a digital imaging system (Image-Pro Plus 4.5; Media Cybernetics Inc., Bethesda, MD). For analysis, 50–60 sections from each animal (nine to 10 slides; six sections/slide) were evaluated, with three to four animals per group.

Hormone measurements

Serum LH levels were determined in a volume of 50 μl using RIA kits supplied by the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program (Torrance, CA) (18, 25). In addition, in selected experimental samples, serum leptin and E2 levels were determined using commercial kits from Linco (St. Charles, MO) and Diagnostic Systems Laboratories (Webster, TX), respectively.

Presentation of data and statistics

Hormone determinations were conducted in duplicate ($n \geq 10$ samples/group). RT-PCR analyses were carried out in duplicate from at least four RNA samples per group. Western blots were carried out in duplicate from at least three independent protein samples per group. Hormonal and semi-Q RNA data are presented as mean ± SEM. Results were analyzed using Student *t* test or ANOVA followed by Student-Newman-Keuls multiple range tests (SigmaStat 2.0; Jandel Corp., San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

Effects of central activation of mTOR on the gonadotropic axis at puberty

Activation of mTOR signaling by i.c.v. administration of L-Leu (31) was first used to monitor its putative role in the control of gonadotropic axis at puberty; LH concentrations being used as surrogate marker. Acute injection of a single bolus (1.3 μg) of L-Leu to peripubertal (35 d old) female rats evoked an increase in serum LH levels, which peaked 15 min and remained elevated at 60 min (Fig. 1A). However, chronic infusion of L-Leu to (pre)pubertal females fed *ad libitum*, at a rate of 1.0 μg/h for 7 d did not overtly accelerate puberty onset (although nonsignificant trends for increased rates of vaginal opening and uterus weights were observed at d 32); neither did it cause a detectable elevation of terminal serum LH levels (supplemental Fig. S1). In addition, continuous i.c.v. infusion of L-Leu was applied to female rats subjected to persistent food restriction along puberty. Female rats were subjected to 30% reduction in daily food intake, starting at d 23 to induce a state of negative energy balance linked to blocked puberty onset and hypoleptinemia (17, 25). This protocol induced a significant drop in serum LH levels in 36-d-old animals, in line with previous references (17, 25). Continuous central infusion of L-Leu ameliorated the hypogonadotropic state of food-restricted animals, LH levels in females infused with L-Leu being double than those in vehicle group (Fig. 1B). Yet L-Leu infusion did not completely normalize serum LH concentrations, nor did it rescue vaginal opening (data not shown).

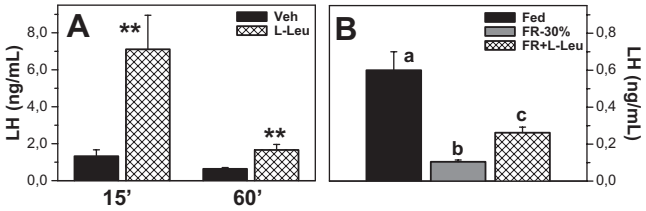

FIG. 1. Central activation of mTOR increases LH secretion at puberty. A, The effects of acute administration of L-Leu on LH secretion in pubertal female rats at 15 and 60 min after i.c.v. injection are shown. Veh, Vehicle. B, Serum LH levels in terminal blood samples from pubertal female rats subjected to 30% food-restriction (FR) and chronic infusion of L-Leu or vehicle are presented. In addition, LH concentrations in pubertal females fed *ad libitum* and infused with vehicle are also shown. Groups with *different superscript letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). **, $P < 0.01$ *vs.* corresponding controls injected with vehicle.

Central inactivation of mTOR delays puberty onset and inhibits LH secretion

The effects of sustained inactivation of central mTOR signaling were also explored in pubertal female rats by means of i.c.v. administration of rapamycin (11). As shown in Fig. 2A, repeated injections of rapamycin between d 30 and d 36 caused a marked delay in the age of vaginal opening (VO; complete VO in 91% of controls *vs.* 33% of rapamycin treated females, at d 36). This effect was linked to a reduction in uterus and ovarian weights, which became detectable already at d 32 (*i.e.* 2 d after beginning of rapamycin administration) and was fully

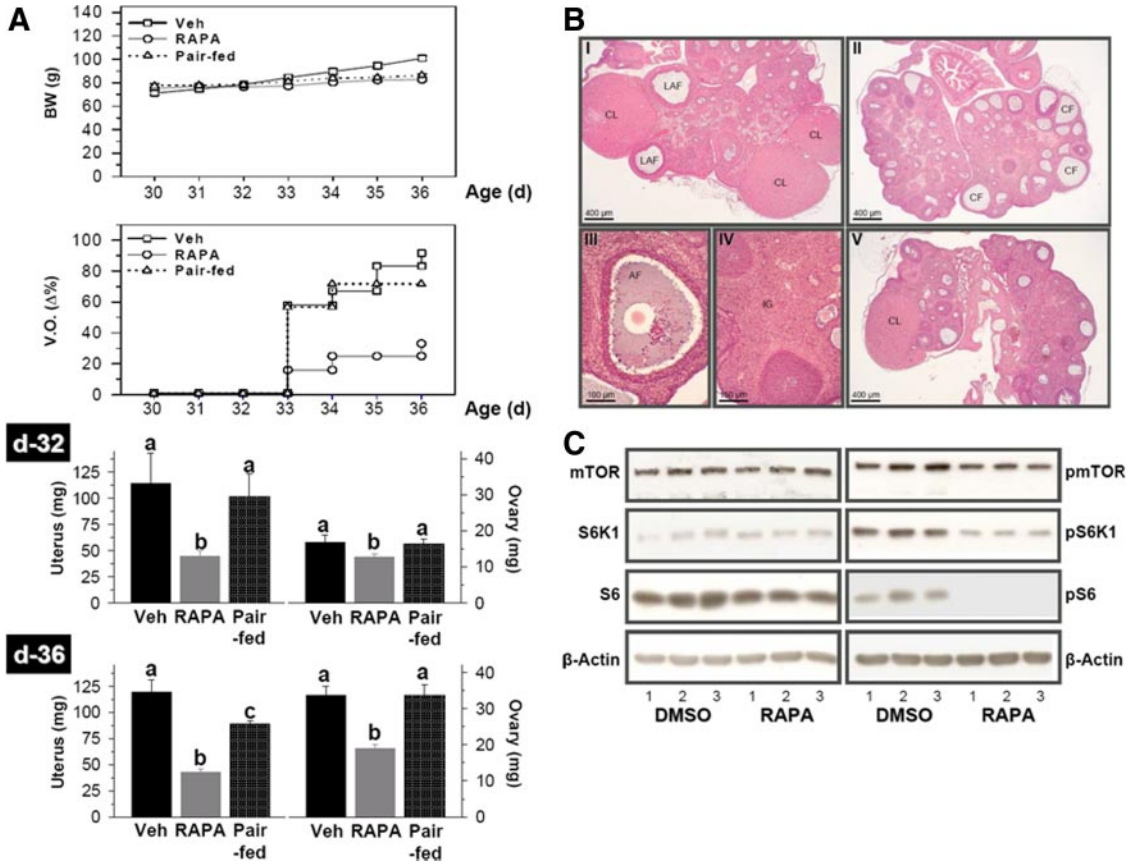

FIG. 2. Inactivation of central mTOR delays puberty onset. In the *left panels* (A), the effects of repeated i.c.v. administration of rapamycin (RAPA) on body weight (BW), VO, and uterus and ovarian weights (at d 32 and 36) in pubertal female rats are presented. Females chronically treated with vehicle [dimethyl sulfoxide (DMSO)] served as controls. Data from pubertal females, pair fed to rapamycin-treated rats, are also shown. Groups with *different superscript letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). In the *right upper panels* (B), the impact of chronic i.c.v. administration of rapamycin on ovarian histology is illustrated. I, A representative ovarian section from the control group is presented, showing abundant corpora lutea (CL) and large antral follicles (LAF). In contrast, CL were absent in most of the sections from rapamycin-treated rats (II-IV), which showed abundant cystic follicles (CF, see II), small atretic follicles (AF, see III), and enlarged interstitial gland (IG; see IV). Signs of ovulation were found in only one of five rapamycin-treated females, yet a single CL was found in ovarian sections from this animal (V). Finally, in the *right lower panels* (C), representative Western blots of the levels of mTOR, its downstream targets S6K1 and S6, and the corresponding phosphor-proteins, at the hypothalamus of pubertal female rats treated with vehicle (DMSO) or rapamycin are presented. Semi-Q analysis of these blots is provided in supplemental Fig. S1.

manifested by the end of treatment (d 36). As additional indices of severe ovarian failure, at d 36, serum E2 levels were significantly decreased in rapamycin-treated animals (14.9 ± 0.82 *vs.* 24.0 ± 3.0 pg/ml in controls; $P \leq 0.05$), and histological analyses revealed the absence of corpora lutea in most ovaries of rapamycin-treated rats, which showed also abundant cystic and small atretic follicles, in contrast with the presence of abundant corpora lutea and large antral follicles in control ovaries (Fig. 2B). Efficiency of blockade of central mTOR signaling was proven by the suppression of phosphorylated S6K1 and S6 levels, downstream targets of mTOR, in the hypothalami of rapamycin-treated rats (Fig. 2C and supplemental Fig. S2).

Initial experiments evidenced that repeated administration of rapamycin caused a decrease in daily food intake and, consequently, body weight gain (Fig. 2A) and serum leptin levels (0.96 ± 0.13 *vs.* 1.67 ± 0.17 ng/ml in vehicle treated rats), which may *per se* have an impact on puberty onset. Thus, age-matched groups of female rats, pair fed to rapamycin-treated animals, were included in our study. Restriction of daily food intake by 15–20% fully mirrored the impact of rapamycin on body weight gain. Yet this protocol of food restriction did not mimic the puberty phenotype induced by mTOR inactivation because the timing of vaginal opening was not substantially delayed and uterus and ovarian weights were not as severely affected as in rapamycin-injected animals. In fact, a modest but significant decrease in uterus weight was detected only at d 36 in pair-fed animals, with ovarian weights being similar to those of controls at both age points (Fig. 2A).

For comparative purposes, similar protocols of central rapamycin administration were applied also to pubertal male rats between d 40 and d 47. As shown in supplemental Fig. S3, central blockade of mTOR signaling failed to significantly modify the timing of balano-preputial separation, as external sign of male puberty, nor did it alter

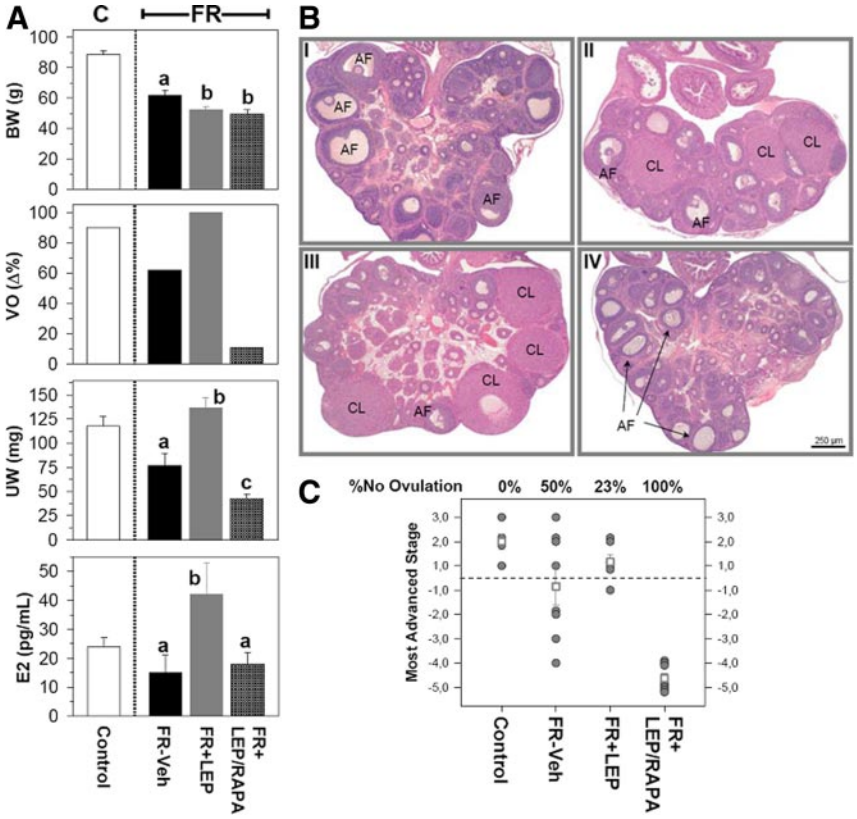

FIG. 3. Inactivation of central mTOR blocks the positive effects of leptin on puberty onset. In the *left panels* (A), body weight (BW), accumulated VO, uterus weight (UW), and serum E2 levels are presented from pubertal female rats under moderate restriction (20%) in daily food intake (FR), submitted to repeated i.c.v. administration of leptin (Lep), alone or in combination with rapamycin (RAPA). Females chronically treated with vehicle, as well as pubertal rats fed *ad libitum*, served as controls. Groups with *different superscript letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). In the *right upper panels* (B), the effects of the above treatments on ovarian histology are illustrated. Representative sections from FR rats treated with vehicle (Veh) are displayed in I-II, showing either absence of ovulation and class 2–4 antral follicles (AF) as the most advanced stage (see I) or newly formed corporal lutea (CL; see II). Most of leptin-treated females had signs of ovulation, with abundant CL (see III). In striking contrast, rapamycin coadministration to leptin-treated rats prevented ovulation and resulted in immature ovaries, with abundant atretic follicles (AF) and small, class 1–2 healthy follicles, as the most advanced stage. A summary of follicular development in the different treatment groups is provided *right lower panel* (C). The time of the first ovulation (at the first transition from proestrus to estrus) was considered point 0 (*dotted line*). Each animal was scored −5 (class 1) to −1 (class 5) when showing class 1–5 follicles as the most advanced stage or +1 to +4 when showing 1- to 4-d-old CL, respectively. The percentage of nonovulating animals is indicated.

testicular weights at the end of treatment. However, chronic rapamycin administration evoked a detectable drop in the weights of epididymis, seminal vesicles, and prostate, thus suggesting the suppression of the gonadotropic axis also in the pubertal male.

Given the above observations, we aimed to explore whether the permissive effects of leptin on puberty onset may depend on proper mTOR signaling, using food-restricted females as experimental model. Moderate restriction (~20%) in daily food intake along puberty partially prevented vaginal opening and ovulation and decreased uterus weights and serum E2 levels. These defects were rescued by repeated central injection of leptin, rescue that was abrogated by concomitant cotreatment with rapamycin. Thus, whereas leptin-treated rats displayed vaginal opening (100%) and ovulation (>75%), with a significant increase in uterus weight and E2 levels, concomitant blockade of central mTOR prevented vaginal opening and suppressed follicular maturation, ovulation rate, uterus weights, and serum E2 levels (Fig. 3).

LH responses to chronic mTOR inactivation were also explored in pubertal females; pre- and postinjection LH levels were monitored in female rats at the end (d 36) of our protocol of chronic rapamycin administration. As shown in Fig. 4A, despite repeated i.c.v. injections of rapamycin, basal LH levels on d 36 were similar to those of control animals. However, an i.c.v. bolus of rapamycin evoked a decline in circulating LH, noticed already at 60 min, which resulted in nearly undetectable levels 180 min after i.c.v. injection.

To rule out the possibility that the above pattern of LH secretion may be due to the unspecific lesion of GnRH neurons induced by rapamycin treatment, LH responses to i.c.v. administration of an effective dose of Kp-10 (1 nmol/rat), as major LH secretagogue (14, 15), were assayed in this model. As shown in Fig. 4B, central injection of Kp-10 induced the expected rise in LH levels in not only control females but also rats chronically treated with rapamycin, the magnitude of LH secretory responses being similar in both groups. In addition, LH secretion after OVX was also studied in rapamycin-injected animals, OVX being a potent stimulus of gonadotropin release, independent of metabolic control. LH levels were markedly elevated in pubertal OVX rats, regardless of chronic administration of rapamycin from d 30 onward (Fig. 4C). In fact, on d 36, serum LH levels were similar in control and rapamycin-treated OVX rats, before (basal) and 60 and 180 min after last injection (data not shown).

Because repeated rapamycin treatment did not reduce LH levels in OVX rats, the influence of E2 in this phenomenon was studied. As shown in supplemental Fig. S4, chronic rapamycin injections to OVX female rats without E2 replacement failed to decrease circulating LH levels at

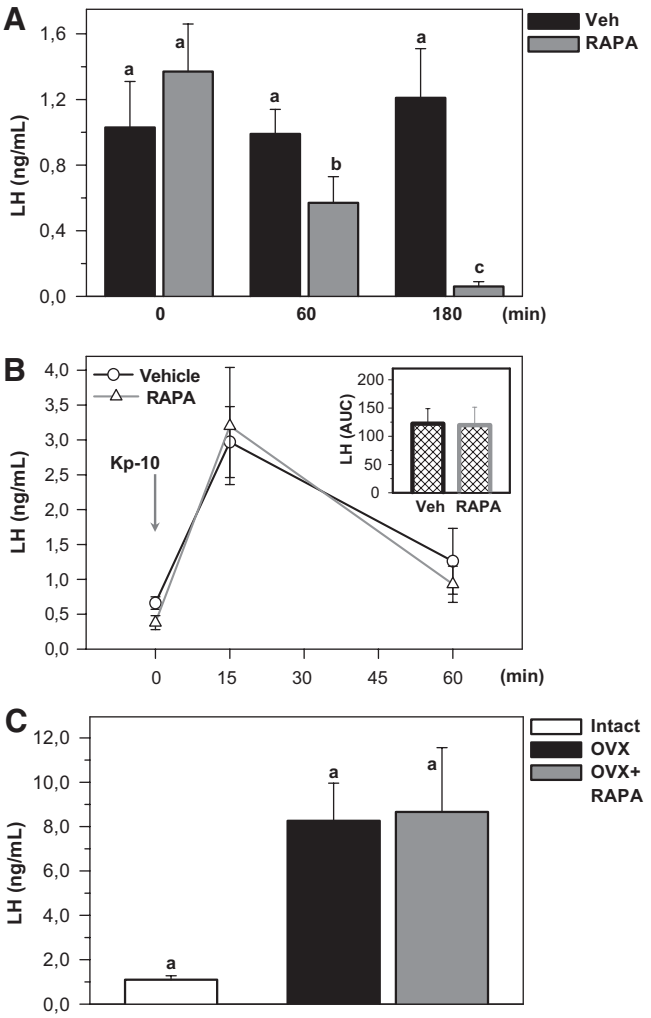

FIG. 4. Secretory profiles of LH after central mTOR inactivation, in basal and stimulated conditions. A, Time course for the effects of rapamycin (RAPA) administration to pubertal female rats; the animals where chronically treated with RAPA or vehicle [dimethyl sulfoxide (DMSO)], and LH levels were assayed immediately before (0 min) and at 60 and 180 min after the last i.c.v. injection. Veh, Vehicle. B, LH responses to an effective i.c.v. dose (1 nmol) of Kp-10 in pubertal female rats chronically treated with vehicle or RAPA. In addition to LH levels at 15 and 60 min after Kp-10 injection, integrated secretory responses (area under the curve over the 60 min after Kp-10) are presented. C, The effects of OVX on LH levels in pubertal female rats, chronically treated with vehicle or RAPA, are shown; LH concentrations at 7 d after OVX are presented. For comparative purposes, LH levels in intact pubertal females are also plotted. Groups with *different superscript letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). AUC, Area under the curve.

the end of the treatment. In contrast, a similar protocol of rapamycin administration resulted in a dramatic drop of serum LH concentrations in OVX rats supplemented with a moderate dose of estradiol (OVX+E2).

Inactivation of mTOR inhibits hypothalamic Kiss1 mRNA expression

The effects of mTOR blockade on the hypothalamic expression of a major gatekeeper of puberty, Kiss1 (14, 15), were also assayed. To avoid the potential influence of changes in sex steroid levels secondary to mTOR inactivation, the impact of chronic rapamycin treatment was explored in OVX rats subjected to hormonal replacement with a moderate dose of estradiol (E2 levels: 10.1 ± 3.0 *vs.* 2.95 ± 0.71 pg/ml in OVX rats) (19, 29). Relative Kiss1 mRNA levels were assayed in whole hypothalamic preparations from OVX and OVX+E2 animals; expression levels in intact and OVX rats without E2 supplementation were also analyzed. OVX resulted in the expected rise in hypothalamic Kiss1 mRNA levels, whereas replacement with estradiol prevented such an increase. In this model, chronic administration of rapamycin evoked a significant decrease of Kiss1 mRNA at the hypothalamus. Serum LH levels closely paralleled those of hypothalamic Kiss1 mRNA, with a dramatic drop in circulating LH levels in OVX+E2 rats chronically treated with rapamycin (Fig. 5). In this group, a significant decrease in hypothalamic GnRH mRNA levels was also detected.

In addition, location of the above Kiss1 mRNA changes within the hypothalamus was monitored by *in situ* hybridization; groups of OVX+E2 females pair fed to ra-

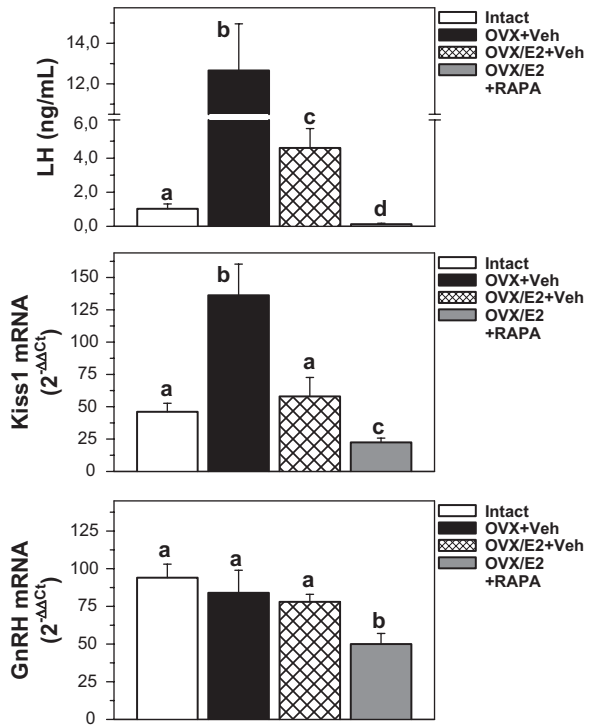

FIG. 5. LH secretion and hypothalamic expression of Kiss1 and GnRH after central mTOR inactivation. Serum LH concentrations and hypothalamic Kiss1 and GnRH mRNA levels in pubertal female rats, subjected to OVX and estradiol replacement, and chronically treated with vehicle [dimethyl sulfoxide (DMSO)] or rapamycin (RAPA), are presented. For comparison, hormonal and RNA levels in intact and OVX pubertal females (not subjected to E2 replacement) are also shown. Groups with *different superscript letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). Veh, Vehicle; Ct, cycle threshold.

pamycin-treated animals were also analyzed. As shown previously (32), two prominent populations of Kiss1-expressing neurons, located at the arcuate nucleus (ARC) and anteroventral paraventricular nucleus (AVPV), were identified. Repeated injections of rapamycin induced a significant suppression of Kiss1 mRNA levels at both sites. Yet the magnitude of such responses was markedly different: whereas Kiss1 mRNA levels were reduced by half at the AVPV, rapamycin treatment virtually nullified Kiss1 mRNA expression at the ARC (Fig. 6). This profile of response was clearly different from that detected in pair-fed animals because Kiss1 mRNA levels at the AVPV were not significantly modified in this group, whereas those at the ARC were reduced by 50% only in food-restricted females.

Discussion

These data are the first to demonstrate the putative involvement of central mTOR signaling in the control of the gonadotropic axis. Our study targeted female (rat) puberty as a relevant developmental stage, which is known to be sensitive to fuel availability and permissive effects of leptin (2), hypothalamic mTOR being proposed as leptin transducer and central gauge for energy balance at whole-body level (11). Our experiments evidenced that central activation of mTOR acutely stimulated LH secretion, as surrogate marker of gonadotropic function. Moreover, stimulatory effects of L-Leu on LH secretion were detected against conditions of persistent negative energy balance linked to defective gonadotropin levels and disturbed puberty onset (17). Yet chronic infusion of low doses of L-Leu to pubertal females fed *ad libitum* failed to further accelerate puberty onset. A tenable explanation for such observation is that, in fed animals, puberty is already proceeding at a maximal pace; thus, further activation of mTOR (as putative transducer of the permissive actions of leptin) is devoid of discernible effects, a phenomenon already described for leptin itself (28).

Conversely, chronic blockade of central mTOR by rapamycin had a dramatic inhibitory impact on puberty onset, as reflected by its effects in terms of vaginal opening, uterus weights, ovarian histology, and circulating LH and E2 levels. Together, these results disclose the positive role of brain mTOR signaling in the control of pubertal activation of the gonadotropic axis, which is compatible with its proposed function as mediator of leptin effects on key neuroendocrine centers at the hypothalamus (11). In keeping with this view, our preliminary results strongly suggest that male (rat) puberty is also sensitive, albeit to a lower extent, to central mTOR inactivation.

Contrary to our initial expectations, chronic inhibition of mTOR resulted in a moderate but sustained decrease in daily food intake and body weight gain. Although the mechanisms behind this observation in pubertal animals are still under active investigation, our initial data suggest that this phenomenon is not related to unspecific taste aversion (article in preparation). Admittedly, decreased body weight and leptin levels might have *per se* a deleterious impact on puberty onset (2). For this reason, groups of rats pair fed to rapamycin-treated females were included in our analyses. Our data evidenced that the reproductive phenotype induced by mTOR inactivation clearly exceeds that of moderate food restriction, as rapamycin-treated rats were more severely compromised in terms of delayed vaginal opening, uterus, and ovarian atrophy and hypothalamic Kiss1 mRNA expression at the ARC, thus ruling out the possibility that this phenotype may merely stem from reduced body weight after rapamycin treatment. On the other hand, the apparent lack of detectable increases in hypothalamic corticotropin-releasing factor mRNA and blood glucose levels in rapamycin-treated rats casts doubts on the possibility that the above

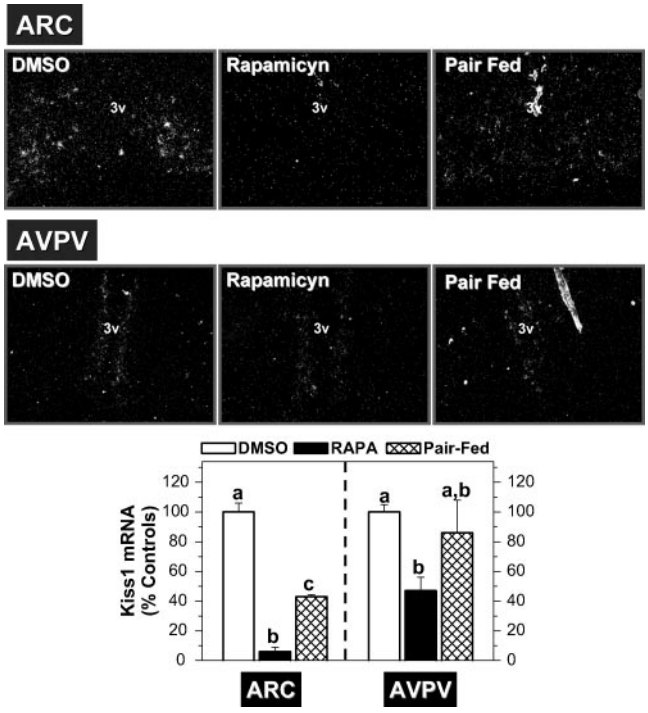

FIG. 6. Pattern of hypothalamic expression of Kiss1 after central mTOR inactivation. In the *upper panels*, dark-field photomicrographs showing *Kiss1* mRNA expression (*white clusters of silver grains*) in representative sections of the ARC and AVPV at the hypothalamus of rat chronically treated with vehicle [dimethyl sulfoxide (DMSO)] or rapamycin (RAPA). Data from females pair fed to rapamycin-treated rats are also shown. In the *lower panel*, quantification of Kiss1 expression data in the above experimental groups is presented, as percentage of levels in controls treated with vehicle. Groups with *different superscript letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). 3V, Third ventricle.

effects could be primarily caused by unspecific stress responses after chronic administration of rapamycin (our unpublished data).

Our results suggest that hypothalamic mTORC1, which is rapamycin sensitive (5, 8, 10), is functionally involved in the central networks governing the activation of the gonadotropic axis at puberty. Of note, chronic inactivation of brain mTOR has been reported to alter synaptic contacts and remodeling (33), a phenomenon that might contribute to our present observations. Yet the fact that rapamycin-treated rats retained full LH responsiveness to gonadectomy and kisspeptin administration strongly suggests that inhibition of the gonadotropic axis in this model is not due to unspecific damage of the neuronal GnRH system but rather to dysregulation of upstream pathways. Moreover, the fact that the suppressive effect of mTOR blockade on LH secretion was absolutely dependent on the presence of estrogens further supports a functional (rather than an organic/lesion) basis for such a phenomenon.

Among the afferents to GnRH neurons, kisspeptins have emerged as essential gatekeepers of puberty onset and fertility due to their ability to activate GnRH secretion (14, 15, 34). Our data conclusively demonstrate that central inactivation of mTOR results in the consistent decrease of hypothalamic Kiss1 expression. Such a suppression of Kiss1 mRNA levels was tightly correlated with concomitant inhibition of circulating LH, which indirectly points out its functional relevance. Blockade of mTOR resulted also in a moderate reduction of GnRH mRNA levels at the hypothalamus. Although these data are compatible with a direct action of mTOR at GnRH neurons to regulate gene expression, in keeping with a previous study in GT1-7 cells (35), it is also plausible that such a decrease in GnRH mRNA levels might be due to consistent suppression of (one of) its major stimulatory afferents, *i.e.* kisspeptins. The occurrence and relative importance of both potential mechanisms is yet to be defined.

Reduction of Kiss1 mRNA levels after central blockade of mTOR was detected at both ARC and AVPV. Yet the magnitude of such an inhibition was much larger at the ARC, in which Kiss1 mRNA expression after rapamycin treatment dropped to nearly negligible levels. This observation is in line with previous reports documenting the prominent expression of phosphorylated mTOR in neuronal populations at the ARC (11) and the ability of leptin (a putative regulator of mTOR signaling) to stimulate Kiss1 mRNA expression at this nucleus (21). Further studies on mTOR signaling at the AVPV are needed to define the mechanisms (direct *vs.* indirect) for the observed decrease of Kiss1 mRNA induced by rapamycin at this site. In any event, the possibility that the above alterations in Kiss1 mRNA levels might be secondary to changes in the sex steroid milieu after rapamycin treatment can be ruled out because these studies were conducted against a fixed, submaximal estrogen background (29). This moderate level of E2 replacement was intended as to allow identification of potential direct effects of mTOR blockade, which might have been obscured by excessive E2 supplementation. Likewise, the above effects on Kiss1 expression at the ARC and AVPV cannot be attributed to partial reduction in food intake associated to rapamycin treatment because the magnitude of Kiss1 mRNA changes was very modest (if not totally absent) in pair-fed animals. Overall, our mRNA data support a genuine primary role of mTOR signaling in the control of hypothalamic expression of Kiss1, preferentially at the ARC.

Very recently the molecular mechanisms for the metabolic control of Kiss1 expression have begun to be deciphered by the demonstration of the positive role of the cAMP response element-binding protein-regulated transcriptional coactivator-1 (Crtc1) in the control of *Kiss1* gene expression (36), Crtc-1 being a putative mediator for the positive action of leptin on Kiss1 mRNA expression (36). In the same line and given the previous demonstration of the stimulatory role of leptin on hypothalamic mTOR signaling, our data are suggestive of a novel leptin-mTOR-Kiss1 pathway, which might contribute to the documented actions of leptin on Kiss1 mRNA expression and puberty onset (18, 21). This hypothesis is supported by our present observations showing that the permissive effects of leptin in terms of puberty onset can be fully blocked by concomitant inhibition of central mTOR. Whether mTOR and Crtc1 signaling pathways are related or independent awaits further investigation. Similarly, whether other CNS fuel-sensing mechanisms are also involved in the regulation of Kiss1 expression at the hypothalamus is yet to be explored. In this context, AMPK has been suggested to reciprocally cooperate with mTOR in the central control of energy homeostasis, AMPK being an appetite-stimulating factor (12, 22). In keeping with this view, our preliminary data suggest that activation of AMPK induced partial delay of puberty onset in female rats (article in preparation), which may be caused, at least partially, by its ability to inhibit mTOR signaling (7, 37).

In summary, we disclose herein the indispensable role of brain mTOR signaling in the control of puberty onset and hypothalamic Kiss1 expression, preferentially at the ARC, a major hypothalamic center for integration of different neuroendocrine functions, including control of energy balance and reproduction. Whereas complementary approaches, such as functional genomics, will be needed to fully define its physiological relevance (not only at puberty but also in adulthood), the proposed role of mTOR as

crossroad for the metabolic gating of cellular and systemic responses (5, 6, 8, 9) and the putative function of kisspeptin neurons as key elements for the metabolic control of fertility (14, 16) make it tempting to hypothesize that this novel pathway may participate in the integrated regulation of energy homeostasis and reproductive function. In addition to their physiological implications, our present findings also may provide novel mechanistic insights for the reported impact of mTOR antagonists, of clinical use in the management of some types of cancer and organ transplantation (6–8) on reproductive function in humans.

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