ABSTRACT Activins are critical components of the signaling network that controls female reproduction. However, their roles in hypothalamus, and the specific functions of their different receptors, have not been elucidated. Here, we investigated the expression and function of the activin receptor ALK7 in the female reproductive axis using Alk7-knockout mice. ALK7 was found in subsets of SF1-expressing granulosa cells in the ovary, FSH gonadotrophs in the pituitary, and NPY-expressing neurons in the arcuate nucleus of the hypothalamus. Alk7-knockout females showed delayed onset of puberty and abnormal estrous cyclicity, had abnormal diestrous levels of FSH and LH in serum, and their ovaries showed premature depletion of follicles, oocyte degeneration, and impaired responses to exogenous gonadotropins. In the arcuate nucleus, mutant mice showed reduced expression of Npy mRNA and lower numbers of Npy-expressing neurons than wild-type controls. Alk7 knockouts showed a selective loss of arcuate NPY/AgRP innervation in the medial preoptic area, a key central regulator of reproduction. These results indicate that ALK7 is an important regulator of female reproductive function and reveal a new role for activin signaling in the control of hypothalamic gene expression and wiring. Alk7 gene variants may contribute to female reproductive disorders in humans, such as polycystic ovary syndrome.—Sandoval-Guzmán, T., Göngrich, C., Moliner, A., Guo, T., Wu, H., Broberger, C., Ibáñez, C. F. Neuroendocrine control of female reproductive function by the activin receptor ALK7. The FASEB J. 26, 4966–4976 (2012). www.fasebj.org

Key Words: ovary • pituitary • hypothalamus • NPY • PCOS

The control of reproductive function involves a highly regulated network of hormonal signals exchanged between the gonads, the anterior pituitary, and the brain. The key regulator of reproduction, gonadotropin-releasing hormone (GnRH), is produced by neurons of the medial preoptic area (MPOA) in the hypothalamus, and acts on gonadotrophs in the anterior pituitary to stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (1). Circulating LH and FSH stimulate the maturation and development of the gonads and the synthesis and secretion of the gonadal steroid hormones. Among the most important signals regulating reproduction are the activins and inhibins, members of the transforming growth factor-β (TGF-β) superfamily, first described for their ability to respectively stimulate or inhibit secretion of FSH from the anterior pituitary (2, 3). Activins were later found to have effects at different levels of the reproductive axis, including the hypothalamus, pituitary, gonads, and placenta (4–8). In accordance with this, activin and inhibin receptors have been found in reproductive and neuroendocrine tissues from fetal stages to adulthood (9–11).

Similar to other TGF-β superfamily members, activin receptor complexes are formed by type I and type II subunits (12–14). Type I receptors become activated by type II receptors on ligand binding, and are the main signaling output of the receptor complex. The main activin type I receptor, activin receptor-like kinase 4 (ALK4), is ubiquitously expressed (15). Mutant mice lacking ALK4 fail to gastrulate and die at preimplantation stages (16). Although both activin A and B are able to signal through ALK4, only activin B can signal through a second type I receptor, ALK7 (17). ALK7 partners with activin type II receptors A and B to mediate signaling by a subset of TGF-β superfamily ligands, including activin B, nodal, growth and differ-

1 Present address: DFG Center for Regenerative Therapies Dresden, Technical University of Dresden, Dresden, Germany.
2 These authors contributed equally to this work.
3 Correspondence: Department of Neuroscience, Karolinska Institute, Stockholm, Sweden. E-mail: carlos.ibanez@ki.se doi: 10.1096/fj.11-199059
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entiation factor 1 (GDF1), GDF3, and GDF11 (17–23). Unlike ALK4-knockout mice, mice lacking ALK7 are viable, but display a range of metabolic abnormalities, including increased insulin serum levels, reduced accumulation of white adipose tissue, and partial resistance to high-fat-diet-induced obesity (21, 22, 24). Male mice deficient in activin B phenocopy the insulin phenotype of ALK7 mutants (22) and breed normally (6). On the other hand, female mice lacking activin B have a marked impairment in reproductive function (6). The role of ALK7 in the control of reproduction is unknown.

In the present study, we analyzed the expression of ALK7 along the female reproductive axis, and investigated the role of this receptor in reproductive function in Alk7-knockout mice, including effects on the estrous cycle, gonadal development, gonadotropin secretion, and hypothalamic gene expression and wiring. Our results indicate that ALK7 is an important regulator of female reproductive function, acting at multiple levels of the reproductive axis to control the onset of puberty and the female estrous cycle.

MATERIALS AND METHODS

Animals

The generation of Alk7-knockout mice has been described previously (24). InhB/−/− mice lacking activin B (6) were obtained as frozen embryos from the Jackson Laboratory (stock no. 002442; Jackson Laboratory, Bar Harbor, ME, USA). Both lines were maintained in a C57/BL6 background. Transgenic mice expressing enhanced green fluorescent protein (EGFP) from a bacterial artificial chromosome (BAC) containing the Alk7 locus (Alk7-GFP) were obtained from the GENSAT project (ref. 25; http://www.gensat.org). All animals were housed under standard conditions with food and water ad libitum. Animal protocols were approved by Stockholm’s Norra Djurförsöksnämnd and in accordance with ethical guidelines of the Karolinska Institute.

Quantitative PCR (qPCR) analysis

On the day of diestrus, brains were removed and placed on a brain matrix to cut 3-mm slices using the optic chiasm as anterior landmark. The hypothalamus was dissected using the end of the optical tract as lateral border and the beginning of the temporal lobe cortex as dorsal border. Pituitaries were also extracted for mRNA studies. The tissue blocks were kept frozen in dry ice until RNA extraction. Total RNA was isolated using a kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s protocol. cDNA was synthesized from 600 ng total RNA primed with random hexamers (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed with MMLV reverse transcriptase (Invitrogen Life Technologies). Quantitative PCR analysis was performed using the StepOnePlus continuous fluorescence detector (Applied Biosystems Research, Foster City, CA, USA). Product amplification was determined by SYBR Green 1 fluorescence detection. Primers used were Cga-upstream, 141–161; Cga-downstream, 312–291; Lhb-upstream, 37–56; Lhb-downstream, 186–166; Fshb-upstream, 56–76; Fshb-downstream, 167–146; Npy-upstream, 222–240; Npy-downstream, 270–289; GnRHR-upstream, 472–491; GnRHR-downstream, 565–584; GnRH-upstream, 73–93; GnRH-downstream, 172–190. Reactions were performed using 2 μl of cDNA, 0.4 μM of forward and reverse primers, and 2× SYBR Green 1 master mix (Applied Biosystems). Standard cycling procedures were employed with annealing temperatures of 60°C. Specific amplicon formation with each primer pair was confirmed by melt curve analysis. Gene expression was quantified relative to standard curves using either TATA box binding protein (TBP) or 18s ribosomal RNA as housekeeping genes. qPCR measurements in hypothalamus were performed in triplicate (samples from 3 animals) and repeated at least twice, often 3 times, with comparable results. In pituitary, 8 animals/group were used in 2 independent experiments.

Antibodies

Antibodies used were as follows: goat anti-GFP (1:300) from Abcam (Cambridge, MA, USA); rabbit anti-FSH (1:500) from AbD Serotec (Kidlington, UK); anti-neuropeptide Y (NPY; 1:200) from Peninsula Labs (Belmont, CA, USA); anti-agouti gene-related protein (AgRP; 1:800) from Millipore (Bedford, MA, USA); anti-goat Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 (1:500–1:1000) from Jackson ImmunoResearch (West Grove, PA, USA) or Invitrogen. According to the manufacturer’s information, the anti-FSH antibodies used here do not show cross-reactivity with thyroid-stimulating hormone.

Hormone measurements

Basal FSH and LH secretion were measured in serum obtained from adult females at diestrus, from males, and from peripubertal animals. Serum FSH and LH concentration were measured in a volume of 25 μl using ELISA kits from BioCode Hycel (Zwölfaxing, Austria) and from Shibayagi (Ishihara, Japan), according to the manufacturer’s protocol. Measurements of serum estradiol levels were commissioned to the Endocrine Technology Services Core Laboratory from the Oregon National Primate Research Center (Beaverton, OR, USA).

Estrous cycle analysis

Vaginal smears were taken daily at 2–3 h after lights on, and the cytology was analyzed after Giemsa staining. One estrous cycle was considered the number of days between two proestrus stages (26). Three to four consecutive cycles were analyzed for each animal (11 mice/group). To date the day of first estrus, vaginal smears were taken from the day of vaginal opening.

Ovary histology

Reproductive tracts were dissected and weighed. Ovaries of wild-type and Alk7-knockout females were sectioned for morphological analysis and stained with standard hematoxylin and eosin (H&E) protocol. Growing follicles were counted in a single section of the ovary at the widest diameter (wild type, n = 8; knockout, n = 9). Staging was performed according to Peters et al. (27). For superovulation, 3 wild-type and 3 Alk7-knockout females [age postnatal day (P) 26 to P30] were injected i.p. with 5 IU PMS-G at 6 h after lights on on d 1 of treatment, and with 5 IU hCG 48 h later. Wild-type and Alk7-knockout control mice received saline injections. At 5 h after the last injection, mice were transcardially perfused with 4% paraformaldehyde (PFA), and their reproductive tracts.
were dissected and postfixed. Cryoprotected ovaries were serially sectioned (20 μm) on a cryostat; sections were thaw-mounted to superfrost slides and stained with H&E. The total number of cumulus oocyte complexes (COCs) was determined from serial sections spaced 120 μm apart from each other (n=3 mice, 6 ovaries/group).

**Pituitary primary culture**

Female mice at random estrous stages were anesthetized with isoflurane and killed by cervical dislocation. Pituitaries were collected in Hank’s solution with 1 mM HEPES. Anterior lobes were dissociated for 50 min at 36.7°C with 0.3% trypsin. Enzymatic dissociation was stopped with FBS and incubated 5 min with DNase. The cell suspension was centrifuged 6 min at 1100 rpm, and the pellet was resuspended in DMEM at washes. Pellets were resuspended in 2 ml of DMEM without phenol red and supplemented with 2% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The dissociated pituitary cells were counted in a hemocytometer and assessed for viability using trypan blue. Only cell preparations with viability >95% were used. Cells were incubated in 48-well dishes in a water-saturated atmosphere, 5% CO₂ and 95% air. After 48 h in culture, cells were washed and preincubated for 6 h with serum-free medium. Medium was changed to DMEM supplemented with 50 ng/ml (1 nM) of recombinant human (rh)-activin A, rh-activin B (R&D Systems), or medium alone. After 24 h, the supernatant was collected and stored at -20°C until processed. All incubations were performed in triplicate. Activin concentration and time of incubation were chosen on the basis of previous studies (3). Wells that were used for immunostaining were washed and fixed with 4% PFA prior to immunocytochemistry as described below.

**Pituitary immunohistochemistry**

Adult female Alk7-GFP mice were transcardially perfused with 4% PFA in phosphate-buffered saline (PBS), and the pituitaries were postfixed overnight at 4°C. Pituitaries cryoprotected in 30% sucrose were coronally sectioned (16 μm) on a cryostat and thaw-mounted on superfrost slides. Blocking, permeabilization, and antibody incubation were carried out in 5% normal donkey serum and 0.2% Triton X-100 in PBS. Primary antibodies were incubated overnight at 4°C, secondary antibodies for 2 h at room temperature. All wash steps were performed with PBS (pH 7.4). Air-dried sections were coverslipped with Dako fluorescence mounting medium (Dako North America, Carpinteria, CA, USA).

**Brain immunohistochemistry**

At the morning of diestrus, animals were anesthetized with pentobarbital sodium and euthanized by transcardial perfusion with 0.1 M phosphate buffer (PB; pH 7.4) followed by a solution of 4% PFA in PB. The brains were dissected out and postfixed overnight with the same fixative and then cryoprotected with ascending concentration of sucrose at 4°C. The brains were sectioned (12 μm thick), mounted on slides, and stored at -80°C until processed. For immunostaining, sections were rinsed with PBS and blocked with 5% normal serum, 10% BSA, and 0.1% Triton X-100 for 1 h before overnight incubation with primary antibody. Sections were rinsed 3 times with PBS and incubated for 2 h with secondary antibody, then rinsed, and coverslipped with Dako fluorescence mounting medium (Dako North America). Quantification of relative optical density of NPY and AgRP immunoreactivity was performed with ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Sections from 5 wild-type and 5 mutant brains stained with cresyl violet were anatomically matched to Paxinos atlas plate 31 for MPOA, plate 38 for paraventricular nucleus (PVN), and plate 43 for arcuate nucleus (ARC) (28). For GnRH immunohistochemistry, brains were sectioned in the coronal plane on a vibratome (40 μm thick). Antibody incubation was performed in 5% normal donkey serum and 0.2% Triton X-100 at 4°C overnight for primary antibodies and at room temperature for 2 h for secondary antibodies. Nuclei were visualized using DAPI. The number of GnRH-expressing cells was analyzed on every sixth section between bregma +1.94 mm and bregma -2.30 mm.

**Colchicine injection**

To detect neuropeptides in cell bodies of the ARC of the hypothalamus, axonal transport was blocked using colchicine. Briefly, colchicine was dissolved in 0.9% NaCl to a final concentration of 30 μg in 5 μl. Female mice at diestrus were anesthetized with a mixture of Hypnorm/midazol (1 ml midasolam 5 mg/ml and 1 ml Hypnorm 2.7 ml/kg). Intracerebroventricular injection coordinates were 0.2 mm posterior from bregma, 0.9 mm lateral from midline, and 2 mm deep from brain surface. Colchicine was loaded in a syringe and injected slowly into the right lateral ventricle. To avoid backflow, the needle was left in place for 5 min before removal. Animals were anesthetized 24 h after colchicine injection with an intraperitoneal injection of sodium pentobarbital (0.15 mg/100 g body weight) and perfused via the ascending aorta with 25 ml of Tyrode’s Ca²⁺-free solution at 37°C, followed by 25 ml of a mixture of 4% PFA and 0.4% picric acid at 37°C, then perfused with 50 ml of the same mixture at 4°C at a constant flow of 10 ml/min. The brains were dissected and postfixed for 90 min, then rinsed in 10% sucrose/PB (pH 7.4) 3 times and left overnight with several changes in between. The brains were sectioned at 12-μm thick sections, mounted on slides, and stored at -80°C until processed.

**NPY in situ hybridization**

Females were deeply anesthetized with intraperitoneal pentobarbital sodium, and decapitated in the morning of diestrus. The brain was rapidly dissected out and frozen on dry ice. Fresh frozen coronal sections (14 μm) were mounted on glass slides. Oligonucleotide probes for Npy were designed as described previously (29). For stereological analysis of the ARC, sections were chosen according to a mouse brain atlas (28) comprising bregma -1.30 to -1.46 mm. The in situ hybridization procedure used has been described previously (29). Antisense probes were labeled with digoxigenin-11-UTP (Roche Diagnostics, Mannheim, Germany). Following 16 h incubation at 42°C, sections were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) overnight. Hybridized probes were visualized through a chromogenic reaction with NBT/BCIP (Life Technologies), resulting in a blue-purple precipitate that was examined under bright-field illumination in a Zeiss Axiosmager M1 microscope. Cell counts were determined every tenth section at ×40 view within the entire ARC anatomically matched between animals (5 animals/group).

**Statistical analysis**

To compare data between groups, we used unpaired Student’s t test or 1-way ANOVA and Tukey’s post hoc test for data following a normal distribution. Comparison of data not
following a normal distribution was performed using Mann-Whitney U test. GraphPad Prism (GraphPad Software, San Diego, CA, USA) and SPSS (IBM, Armonk, NY, USA) were used for data analysis. Values of \( P < 0.05 \) were considered statistically significant.

RESULTS

ALK7 expression in the reproductive axis

Although earlier studies had reported Alk7 mRNA expression in ovary, pituitary, and hypothalamus (11, 30–32), the cell types expressing ALK7 within these structures have remained undefined. ALK7-expressing cells were identified by immunostaining for EGFP in a transgenic mouse line expressing EGFP from a BAC encompassing the entire Alk7 locus (25), which recapitulates endogenous ALK7 expression. In the ovary, ALK7 expression was detected in granulosa cells expressing steroidogenic factor-1 (SF-1; Fig. 1A). In the anterior pituitary, Alk7-GFP expression was detected in FSH-expressing gonadotrophs (Fig. 1B). There is a high overlap between FSH and LH expression in mouse pituitary (33); hence, ALK7 is also expected to be expressed by some LH gonadotrophs. In the hypothalamus, Alk7-GFP expression could be localized to cells immunopositive for NPY in the ARC of colchicine-treated brains (Fig. 1C). Most (≥85%) Alk7-GFP cells expressed NPY, but only about half (51.0±10.2%, \( n=5 \)) of NPY-expressing cells also expressed ALK7. β-Endorphin cells, the second major ascending cell population in the ARC, did not express Alk7-GFP (Fig. 1C, bottom panels). Together, these results indicate that ALK7 is expressed in distinct subpopulations of cells at all levels of the hypothalamic-pituitary-gonadal axis that controls female reproduction.

Delayed puberty onset and abnormal estrous cyclicity in female mice lacking ALK7

Vaginal opening occurs in response to estrogen and is one of the first pubertal markers. There was no significant difference in the onset of vaginal opening between wild-type and Alk7-knockout mice (Fig. 2A). Analysis of estradiol serum levels at estrus and diestrus showed no significant difference between the two genotypes (Fig. 2B), although values in the mutant were considerably more variable. After vaginal opening, daily vaginal smears were analyzed from P40 to P60 to assess the day of the first estrus. There was a significant delay in the appearance of the first estrus in Alk7-knockout mice (Fig. 2C), indicating a delayed onset of puberty in the absence of ALK7. Detailed analysis of the estrous cycle revealed a significantly lower number of days in estrus and metestrus, as well as longer diestrus in Alk7-knockout mice (Fig. 2D). There was also a tendency to longer estrous cycles in the mutants that did not reach statistical significance. Together, these data indicated altered female reproductive function in the absence of ALK7.

Early follicle degeneration, delayed ovulation, and reduced ovulatory competence in ovaries of mice lacking ALK7

Sections of ovaries of P21, P40, and adult mice were analyzed by H&E staining using the follicle classification developed by Peters et al. (27) (Fig. 3A). At P21, ovaries of wild-type animals were still compacted, with growing follicles of types 5a, 5b, and 6, along with a few degenerating follicles, as is normal during this stage. Although follicles in stages 5a, 5b, and 6 could also be seen in ovaries of Alk7-knockout mice, there were more degenerating follicles without an oocyte, suggesting that degeneration was occurring prematurely in mutant ovaries (Fig. 3A). At P40, ovaries of wild-type animals have a more mature

Figure 1. ALK7 expression in the reproductive axis. A) Alk7-GFP immunoreactivity colocalizes with SF-1+ cells in adult ovary. B) Alk7-GFP immunoreactivity in adult anterior pituitary colocalizes with FSH. C) Top panels: Alk7-GFP (green) and NPY (red) expression in the ARC of diestrous female mice injected with colchicine. Bottom panels: Alk7-GFP does not colocalize with β-endorphin in the ARC. V3, third ventricle. ME, median eminence. Scale bars = 50 μm.
appearance, with presence of corpora lutea (CL), a sign that ovulation has occurred. In contrast, no CL could be found at P40 in the 5 mutant ovaries examined (Fig. 3A), in agreement with delayed ovulation in females lacking ALK7. At this age, mutant ovaries also displayed higher numbers of degenerating follicles, as in more juvenile stages. At adult stages, ovaries of Alk7 mutant mice had significantly fewer growing follicles than their wild-type counterparts (7.6±1.3 vs. 13.3±1.2, P<0.05, n=8–9). These data suggest premature depletion of follicles and oocyte degeneration in female mice lacking ALK7. In contrast, no histological abnormalities could be detected in testis or epididymi of Alk7-knockout male mice (Supplemental Fig. S1).

Ovarian function was investigated in juvenile wild-type and knockout females by induction of superovulation through administration of exogenous gonadotropins (PMSG/hCG). The total number of COCs was determined from serial sections of control and gonadotropin-treated wild type and Alk7-mutant mice. COCs were significantly reduced in ovaries from mice lacking ALK7 (Fig. 3B, C), indicating impaired ovulatory competence in the mutants.

Figure 2. Delayed pubertal onset and abnormal estrous cyclicity in Alk7-knockout female mice. A) Age of vaginal opening in wild-type (WT) and Alk7-knockout (Alk7<sup>−/−</sup>) mice. Results are expressed as means ± SE (n=11). B) Estradiol serum levels at estrus (E) and diestrus (DE) in WT and Alk7<sup>−/−</sup> adult mice. Results are expressed as means ± SE (n=11 for measurements at E; n=7 WT and n=9 Alk7<sup>−/−</sup> for measurements at DE). C) Age of first estrus. Results are expressed as means ± SE (11 animals/group). *P < 0.05; 1-way ANOVA and Tukey-Kramer HSD test. D) Estrous cycle analysis. Days per cycle in proestrus (PE), estrus (E), metestrus (ME), and diestrus (DE) were counted in 4 consecutive cycles in adult WT and Alk7<sup>−/−</sup> female mice. (One knockout mouse was observed for only 3 cycles because they were too long.) Total cycle length is also indicated. Results are presented as median ± interquartile range. P = 0.027 (E), P = 0.33 (ME) and P = 0.0001 (DE); Mann-Whitney’s U test (11 animals/group). *P < 0.05.

Figure 3. Histological analysis of prepubertal, midpubertal, adult, and superovulated ovaries of wild-type and Alk7-knockout mice. A) Representative sections of ovaries from P21, P40, and adult wild-type and Alk7-knockout mice stained with H&E. Wild-type ovaries have developing follicles of types 5a, 5b, and 6, as well as degenerating follicles (d). In contrast, ovaries from Alk7-knockout mice have more degenerating follicles and are more vascularized. Wild-type P40 and adult ovaries already have corpora lutea (CL), a sign that ovulation has occurred. Ovaries from Alk7-knockout females lacked CL. B) Analysis of superovulation induced by exogenous administration of gonadotropins (PMSG/hCG) in wild-type and Alk7-knockout mice. Representative H&E stainings are shown. Scale bars = 200 μm (A); 250 μm (B). C) Quantification of COCs after superovulation of wild-type and Alk7-knockout mice with PMSG/hCG. Results are shown as average ± SE COC number per ovary (n=3). *P < 0.05; Student’s t test.
Abnormal pituitary function in Alk7-knockout mice

To assess pituitary function, we measured the serum concentration of FSH in prepuberty (P21), midpuberty (P40), and adult animals. Although somewhat higher than normal at prepuberty, serum FSH levels were found to decline with age in mutant females, and they were significantly lower than in wild-type animals at both midpubertal and adult (diestrus) stages (Fig. 4A). Serum FSH levels were normal in adult males lacking ALK7 (Supplemental Fig. S1B). On the other hand, LH levels in serum were higher in adult Alk7 mutant females than in wild-type controls at diestrus (Fig. 4B). It has been previously reported that deletion of the Fshβ gene results in elevated LH levels in mice (34). At the mRNA level, a modest increase in Lhβ mRNA was detected by quantitative PCR in the pituitary from mice lacking ALK7 (Fig. 4C). No change was found in either Cga or Fshβ mRNA levels between wild-type and mutant pituitary (Fig. 4C). Next, we assessed secretion of FSH and LH in cultures of pituitary cells dissociated from the adult pituitary. As in the intact gland, a proportion of FSH gonadotrophs also expressed ALK7 in culture (Fig. 4D). However, there was no difference in either basal or activin-stimulated FSH secretion between wild-type and mutant animals (4 animals/group). *Student’s t test.}

Reduced Npy expression and Npy neuron number in the ARC of Alk7-knockout mice

Given the overlap between ALK7 and NPY expression in neurons of the ARC of the hypothalamus (Fig. 1C) and the important roles played by arcuate NPY neurons in the control of female reproduction, we investigated whether lack of ALK7 had any effect on Npy mRNA expression and Npy neuron number in the arcuate. qPCR analysis revealed that levels of Npy mRNA were significantly reduced (by ~40%) in the ARC of Alk7-knockout female mice (Fig. 5A). Interestingly, mutant mice lacking activin B (InhbB+/−) also showed reduced Npy mRNA expression in the ARC (Fig. 5A), raising the possibility that activin B acts through ALK7 to positively regulate Npy mRNA expression or Npy neuron number in this nucleus. To address this latter possibility, we analyzed the distribution of neurons expressing Npy mRNA in the ARC by in situ hybridization (Fig. 5B). Eight anatomically matched sections from wild-type and mutant animals (4 animals/group) were analyzed.

*Figure 4. Basal FSH secretion in serum and dissociated pituitary cells from wild-type (wt) and Alk7-knockout (ko) mice. A) Basal levels of FSH in serum of prepubertal (P21), midpubertal (P40), and adult females (on the morning of diestrus) as detected by ELISA. FSH concentration is expressed in nanograms per milliliter. Data are expressed as means ± se (10 animals/group). *P < 0.05; Student’s t test. B) Basal serum levels of LH in adult females (diestrus) as detected by ELISA. Data are expressed as means ± se (10 animals/group). *P < 0.05; Student’s t test. C) Analysis of Cga, Fshβ, and Lhβ mRNA levels in pituitaries of adult wild-type and Alk7-knockout mice by quantitative PCR. Data are expressed as means ± se (8 animals/group). *P = 0.04; Student’s t test. D) ALK7 immunoreactivity in primary cultures of pituitary cells. All ALK7-expressing cells are also positive for FSH, identifying them as gonadotrophs. Not all FSH⁺ cells express ALK7. Scale bar = 20 μm. E) Pituitary primary cultures were stimulated with 50 ng/ml (1 nM) of activin A (ActA), activin B (ActB), or medium for 24 h. Supernatant was collected to quantify secretion of FSH by ELISA. Data are expressed as mean ± se (n=5). *P < 0.05; Student’s t test.
and cells positive for Npy mRNA expression were counted. The highest cell count was observed in the central region of the nucleus in both groups. Interestingly, the anterior and medial regions of the arcuate central region of the nucleus in both groups. Interest-
counted. The highest cell count was observed in the number of Npy neurons in the ARC compared to wild-type controls.

Reduced NPY and AgRP innervation of hypothalamic medial preoptic area in mice lacking ALK7

NPY fibers originating in the ARC project anteriorly to the PVN and to the MPOA of the hypothalamus. NPY innervation of the MPOA plays an important role in regulating the production and release of GnRH (35–37). We investigated whether the absence of ALK7 affected the NPY projection from the arcuate to these nuclei by immunohistological labeling of NPY fibers in brain sections of wild-type and Alk7 knockout mice. In the PVN, there was a normal complement of NPY fibers in sections of the Alk7 knockout (Fig. 6A, C). In contrast, the density of NPY fibers was greatly reduced (by ~70%) in the dorsal aspect of the MPOA in the mutants (Fig. 6B, C), suggesting a selective deficiency in NPY innervation of this target of the arcuate. AgRP is exclusively expressed by arcuate NPY neurons and has been used as a specific marker of NPY projections from the arcuate (29). To assess whether the paucity of NPY fibers in the mutant MPOA was indeed due to a deficit in the projection from the arcuate, immunohistochemistry for AgRP was performed in the MPOA of wild-type and Alk7-mutant female mice (Fig. 6D). AgRP fiber density was found to be reduced by 40% in the MPOA of the mutants (Fig. 6E), indicating that the NPY/AgRP projection from the arcuate to the MPOA is abnormal in Alk7-knockout mice. No difference was found in the number of neurons expressing GnRH (80.8 ± 23.2 vs. 76.0 ± 18.2 GnRH cells; n = 5; P = 0.97) or in GnRH mRNA levels (data not shown) in the MPOA of female mice lacking ALK7 compared to wild types.

DISCUSSION

The control of female reproduction is accomplished by a complex network of feedforward and feedback regulatory interactions between the gonads, the pituitary, and the hypothalamus. Activins play crucial roles in this network at different levels of the reproductive axis and have been widely studied for >2 decades. Although their expression and function in ovary and pituitary gland have been well documented, their actions on central hypothalamic nuclei are not well understood. Because of the embryonic lethality of mice lacking ALK4, elucidation of the specific role of this activin receptor in reproduction awaits the generation and analysis of conditional mutants. The second type I activin receptor, ALK7, binds activin B but not activin A, in addition to other ligands of the TGF-β superfamily. Mutant mice lacking ALK7 are viable and, in this study, we report the first characterization of the reproductive axis of mice lacking this receptor.

In agreement with the phenotype of mice lacking
activin B, female (but not male) mice lacking the ALK7 receptor displayed reproductive function defects. However, the abnormalities observed in Alk7-knockout females were of a different character, suggesting different roles for the ALK7 and ALK4 receptors in mediating the actions of activin B during reproduction control. Activins are expressed throughout ovarian development (9) and contribute to the formation of the initial follicle pool (38). Activins are also important regulators of follicle growth in mature ovaries (39). Our results suggest that ALK7 may mediate some of the effects of activin B during reproduction control. Activins are expressed throughout ovarian development (39, 41), our expression of activin B, Smad2, Smad3, Smad7, and the components of the activin signaling pathway, including expression of activin B, Smad2, Smad3, Smad7, and the endogenous activin inhibitor follistatin (45). They are also thought to regulate FSH production, in part, through the activin signaling system. It is possible that the different actions of female and male steroids on the activin signaling system underlie the dimorphic effects of the Alk7 mutation on reproductive function.

In addition to cell-autonomous defects in the ovaries of mice lacking ALK7, our results also point to abnormal gonadotropin levels as one of the primary abnormalities underlying the reduced fertility of females lacking ALK7. LH hypersecretion by mutant pituitary cells correlated with higher LH serum levels in Alk7-knockout females, suggesting a defect in mutant gonadotrophs. On the other hand, mice lacking ALK7 showed significantly reduced FSH levels at puberty and adult stages compared to wild-type controls. Reduced FSH production, as in mice deficient in FSH-subunit, has been shown to result in increased serum LH levels (34). Although pituitary derived activin B contributes to the tonic, autocrine regulation of basal FSH secretion by gonadotrophs (8, 46–48), we found that neither basal nor activin-stimulated FSH secretion from pituitary cultures of Alk7-knockout mice was different from wild-type mice, suggesting that the ALK4 receptor, which can bind both activins and is also present in gonadotrophs, can compensate for the lack of ALK7 in

Figure 6. Reduced NPY innervation of MPOA in Alk7-knockout mice. A) NPY-immunoreactive fibers in hypothalamic PVN of wild-type (WT) and Alk7-knockout (KO) mice. V3, third ventricle. B) NPY-immunoreactive fibers in the dorsal region of MPOA of WT and KO mice. C) Quantification of NPY-immunoreactive fibers innervating PVN, ventral MPOA, and dorsal MPOA. Animals were perfused intracardially the morning of diestrus, and matched sections from WT and KO animals were used for analysis. Data are presented as means ± se (n=5). *P < 0.05, Student’s t test. D) AgRP-immunoreactive fibers in the MPOA of WT and KO mice. Scale bars = 100 μm (A, D); 50 μm (C). E) Quantification of AgRP-immunoreactive fibers innervating the MPOA. Data are presented as means ± se (n=5). *P < 0.05; Student’s t test.
these cells. In vivo FSH levels are regulated by complex feedback and feedforward systems involving the action of steroids, gonadotropins, GnRH, and activins/inhibins on ovaries, pituitary, and hypothalamus. As shown here, ALK7 is not only expressed in pituitary, but also hypothalamus and ovary, and so it is likely that the combined loss of ALK7 signaling in those tissues underlies the apparent discrepancy between the in vivo and in vitro effects of ALK7 loss on FSH levels.

ALK7 was localized to NPY-expressing neurons in the ARC. In this structure, mutant mice lacking ALK7 showed reduced expression of Npy mRNA and lower numbers of Npy-expressing neurons compared to wild-type controls. In addition, a similar reduction in Npy mRNA expression was also observed in InhβB knockout animals, lacking the ALK7 ligand activin B. Together, these data suggest that activin B signaling through ALK7 functions cell-autonomously to regulate Npy mRNA expression and/or Npy neuron number in the ARC. It should also be noted that insulin can reduce Npy transcription following central administration (49). Because both Alk7- and InhβB-mutant mice have increased serum insulin levels, it is also possible that this contributes to the reduced levels of Npy expression in the ARC of these mutants. At this point, it is unclear whether a subset of Npy-expressing neurons is lost in Alk7 mutants or has simply down-regulated Npy expression to undetectable levels. In any case, it is clear that less NPY is being made in the arcuate of Alk7-knockout animals.

The arcuate NPY system provides direct input to GnRH cell bodies of the MPOA (50–52), where it contributes to the regulation of GnRH secretion (37, 53). Interestingly, NPY fiber density was drastically reduced in the dorsal MPOA of mutants lacking ALK7. Although the MPOA is thought to receive NPY innervation from other structures (i.e., brain stem) in addition to the ARC (52), the parallel reduction in AgRP+ fibers, all of which originate from NPY arcuate neurons (54, 55), indicates impaired arcuate NPY/AgRP innervation of GnRH neurons in Alk7-knockout mutants. Reduced NPY/AgRP innervation of the MPOA is expected to affect the pulsatile release of GnRH at the median eminence (36, 36, 37, 37, 53, 53, 56). In this regard, it should be noted that, although NPY has generally been proposed to have a stimulatory effect on LH secretion, deletion of the Npy gene has been shown to attenuate the preovulatory LH surge but to have little effect on basal LH levels at metestrus (57). Alk7-mutant mice have lost 27% of arcuate NPY neurons, but not all, and it is possible that an imbalance in MPOA innervation leads to abnormal GnRH stimulation of pituitary gonadotrophs and regulation of FSH/LH secretion.

In contrast to the MPOA, no reduction in NPY fiber density was observed in the mutant PVN, a second major rostral target of arcuate NPY fibers (29, 58). This indicates that the NPY deficits observed in the ARC of mice lacking ALK7 did not propagate across all arcuate NPY projections. The selective loss of arcuate NPY/AgRP fibers in the MPOA suggests that ALK7 plays a distinct role in hypothalamic wiring in addition to regulation of arcuate Npy mRNA levels and neuron number. Notably, ALK7 is not expressed in either MPOA or PVN (data not shown). The loss of NPY/AgRP fibers in the MPOA could thus be explained by a selective dependence of a subpopulation of MPOA-projecting arcuate NPY neurons on ALK7. There is evidence supporting neurotrophic functions of activins as stimulators of both synaptic connectivity (59) and neuronal survival (60). It is currently unclear whether different subsets of arcuate NPY neurons project to MPOA and PVN or whether the same neurons send collaterals that terminate in both targets. It is, therefore, possible that ALK7 regulates the differential innervation of these nuclei by the arcuate by, for example, mediating neurotrophic functions of MPOA-derived activins. The study of mice lacking ALK7 opens an opportunity to address several fundamental questions about hypothalamic connectivity.

We note that female mice lacking ALK7 show several of the abnormalities found in patients with the human condition known as polycystic ovary syndrome (PCOS), including abnormal estrous cyclicity, ovulatory dysfunction, LH hypersecretion, increased LH/FSH ratio, hyperinsulinemia, and insulin resistance (22, 61). Genetic factors are known to influence the development of PCOS, and several gene variants have been found to be associated with this syndrome. A variant that has been consistently replicated in different studies is present in the gene encoding the human Activin type IIA receptor (61, 62), indicating that abnormal activin signaling may contribute to PCOS. No single gene variant is expected to account for all the features of PCOS, and it is, therefore, not surprising that ALK7-mutant mice phenotype only some of the abnormalities found in this syndrome. On the basis of our results, we suggest that gene variants in Acrnel (the locus encoding ALK7) may represent risk factors for human PCOS.

In summary, our results indicate that ALK7 is a crucial regulator of female reproductive function and reveal a new role for activin signaling in the control of hypothalamic gene expression and wiring.

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REFERENCES


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