Cellular/Molecular

Hypothalamic Dysregulation and Infertility in Mice Lacking the Homeodomain Protein Six6

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The hypothalamus, pituitary, and gonads coordinate to direct the development and regulation of reproductive function in mammals. Control of the hypothalamic–pituitary–gonadal axis is dependent on correct migration of gonadotropin-releasing hormone (GnRH) neurons from the nasal placode to the hypothalamus, followed by proper synthesis and pulsatile secretion of GnRH, functions absent in patients with hypogonadal hypogonadism. In this study, we identify sine oculis-related homeobox 6 (Six6) as a novel factor necessary for proper targeting of GnRH expression to the limited population of GnRH neurons within the adult mouse hypothalamus and demonstrate that it is required for proper reproductive function in both male and female mice. Female Six6-null mice exhibit a striking decrease in fertility, failing to progress through the estrous cycle normally, show any signs of successful ovulation, or produce litters. Although basal gonadotropin production in these mice is relatively normal, analysis of GnRH expression reveals a dramatic decrease in total GnRH neuron numbers. We show that expression of Six6 is dramatically increased during GnRH neuronal maturation and that overexpression of Six6 induces GnRH transcription in neuronal cells. Finally, we demonstrate that this induction in GnRH expression is mediated via binding of Six6 to evolutionarily conserved ATTA sites located within the GnRH proximal promoter. Together, these data indicate that Six6 plays an important role in the regulation of GnRH expression and hypothalamic control of fertility.

Introduction

Mammalian reproduction is mediated by the pulsatile release of gonadotropin-releasing hormone (GnRH) from a distinct population of neurons within the hypothalamus. GnRH stimulates the anterior pituitary gland to secrete gonadotropins, which, in turn, act on the gonads to control gametogenesis. GnRH neurons have a unique point of origin within the olfactory placode (OP) at embryonic day 11.5 (E11.5) (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Schwanzel-Fukuda et al., 1992). From the OP, they migrate across the cribriform plate and through the basal forebrain to arrive within the presumptive hypothalamus by E17.5. They then extend their axons to the median eminence (ME) to allow secretion of GnRH into the hypophyseal portal system.

Received April 1, 2010; revised Oct. 19, 2010; accepted Oct. 25, 2010.

This work was supported by National Institutes of Health (NIH) Grants R01 DK044838 and R01 HD020377 (P.L.M.) and by National Institute of Child Health and Human Development–NIH through Cooperative Agreement U54 HD012303 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research (P.L.M.). This work was also supported by NIH Grants P30 DK063491, P30 CA023100, and P42 ES010337. D.D.C. was supported in part by NIH Grants T32 GM008666 and T32 DK007541 and was a student in the University of California, San Diego (UCSD), Biological Sciences Graduate Program. N.L.G.M. was supported in part by NIH Grant T32 GM08666 and was a student in the UCSD Biomedical Sciences Graduate Program. The LHβ antibody was provided by Dr. A. F. Parlow from the National Hormone and Pituitary Program, Harbor–University of California, Los Angeles, Medical Center. DNA sequencing was performed by the DNA-Sequencing Shared Resource, UCSD Cancer Center, which is funded in part by National Cancer Institute Cancer Support Grant P30 CA023100. Serum hormone assays were performed by University of Virginia Ligand Assay Core Laboratory, which is supported through National Institute of Child Health and Human Development Grant U54 HD028934. We thank Jonathan Aderinto, Susan Mayo, and Jason Meadows for technical assistance and Kirsten McTavish for assistance with interpretation of ovarian histology. We also thank Darren Logan for assistance with bioinformatic analysis and critical reading of this manuscript, Djurdjica Coss and Kellie Breen for critical reading of this manuscript, and members of the Mellon Laboratory for helpful discussion and support throughout this work.

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DOI:10.1523/JNEUROSCI.1688-10.2011

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Since adult mice have only ~800–1000 GnRH neurons, dispersed throughout the septohypothalamic region (Wray et al., 1989), analysis of GnRH transcriptional regulation has benefited from the generation of immortalized cell lines (Mellon et al., 1990; Radovick et al., 1991). GT1-7 cells represent a fully differentiated GnRH neuron that secretes high levels of GnRH in a pulsatile manner (Wetsel et al., 1991, 1992). In contrast, GN11 cells represent a developmentally earlier, migratory GnRH neuron that expresses low levels of GnRH and responds to migratory cues (Radovick et al., 1991). Using these cell lines, enhancer and promoter elements have been characterized (Kim et al., 2002, 2007; Lawson et al., 2002; Givens et al., 2004; Iyer et al., 2010) and several transcription factors that regulate GnRH gene expression have been elucidated (Clark and Mellon, 1995; Lawson et al., 1996; Wierman et al., 1997; Fang et al., 1998; Kelley et al., 2000; Wolfe et al., 2002; Rave-Harel et al., 2004, 2005; Pierce et al., 2008; Larder and Mellon, 2009).

Recently, we reported necdin, a Prader–Willi syndrome candidate gene, as the most differentially expressed transcript from a comparative microarray screen of GN11 and GT1-7 cell mRNA (Miller et al., 2009). We identified an additional ~2000 transcripts that were significantly upregulated in the more mature GT1-7 cell line (our unpublished observations). One of the most differentially expressed transcripts corresponded to sine oculis-related homeobox 6 (Six6), a homeodomain protein and vertebrate homolog of *Drosophila optix* (Jean et al., 1999). Mammalian Six proteins have two highly conserved domains; a homeodomain and a "Six" domain and are classified into three subfamilies based on the homology of these domains (Seo et al., 1999). Although Six1, Six2, Six4, and Six5 all show broad expression during embryogenesis, Six3 and Six6 are restricted to the developing

eye and brain (Oliver et al., 1995; Jean et al., 1999). However, despite their initial overlapping pattern, their expression becomes segregated in the postnatal brain, with Six6 expression becoming confined to the adult hypothalamus (Conte et al., 2005). With our identification of Six6 as one of the most upregulated genes in the GT1-7 cells compared with the GN11, we sought to determine its role in the control of GnRH neuronal maturation and its contribution to fertility *in vivo*.

Materials and Methods

Microarray data analysis and PCR. Extraction of RNA for microarray experiments and analysis of microarray data were performed as previously described (Miller et al., 2009). Adult mouse hypothalamus, pituitary, testes, ovary, and eye cDNA were purchased from Zyagen. Preparation of cDNA from cultured cells and reverse transcription (RT)-PCR were performed as previously described (Larder et al., 2004). Standard PCR conditions were used and were identical for all primers. Quantitative RT-PCR (Q-RT-PCR) amplification used GN11 or GT1-7 cDNA, IQ SYBR Green Supermix (Bio-Rad), specific primer sequences (supplemental Table S1, available at www.jneurosci.org as supplemental material), and an IQ5 real-time PCR instrument (Bio-Rad). Q-RT-PCR was performed as previously described (Larder and Mellon, 2009).

Mouse breeding and genotyping. Mouse colonies were maintained in agreement with protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. All animals were housed under a 12 h light/dark cycle and provided with food and water *ad libitum*. Six6-null mice were generated as previously described (Li et al., 2002) and were kindly provided by Dr. Xue Li (Children's Hospital of Boston, Harvard Medical School, Boston, MA). All mice were on a mixed 129/Sv and C57BL/6J genetic background.

Determination of day of vaginal opening and phase of estrous cycle. After weaning (20 d), all female mice were inspected daily and the age at vaginal opening recorded. To assess estrous cyclicity, vaginal smears were obtained from 4- to 6-month-old mice by vaginal lavage, over a period of at least 21 continuous days. Vaginal lavage was performed daily (at 10:00 A.M.) by flushing the vagina with distilled H_2O. Collected smears were mounted on glass slides and examined microscopically for cell type (Becker, 1995).

Vaginal plug formation. To monitor plug formation, a fertile C57BL/6J female mouse was housed with either a wild-type, heterozygous, or homozygous Six6-null male (8–12 weeks of age). The following morning, females were checked for the presence of vaginal plugs. If a plug was present, the female was removed to a separate cage. If no plug was detected, the female remained in the cage with the male. Plug checks were performed for 10 consecutive days.

Fertility assessment and hormone measurements. At 8 weeks of age, wild-type, heterozygous, or homozygous Six6-null mice were housed singly with an 8-week-old C57BL/6J mouse. The numbers of litters born and the number of pups per litter were recorded over a period of 90 d. For serum hormone analysis, mice were killed by overdose of 5% Avertin, and blood was collected by cardiac puncture. Serum was separated by centrifugation and stored at −20°C before radioimmunoassay (RIA) analysis at the Center for Research in Reproduction Ligand Assay and Analysis Core at the University of Virginia. All females were killed when they were in the diestrus stage of the estrous cycle.

GnRH dose–response and pituitary stimulation tests. To determine the appropriate dose of GnRH required to evoke a significant increase in serum luteinizing hormone (LH) levels, adult C57BL/6J male mice ($n = 3$) were injected subcutaneously with varying concentrations (100, 200, or 400 ng/kg) of GnRH (Sigma-Aldrich) dissolved in 0.9% saline, or with vehicle. Exactly 10 min after the injection, blood was collected by cardiac puncture. Serum was separated by centrifugation and stored at −20°C before analysis using the LUMINEX system at the Center for Research in Reproduction Ligand Assay and Analysis Core at the University of Virginia. From this preliminary experiment, we established that 200 ng/kg GnRH was an appropriate submaximal dose for determining the pituitary responsiveness of Six6KO males. From experimental wild-type ($n = 3$) or homozygous Six6-null ($n = 3$) mice, a small sample of blood (100 μl) was taken, via tail bleed, to serve as a baseline for LH levels. After 3 d of recovery, mice were given an injection of either 200 ng/kg GnRH or vehicle (saline). Exactly 10 min after the injection, blood was collected by tail bleed and serum analyzed as stated above.

Gonadal histology. Ovaries and testes were dissected and weighed from animals for each of the three genotypes. Ovaries were fixed for 1–2 h at room temperature in 4% paraformaldehyde (PFA) (Thermo Fisher Scientific) in PBS. Testes were fixed for 8 h in Bouin's fixative (Sigma-Aldrich) at room temperature. Gonads were paraffin embedded, serially sectioned at 10 μm, and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich). Histology was examined and the presence or absence of corpus lutea in the ovaries recorded.

LHβ immunohistochemistry. Pituitaries were dissected out and fixed for 1–2 h at room temperature in 4% PFA. After processing for paraffin embedding, they were serially sectioned at 7 μm. The primary antibody used was rabbit anti-rat LHβ (anti-rat β-LH-IC-3; 1:1000 dilution in 5% goat serum/0.3% Triton X-100). Biotinylated goat-anti-rabbit IgG (Vector Laboratories; 1:300 dilution) was used as a secondary antibody and LHβ peptide was visualized using the Vectastain ABC elite kit and vasoactive intestinal peptide (VIP) peroxidase kit (Vector Laboratories). To quantify the LHβ expression, a single section through the middle of each pituitary was visualized down the microscope, and all positive cells within the section were counted ($n = 4$ for each genotype).

Hypothalamic GnRH content. Hypothalami were dissected from 4-month-old female mice in diestrus, snap frozen, and stored at −80°C until processed. RNA was extracted using Trizol (Invitrogen) according to manufacturer's instructions and reverse transcribed using First-Strand cDNA Synthesis Kit (GE Healthcare) according to the manufacturer's instructions. Q-RT-PCR was performed as previously described (Larder and Mellon, 2009). For primer sequences, see supplemental Table S1 (available at www.jneurosci.org as supplemental material).

Embryo collection. Embryos were generated through timed-breeding of adult heterozygotes with E0.5 being noon of the day the vaginal plug was detected. Plugged females were killed, and embryos were harvested at E13.5. A small amount of the tail was removed from each embryo and used to extract DNA for genotyping (for primers, see supplemental Table S1, available at www.jneurosci.org as supplemental material). Whole embryos were fixed in 10% acetic acid, 30% formaldehyde, 60% ethanol, overnight at 4°C, and dehydrated in 70% EtOH before embedding in paraffin. Sagittal sections (10 μm) were floated onto SuperFrost Plus slides (Thermo Fisher Scientific). Approximately 150 sections were processed and stained per embryo.

Embryonic GnRH immunohistochemistry. Immunohistochemistry was performed as previously described (Miller et al., 2009). The primary antibody used was anti-GnRH antibody (Affinity Bioreagents; PA1-121; 1:1000 dilution in 5% goat serum/0.3% Triton X-100). Biotinylated goat anti-rabbit IgG (Vector Laboratories; 1:300 dilution) was used as a secondary antibody and GnRH peptide was visualized using the Vectastain ABC elite kit and VIP peroxidase kit (Vector Laboratories). Sections were counterstained using methyl green (Vector Laboratories). GnRH neurons were counted in three or more embryos of each genotype analyzed. Cells were divided into nasal, cribriform plate, and brain regions, and the mean was calculated.

Adult brain immunohistochemistry. Adult mice (4–5 months of age) were anesthetized with 5% Avertin, and then transcardially perfused with 20 ml of PBS followed by 20 ml of 4% PFA, pH 7.4. Adult females were killed during diestrus. Postnatal day 1 (P1) mice were killed at birth by decapitation. Brains were dissected out and incubated in 4% PFA overnight at 4°C. After embedding, each brain was serially sectioned in 12 μm coronal sections onto GOLD Superfrost Plus slides (Thermo Fisher Scientific). Approximately 250 sections were processed per mouse. GnRH immunohistochemistry was performed as described above. Tyrosine hydroxylase immunohistochemistry was performed as described above using a rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (Pel-Freez Biologicals; P40101; 1:1000 dilution in 5% goat serum/0.3% Triton X-100). Secondary antibody and chemical visualization steps were performed as for GnRH immunohistochemistry.

Bioinformatic analysis. Approximately 2000 bp of GnRH upstream regulatory region sequence was obtained for rat, mouse, human, dog, horse, cow, shrew, and bat using Ensembl. Sequences were aligned using the ClustalW program (available from the European Bioinformatics In-

stitute) and imported into GeneDoc (available from National Resource for Biomedical Supercomputing) for annotation.

Expression plasmids. Mouse Six6 was amplified by RT-PCR from GT1-7 cell cDNA and the product was TOPO-cloned into pCR2.1 (Invitrogen). After sequence verification, Six6 was subcloned into pSG5 (Stratagene; pSG5-Six6) and p3xFLAG-CMV7.1 (Sigma-Aldrich; pCMV-Six6-FLAG). Mouse Six3 expression plasmid (pCMV-HA-Six3) was obtained from G. Olivier (University of Tennessee, Memphis, TN) and subcloned into pSG5 (pSG5-Six3). Six3DelN expression plasmid (pSG5-Six3DelN) was generated by PCR from the wild-type plasmid and corresponds to amino acids 80–333 (primers in supplemental Table S1, available at www.jneurosci.org as supplemental material). The mouse Msx1 expression plasmid (pCB6+Msx1) was obtained from C. Abate-Shen (Columbia Univeristy, New York, NY), the mouse Dlx5 expression plasmid (pcDNA3-Dlx5) was obtained from J. L. R. Rubenstein (University of California, San Francisco, San Francisco, CA), and the mouse Otx2 expression plasmid (pSG5-Otx2) was obtained from A. Simeone (Institute of Genetics and Biophysics, Napoli, Italy).

Cell culture and transient transfections for luciferase reporter assays. All cells were cultured in DMEM (Mediatech) containing 10% fetal calf serum (Gemini Bio-Products) and 1% penicillin/streptomycin (Invitrogen) in a humidified 5% CO_2 incubator at 37°C. Cells were seeded into 24-well plates and incubated overnight at 37°C before being transiently transfected using FuGENE reagent (Roche Applied Science). Luciferase reporters were pGL3-GnRHe/p [rat GnRH enhancer (−1863 to −1571 bp) fused to the rat GnRH promoter (−173 to +112 bp)]; pGL3–5kb-GnRH (5 kb of the rat GnRH regulatory region); pGL3-ATTA-multimer [five copies of −48 to −55 bp of the rat GnRH promoter fused to a herpes simplex virus thymidine kinase (TK) promoter]; pGL3-GnRHe/p-E+Pdel [GnRHe/p with all four ATTA sites deleted]; pGL3-GnRHe/p-EDel [GnRHe/p with both enhancer ATTA sites deleted]; pGL3-GnRHe/p-Pdel [GnRHe/p with both promoter ATTA sites deleted]. Cells were transfected with 200 ng of expression plasmid, 400 ng of luciferase-reporter plasmid, and 100 ng of the internal-control TK −109 bp promoter on β-galactosidase. For titration experiments (see Fig. 6*D*), cells were cotransfected with 200 ng of Six3 expression plasmid and either 50, 100, or 200 ng of Six6 expression plasmid along with 400 ng of pGL3-GnRHe/p luciferase-reporter plasmid and 100 ng of the internal control TK −109 bp promoter. Cells were harvested after 48 h, lysed, and then assayed for luciferase and β-galactosidase as previously described (McGillivray et al., 2005). Luciferase values were divided by β-galactosidase values to control for transfection efficiency. All experiments were performed in triplicate and repeated a minimum of three times.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assay (EMSA) oligonucleotides (supplemental Table S1, available at www.jneurosci.org as supplemental material) were annealed, end-labeled, and then purified as previously described (Cherrington et al., 2008). Binding reactions used 2 fmol of ^{32}P-labeled oligonucleotide and either 2 μg of GT1-7 nuclear protein, 1 μg of Cos cell nuclear protein transfected with pCMV-3XFLAG, or 1 μg of nuclear protein from Cos cells transfected with p3XFLAG-CMV7.1. After addition of the probe, binding reactions were incubated for 10 min at room temperature before electrophoresis on a 5% polyacrylamide gel in 0.25× TBE (Tris–borate–EDTA). Competition assays were performed by preincubating the reactions with 500-fold excess of unlabeled oligonucleotide for 5 min before addition of probe. For supershift assays, 2.5 μg of an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich), or normal mouse IgG control (Santa Cruz Biotechnology), was added to the reaction. Gels were electrophoresed at 250 V for 2 h, and then dried, under vacuum, and exposed to film.

Statistical analysis. Raw data were analyzed by one-way ANOVA, followed by *post hoc* comparisons with the Tukey–Kramer honestly significant difference test using statistical package JMP 8.0 (SAS). Significant differences were designated as $p < 0.05$.

Results

Six6 expression is dramatically induced in a mature GnRH neuronal cell line

To identify candidate genes critical for the maturation of the GnRH neuronal phenotype, we performed an Affymetrix mi-

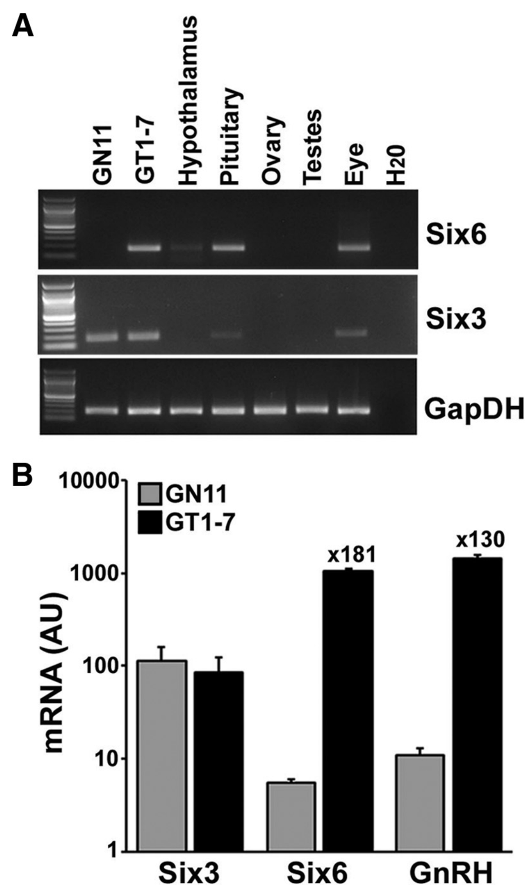

Figure 1. Six6 expression is dramatically increased in the mature GnRH neuronal GT1-7 cell line. ***A***, RT-PCR analysis of Six6 and Six3 expression in various adult mouse tissues and two GnRH neuronal cell lines. GapDH was used as a positive control. ***B***, Quantitative RT-PCR analysis of Six6, Six3, and GnRH mRNA extracted from GN11 and GT1-7 cells. Results are expressed as arbitrary units (AU) of GnRH mRNA levels normalized against GapDH mRNA levels and are the mean of three separate experiments performed in triplicate. Results shown are average ± SEM. Numbers above the GT1-7 bar indicate fold increase in mRNA levels in GT1-7 cells compared with GN11 cells.

croarray screen comparing RNA from GN11 cells (representing an early stage of development) and GT1-7 cells (representing a more mature hypothalamic neuron). Analysis of the results determined that >2000 transcripts showed significantly higher levels of expression in the more mature, GT1-7 cells versus the immature GN11 cells (data not shown). Unsurprisingly, several of these differentially regulated transcripts have previously been reported as being important for regulation of GnRH transcription such as Otx2 (Kelley et al., 2000; Larder and Mellon, 2009), Dlx5 (Givens et al., 2005), Gata4 (Lawson and Mellon, 1998), Msx1 (Givens et al., 2005), and Pbx1 (Rave-Harel et al., 2004). Intriguingly, we observed that Six6, a homeodomain-containing protein, known to be expressed in the pituitary and hypothalamus (Jean et al., 1999), was one of the most differentially regulated transcripts on the microarray (~208-fold higher expression in GT1-7 cells compared with GN11 cells). Semiquantitative RT-PCR validated the differential expression of Six6 mRNA between the two cell lines (Fig. 1*A*) and confirmed previous reports demonstrating that Six6 is expressed within adult mouse pituitary, hypothalamus, and eye (Li et al., 2002; Conte et al., 2005). In addition, quantitative RT-PCR confirmed the differential expression profile of Six6 and Six3 in the two model cell lines (Fig. 1*B*), with Six6 upregulated to a similar degree to that seen in the mi-

Table 1. Male fertility assessment

	No. litters in 3 months	No. days until first litter	No. pups per litter
WT	2.8 ± 0.2 (*n* =6)	23 ± 1.0 (*n*=6)	8.3 ± 0.8 (*n*=16)
HET	2.8 ± 0.4 (*n* =5)	25 ± 1.6 (*n*=5)	8.8 ± 0.6 (*n*=14)
KO	1.8 ± 0.3* (*n*=6)	34 ± 5.6 (*n*=6)	6.9 ± 0.8 (*n*=12)

Results shown are average ± SEM.

ANOVA with *post hoc* Tukey's test was performed on WT versus HET, WT versus KO, and HET versus KO, and established statistical significance as $^*p < 0.05$.

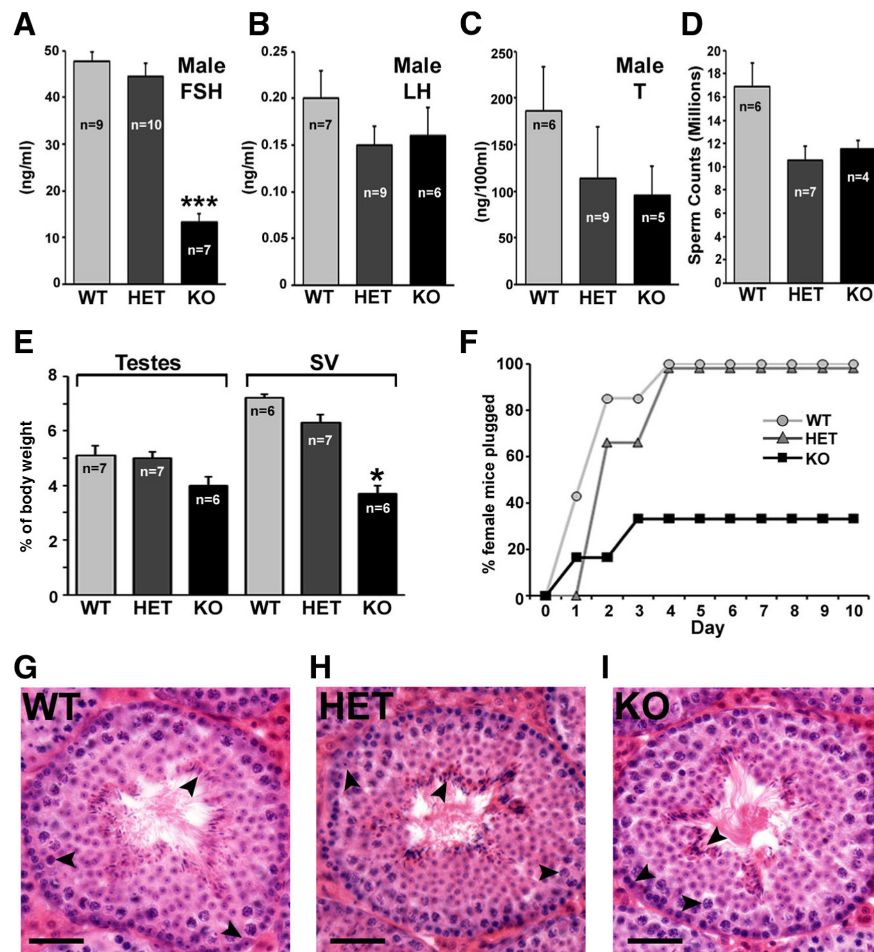

Figure 2. Reproductive phenotype of Six6-null male mice. ***A***, Average serum FSH levels of 4- to 6-month-old WT, HET, and KO male mice. ***B***, Average serum LH levels of 4- to 6-month-old WT, HET, and KO male mice. ***C***, Average serum T levels of 4- to 6-month-old WT, HET, and KO male mice. ***D***, Average sperm count of 4- to 6-month-old WT, HET, and KO male mice. ***E***, Average relative testis and SV weight of 4- to 6-month-old WT, HET, and KO mice. ***F***, Percentage of wild-type females that plug after 10 consecutive days of mating with either a WT, HET, or KO male mouse. ***G–I***, H&E-stained testes sections from WT (***G***), HET (***H***), and KO (***I***) Six6-null mice. Sertoli cells, primary spermatagonia, and elongated spermatids are indicated with arrowheads in each section. No abnormal histology was observed between the three genotypes examined. Magnification, 40×. Scale bars, 10 μm. Results shown are average ± SEM. ANOVA with *post hoc* Tukey's test was performed on WT versus HET, WT versus KO, and HET versus KO, and established statistical significance as follows: $^*p < 0.05$ and $^{***}p < 0.001$.

croarray (~180-fold). Although Six3 is in the same subfamily as Six6 (Kumar, 2009), it is not differentially expressed between the two cell lines and, as previously shown (Conte et al., 2005), is not detected in the adult hypothalamus (Fig. 1*A*,*B*). Given that the observed increase in Six6 expression correlated with an increase in GnRH expression, we hypothesized that Six6 may regulate GnRH gene expression and thus play an important role in hypothalamic control of fertility.

Loss of Six6 expression disrupts male fertility because of decreased follicle-stimulating hormone release

Initial reports characterizing the Six6-null mouse focused on its role during eye development. Although the knock-out mice were reported to have hypoplastic pituitary glands, the presence of all the pituitary hormones was confirmed and the fertility of the mice was not reported (Li et al., 2002). To determine whether loss of Six6 expression results in compromised fertility, we performed a fertility assessment of wild-type, heterozygous, and homozygous Six6-null littermates. At 8 weeks of age, each male mouse was paired with an 8-week-old C57BL/6J female and the frequency and size of any litters born recorded for 3 months. Although homozygous (KO) males were fertile, they produced significantly reduced numbers of litters during the 3-month assessment period when compared with their wild-type (WT) and heterozygous (HET) littermates (Table 1). The number of pups per litter was not significantly different between the three genotypes, suggesting comparable embryonic viability (Table 1).

Changes in gonadotropin hormone release in these mice were measured by RIA. Although all serum gonadotropins [follicle-stimulating hormone (FSH), LH, and testosterone (T)] were reduced in the KO males, compared with their WT littermates, only the decrease in FSH levels reached statistical significance (Fig. 2*A–C*). With KO mice showing both decreased fertility and serum gonadotropins, we sought to determine whether Six6-null males had any obvious gonadal abnormalities. Comparison of sperm counts (Fig. 2*D*) and testes weights (Fig. 2*E*) revealed no significant differences between the three genotypes; however, seminal vesicle (SV) weight was significantly reduced in the KO males (Fig. 2*E*). This result is consistent with the decreased testosterone levels seen in these animals (Fig. 2*C*) since SV tissue is responsive to T (Shima et al., 1990). Given that histological examination of WT, HET, and KO testes revealed no striking differences in either architecture or spermatogenesis (Fig. 2*G–I*), we analyzed other reproductive functions in these mice. We hypothesized that the lowered SV weight in these mice might lead to a decrease in seminal fluid volume (Bradshaw and Wolfe, 1977). Analysis of the ability of WT, HET, and KO males to produce vaginal plugs in wild-type females confirmed this hypothesis: only 33% of females housed with a KO male formed a visible plug after 10 d of cohabitation compared with 100% of females housed with either a WT or HET male (Fig. 2*F*).

Loss of Six6 expression severely disrupts female fertility

The fertility of female Six6-null mice was also assessed over a 3 month period. As expected, wild-type and heterozygous females demonstrated normal reproductive behavior (Table 2). In con-

Table 2. Female fertility assessment

	No. litters in 3 months	No. days until first litter	No. pups per litter	Day of vaginal opening
WT	2.6 ± 0.3 ($n = 7$)	24 ± 1.9 ($n = 7$)	8.3 ± 0.7 ($n = 18$)	27 ± 0.3 ($n = 12$)
HET	2.1 ± 0.5 ($n = 7$)	26 ± 2.7 ($n = 6$)	7.1 ± 0.8 ($n = 16$)	27 ± 0.5 ($n = 12$)
KO	0.13 ± 0.1*** ($n = 8$)	27 ($n = 1$)	5 ($n = 1$)	28 ± 0.2 ($n = 5$)

Results shown are average ± SEM.

ANOVA with *post hoc* Tukey's test was performed on WT versus HET, WT versus KO, and HET versus KO, and established statistical significance as $^{***}p < 0.001$.

trast, the fertility of KO females was severely compromised with only one of the eight females assessed (12.5%) producing a single litter of five pups, 27 d after being paired with a male (Table 2).

Six6-null females display normal puberty but have disrupted estrous cycles

In female rodents, normal initiation of puberty results in vaginal opening followed by the beginning of regular estrous cycles. To assess puberty in Six6-null mice, female pups were observed daily for vaginal opening beginning at time of weaning (20 d). No significant differences in day of vaginal opening were observed between the three genotypes examined (Table 2), suggesting that loss of Six6 expression does not alter onset of puberty. To assess estrous cyclicity, vaginal cytology of WT, HET, and KO Six6-null littermates was examined daily by vaginal lavage. Mean cycle length was doubled in KO mice compared with their WT and HET littermates resulting in the KO females spending significantly more of the cycle in estrus (Fig. 3*A*–*C*).

Ovarian histology is abnormal in Six6-null females

To examine folliculogenesis and ovulation in Six6-null mice, diestrus ovaries from 4- to 6-month-old mice were inspected for developing follicles and corpora lutea (CL). Ovaries from KO mice were significantly smaller (Fig. 3*G*) and had significantly fewer CL (Fig. 3*H*) than ovaries from WT or HET littermates. Histological analysis revealed that ovaries from WT and HET mice displayed normal folliculogenesis (Fig. 3*D*,*E*,*H*). In contrast, of the five ovaries analyzed from KO mice, CL were only detected in the ovaries of the single Six6-null mouse that produced one litter during the fertility assessment (Fig. 3*H*). Furthermore, closer examination of all the KO ovaries revealed the presence of abnormal stromal tissue consisting of cells with enlarged cytoplasm, indicative of a failed attempt to transform granulosa cells into luteal tissue (Fig. 3*I*,*J*). In addition, large cystic follicles (CFs) (Fig. 3*F*) were also commonly observed in the ovaries of Six6-null animals.

Basal serum gonadotropin levels are not significantly altered in Six6-null females

Because Six6 has been reported to be expressed in both the developing and mature pituitary (Jean et al., 1999; Li et al., 2002) and Six6-null females appear to have defective ovulation (Fig. 3*F*), we wanted to determine whether Six6-null mice have normal expression of LHβ from gonadotrope cells within the anterior pituitary. Immunohistochemistry revealed no significant difference in either gonadotrope cell number or expression of LHβ when pituitaries from wild-type mice were compared with those from homozygous Six6-null littermates (Fig. 3*K*,*L*) (WT, 252 ± 27 positive cells, vs KO, 297 ± 48 positive cells; $p = 0.44$, *t* test). This suggests that loss of Six6 expression does not affect the ability of the gonadotrope to produce LHβ. RIA measurement of serum gonadotropins revealed that, whereas neither LH or FSH concentrations were significantly different between the three genotypes, serum LH levels were almost doubled in KO females, although this did not reach statistical significance (Fig. 3*M*); increased serum LH is consistent with the cystic follicles seen in these animals (Fig. 3*F*).

Six6-null mice have significantly decreased hypothalamic GnRH expression

Since Six6-null ovaries showed no evidence of ovulation, and females had increased expression of pituitary LHβ, it was important to determine the levels of GnRH expression in the hypothalami of these mice. Analysis of protein expression using a GnRH antibody revealed that Six6KO mice had dramatically reduced numbers of GnRH neurons (Fig. 4*A*,*B*). Cell counts from coronal sections extending from the medial septum through to the anterior hypothalamus revealed an 89% decrease in total GnRH neuron numbers in 4-month-old KO females when compared with their WT littermates (Table 3). A similar decrease was seen in 4- to 5-month-old Six6KO male mice (Table 3). The location of the GnRH neurons was mapped within the brain, revealing that the significant decrease in neuron numbers affected all the brain regions analyzed (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Immunostaining of the ME showed a similar marked decrease in GnRH neuron axon density (Fig. 4*C*,*D*). The decrease in GnRH protein expression was then confirmed at the mRNA level by Q-RT-PCR analysis of hypothalami from adult WT and KO mice. Loss of Six6 resulted in an ~83% decrease in GnRH mRNA expression (Fig. 4*I*). To determine whether other neuronal cell types are also affected by the loss of Six6, expression of TH throughout the brain of WT and Six6KO animals was also analyzed. TH is the first enzyme in the catecholamine (CA) biosynthesis pathway catalyzing the conversion of L-tyrosine to L-DOPA and is, therefore, a useful marker of all CA-positive neurons. Our analysis revealed that female Six6-null mice have decreased numbers of TH-positive neurons within the anteroventral periventricular (AVPV) region (Fig. 4*E*,*F*) and also show decreased targeting to the ME (Fig. 4*G*,*H*). The rodent AVPV is sexually dimorphic, with females showing a greater number of TH-positive neurons than males (Simerly et al., 1985). Whether the observed decrease in TH-positive AVPV neurons in female Six6KO mice represents a deficit in the sexual dimorphism of the brain, or represents a more general hypothalamic defect, awaits additional investigation; however, our analysis of vaginal opening (Table 2) suggests the latter and indicates that the hypothalamic effects of deleting Six6 may not be confined to the GnRH neuronal population.

The decrease in GnRH neuron numbers in Six6KO mice occurs during late embryonic development

GnRH neurons are first observed within the olfactory placode at around E10.5. From there, they migrate across the cribriform plate and through the basal forebrain to arrive at the presumptive hypothalamus at around E17.5. To establish whether the loss of GnRH neurons observed in Six6-null adults occurs during embryonic development, the total number and distribution of GnRH-positive neurons was evaluated in E13.5 and P1 animals. WT and KO embryos were sagittally sectioned, stained for GnRH protein, and positive neurons were counted. In contrast to the results observed in adult mice, total numbers of GnRH neurons at E13.5 were not significantly different between WT and KO littermates (Table 3). Furthermore, there was no disruption to the progress of the GnRH neurons along their migratory path (Fig. 4*K*,*L*; supplemental Fig. S2, available at www.jneurosci.org as supplemental material). However, similar to our observations

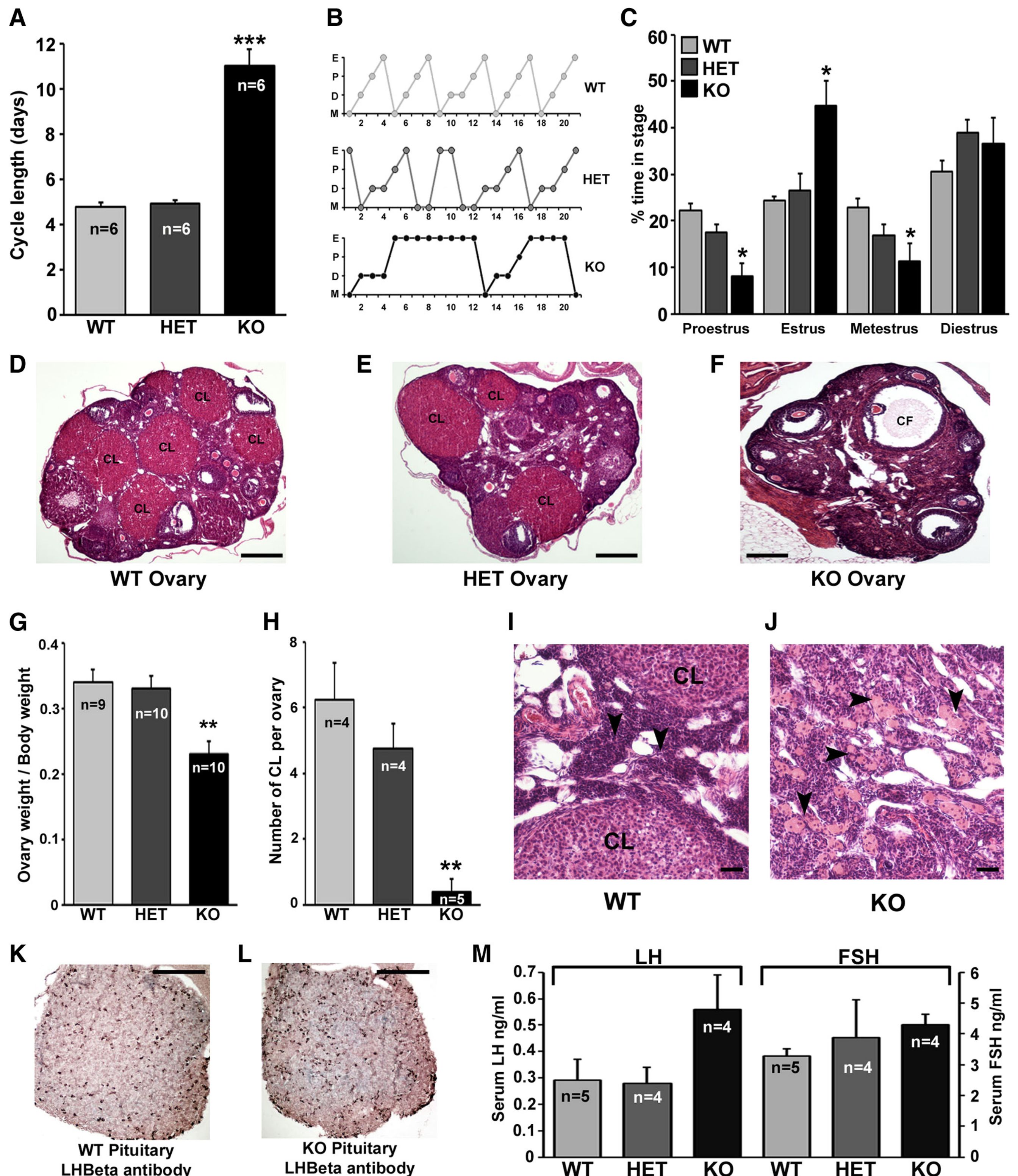

Figure 3. Reproductive phenotype of Six6-null female mice. ***A***, Average estrous cycle length of WT, HET, and KO females. ***B***, Representative estrous cycles as measured by vaginal cytology. E, Estrus; P, proestrus; D, diestrus; M, metestrus. ***C***, Percentage of time during a single estrus cycle that is spent in each stage. ***D–F***, H&E-stained ovarian sections from WT (***D***), HET (***E***), and KO (***F***) Six6-null mice. KO ovaries exhibit large CFs and no CL. Magnification, 4×. Scale bars, 100 μm. ***G***, Average relative ovary weight of 4- to 6-month-old WT, HET, and KO mice. ***H***, Average number of CL in ovaries of WT, HET, and KO Six6-null females. ***I***, ***J***, H&E-stained ovaries from WT and homozygous Six6-null mice (KO) showing stromal tissue. Extensive areas of cells with enlarged cytoplasm (arrowheads) are seen in the stroma of KO mice. Magnification, 20×. Scale bars, 10 μm. ***K***, ***L***, LH β-subunit (LHβ) staining of the anterior pituitaries of 6-month-old WT and KO female mice. Magnification, 10×. Scale bars, 10 μm. ***M***, Serum LH and FSH levels of 4- to 6-month-old, diestrus stage, WT, HET, and KO female mice. KO mice had increased levels of LH compared with WT and HET littermates ($p = 0.056$). There was no difference in FSH levels between the three genotypes. Results shown are average ± SEM. ANOVA with *post hoc* Tukey's test was performed on WT versus HET, WT versus KO, and HET versus KO, and established statistical significance as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

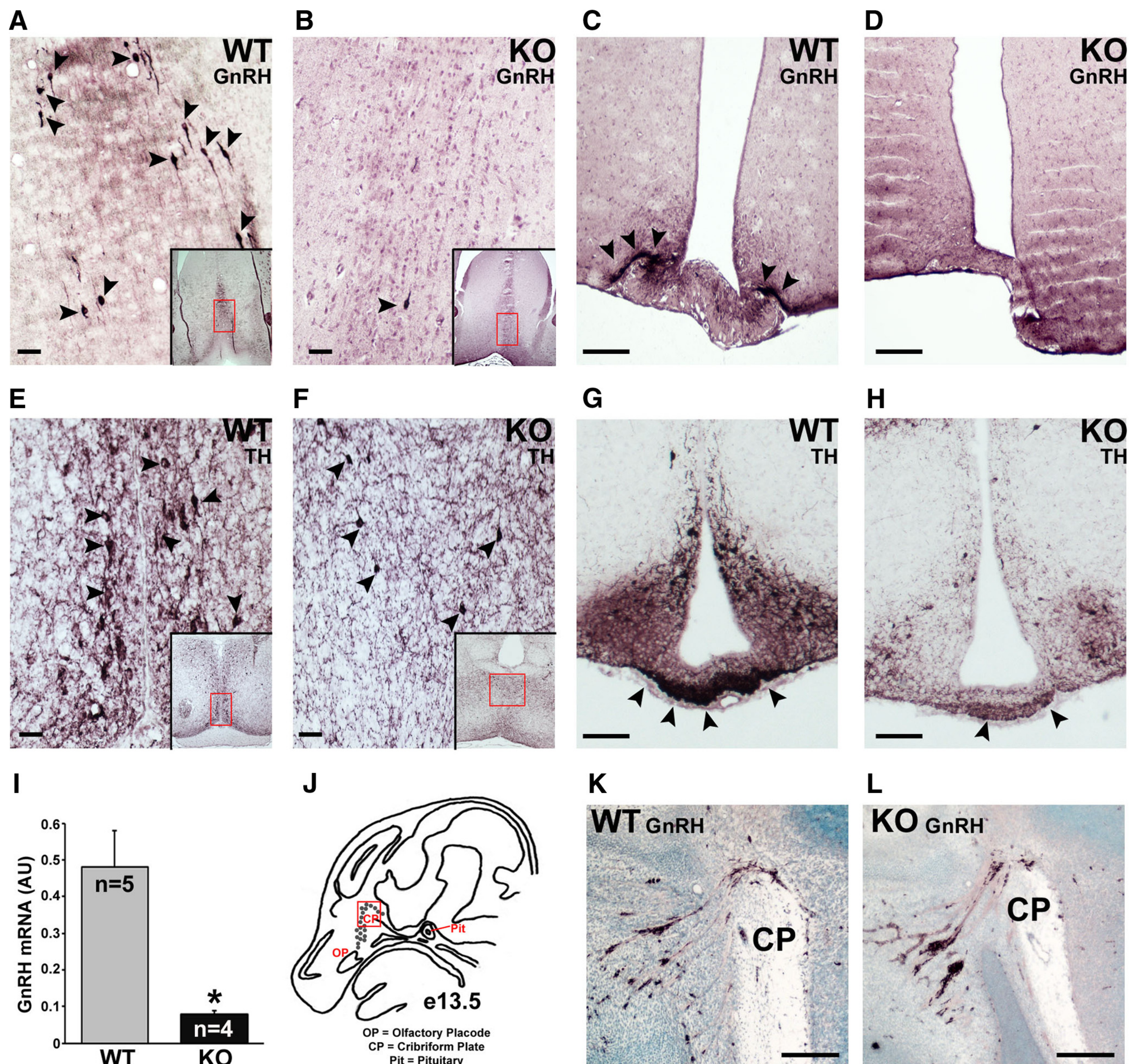

Figure 4. Adult Six6-null mice have a significant reduction in GnRH neuron numbers. ***A–D***, Coronal brain sections showing GnRH staining in the hypothalamus (***A***, ***B***) and median eminence (***C***, ***D***). The red box in ***A*** and ***B*** indicates the area of the section that has been enlarged. The black arrowheads indicate GnRH neurons in ***A*** and ***B*** and GnRH axons in ***C***. ***E–H***, Coronal brain sections showing TH staining in the AVPV nucleus (***E***, ***F***) and median eminence (***G***, ***H***). The red box in ***E*** and ***F*** indicates the area of the section that has been enlarged. The black arrowheads indicate TH neurons in ***E*** and ***F*** and TH axons in ***G***. Scale bars: ***A***, ***B***, ***E***, ***F***, 10 μm; ***C***, ***D***, ***G***, ***H***, 100 μm. ***I***, Quantitative RT-PCR analysis of hypothalamic GnRH mRNA in 4- to 6-month-old WT and KO female mice. Results are expressed as arbitrary units (AU) of GnRH mRNA levels normalized against GapDH mRNA levels and are the mean of three separate experiments performed in triplicate. ANOVA with *post hoc* Tukey's test established statistical significance as $^{*}p < 0.05$. ***J***, Diagram of E13.5 mouse head anatomy in a sagittal section showing the location of migrating GnRH neurons. The red box indicates the field shown in ***K*** and ***L***. ***K***, ***L***, Immunohistochemical staining for GnRH in E13.5 WT (***K***) and Six6-null (KO) (***L***) embryos. Magnification, 10×. Scale bars, 50 μm. CP, Cribriform plate.

Table 3. GnRH neuron counts

	Female adult	Male adult	Postnatal day 1	Embryonic day 13.5
WT	442 ± 27 ($n = 3$)	488 ± 51 ($n = 3$)	254 ($n = 1$)	449 ± 41 ($n = 3$)
KO	47 ± 12*** ($n = 3$)	77 ± 15*** ($n = 3$)	96 ± 30 ($n = 3$)	425 ± 28 ($n = 3$)

Results shown are average ± SEM.

ANOVA with *post hoc* Tukey's test was performed on WT versus HET, WT versus KO, and HET versus KO, and established statistical significance as $^{***}p < 0.001$.

in adult mice, a substantial decrease in GnRH neuron numbers was seen in Six6-null animals at P1 (Table 3), leading us to hypothesize that a similar decrease in GnRH mRNA would also been seen at this time point. Together, these data suggest that Six6 is not required for either the birth or initial migration of GnRH neurons, nor is it essential for initiation of GnRH expression. However, as observed in the case of AP-2 (activator protein-2) expression (Kramer et al., 2000), as the GnRH neuron matures during adulthood, and GnRH expression increases, expression of Six6 is required to ensure that normal adult levels of GnRH transcription are achieved and maintained.

Six6KO null males are able to generate a normal LH surge in response to GnRH

Given that Six6 is expressed in the pituitary (Fig. 1*A*), it is possible that the fertility effects observed in these animals may be

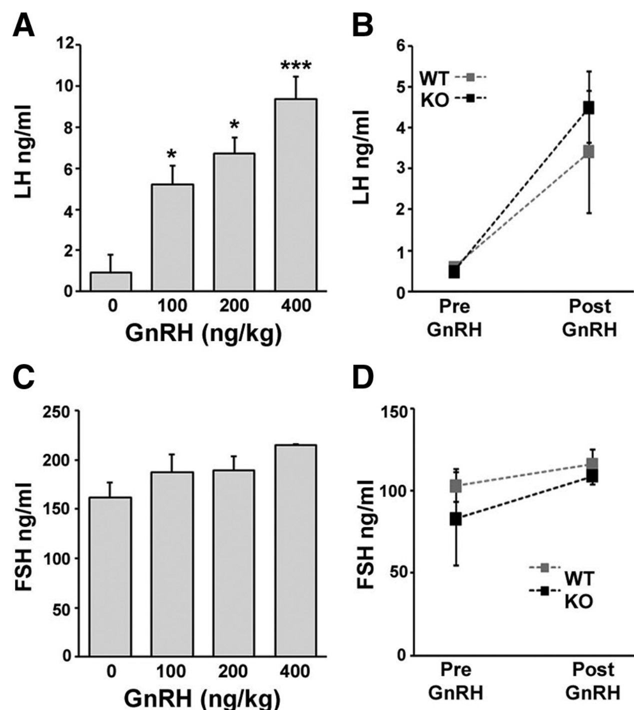

Figure 5. Six6KO male mice respond normally to a surge of GnRH. ***A***, Effect of increasing doses of GnRH on WT LH serum levels. ***B***, LH serum levels of WT and KO male sib pairs before, and 10 min after, injection of 200 ng/kg GnRH. ***C***, Effect of increasing doses of GnRH on FSH serum levels. ***D***, FSH serum levels of WT and KO male sib pairs before, and 10 min after, injection of 200 ng/kg GnRH. Results shown are average ± SEM. ANOVA with *post hoc* Tukey's test comparing each GnRH treatment to the control (saline) established statistical significance as follows: $*p < 0.05$ and $***p < 0.001$.

attributable, in part, to the loss of Six6 expression within gonadotrope cells. Therefore, it was important to establish whether the pituitaries of these animals were functioning normally with respect to gonadotropin release in response to GnRH. Male mice were injected with a bolus of GnRH and blood collected 10 min later for analysis of serum gonadotropins levels. All analysis was performed on male mice to rule out any complications attributable to the stage of the estrus cycle and steroid feedback from the ovaries. Analysis revealed that all doses of GnRH used (100, 200, or 400 ng/kg) resulted in a significant increase in LH serum levels (Fig. 5*A*). Furthermore, a small, but not significant, increase in FSH serum levels was also observed at all concentrations tested (Fig. 5*C*). Given this, we chose 200 ng/kg GnRH as an appropriate dose to determine whether a significant increase in serum LH would also be observed in Six6-null animals. Although no difference in basal LH serum levels was detected between the two genotypes (because of the sensitivity of the assay being 0.48 ng/ml), both WT and KO males showed a dramatic increase in LH serum levels 10 min after injection with 200 ng/kg GnRH (Fig. 5*B*), suggesting that the pituitaries of Six6KO mice can respond appropriately when presented with GnRH. Finally, whereas a difference in baseline FSH levels was observed between the two genotypes, confirming data shown in Figure 2, both WT and KO mice showed a small increase in FSH secretion in response to GnRH (Fig. 5*D*).

Overexpression of Six6 increases GnRH transcriptional activity in both GnRH neuronal cell lines

Given that Six6-null adults have such a dramatic decrease in both GnRH mRNA and protein expression, we next investigated the

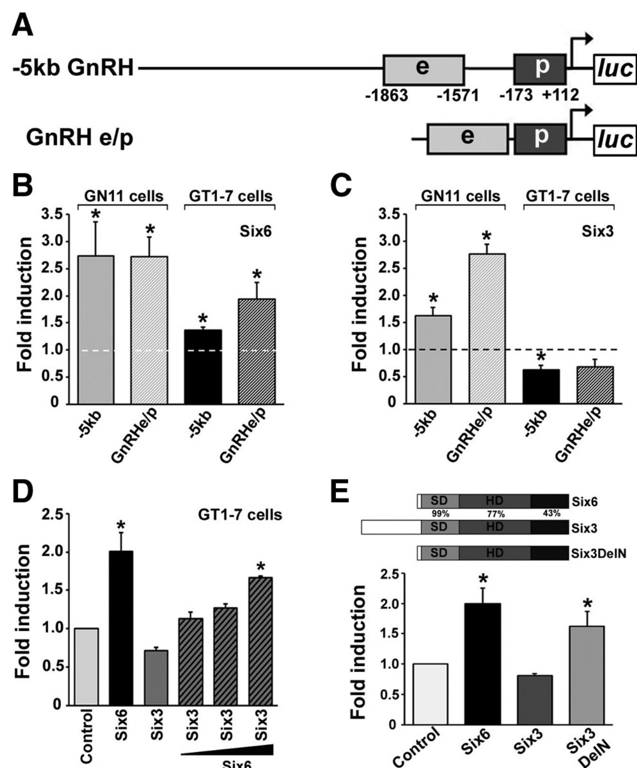

Figure 6. Six6 induces GnRH promoter activity. ***A***, Schematic diagram showing the luciferase reporter constructs used in transient transfection assays. Numbers indicate the position of nucleotides relative to the transcription start site: promoter (p), enhancer (e), luciferase (luc). ***B***, ***C***, Effect of overexpression of Six6 (***B***) or Six3 (***C***) on the −5 kb GnRH and GnRHe/p luciferase reporters in GN11 and GT1-7 cells. Fold induction compared with "empty" vector alone (dashed line) is indicated, corrected for β-galactosidase, which was used as an internal control. Overexpression of Six6 resulted in significant fold induction of both reporter constructs. Activation of promoter activity by Six3 was only seen in GN11 cells. ***D***, Cotransfection of 200 ng of Six3 expression plasmid and increasing amounts (50 –200 ng) of Six6 expression plasmid relieves the repression of GnRHe/p promoter activity seen with Six3 alone. ***E***, Overexpression of a mutant Six3 expression plasmid (Six3DelN) resulted in significant activation of GnRHe/p promoter activity in GT1-7 cells. SD, Six domain; HD, homeodomain. All experiments were performed in triplicate and repeated three times. Results shown are average ± SEM. One-way ANOVA with *post hoc* Tukey's test established statistical significance as $p < 0.05$. *Significant activation versus empty vector "control."

role of Six6 in transcriptional control of GnRH gene expression. Six6 was overexpressed by transient transfection in GN11 and GT1-7 cells along with either −5 kb of the rat GnRH regulatory region or the previously characterized rat GnRH enhancer and promoter (GnRHe/p), fused upstream of a luciferase reporter gene (Lawson et al., 2002) (Fig. 6*A*). Overexpression of Six6 significantly increased the activity of both of the luciferase reporter genes tested, in both cell lines (Fig. 6*B*). When the same experiments were performed using a Six3 expression plasmid, contrasting results were observed. Six3 significantly activated both luciferase reporters in GN11 cells; however, in the GT1-7 cells, overexpression of Six3 resulted in significant repression of GnRH promoter activity (Fig. 6*C*). Additional experiments revealed that the repressive effects of Six3 on GnRH transcription in GT1-7 cells could be relieved by titrating in increasing amounts of Six6 (Fig. 6*D*), indicating a potential competition between the two factors for binding sites in the GnRH regulatory region.

N terminus truncation of Six3 results in activation of the GnRH promoter

The homeodomain and Six domain are highly homologous between Six3 and Six6 proteins [Fig. 6*E*, homeodomain (HD), 77%

identical; Six domain (SD), 99% identical]. The main divergence between the two proteins is seen in the C terminus region common to both proteins (43% identical) and by the presence of an 80 aa, glycine-rich region, at the N terminus of the Six3 protein. To determine whether this additional N terminus region was mediating the repressive effects of Six3 on the GnRH promoter in GT1-7 cells, we generated a mutant Six3 protein (Six3DelN) with a deletion of this 240 bp sequence (Fig. 6*E*). In contrast to the results observed with wild-type Six3, overexpression of Six3DelN in GT1-7 cells resulted in similar induction in GnRH promoter activity to that seen in response to Six6 (Fig. 6*B*). This suggests that the unique N terminus region of Six3 is required for the repressive activity of Six3 in GT1-7 cells.

Six6 binds to conserved ATTA sites within the rat GnRH enhancer and promoter

In recent years, the regulatory target sequences of several Six proteins have been reported. Six1, Six2, Six4, and Six5 have been shown to bind to a TCAGGT sequence motif (Spitz et al., 1998; Kawakami et al., 2000). In contrast, Six3 and Six6 have been shown to bind to the classical homeodomain core sequence ATTA (Zhu et al., 2002; Hu et al., 2008), indicating that the DNA-binding specificity of the Six3/6 class of Six proteins differs from other members of the gene family. We have previously characterized a 173 bp promoter element and a 300 bp enhancer element within the rat GnRH regulatory sequence, both of which are essential for correct neuronal expression of GnRH (Lawson et al., 2002). Within these regions, several important transcription factor-binding sites have been identified including four ATTA sites that have previously been shown to be essential for cell-specific expression of the GnRH promoter (Nelson et al., 2000; Kelley et al., 2002). Bioinformatic analysis revealed a high level of conservation in the ATTA sites found in these regions between rat, mouse, and human (Givens et al., 2004). To determine whether these ATTA binding sites are conserved across other species, the DNA sequences from −65 to −22 bp and from −1642 to −1603 bp of the rat GnRH regulatory sequence were aligned with the GnRH regulatory sequences from various mammals (Fig. 7*A*). The alignments show that all four ATTA motifs are well conserved, suggesting that all four sites are important for proper regulation of GnRH transcription. To analyze the affinity of Six6 for these ATTA binding sites, we performed EMSAs with radioactively labeled probes containing the −41, −53, −1622, or −1635 bp sites (see supplemental Table S1, available at www.jneurosci.org as supplemental material). Nuclear protein extracts from COS-1 cells transiently transfected with FLAG-tagged Six6 formed a specific complex when incubated with any of the four probes (Fig. 7*B*, lanes 2, 9, 16, and 23). This complex was not observed when nuclear extracts from COS-1 cells transfected with empty FLAG vector were incubated with the probes (lanes 1, 8, 15, and 22). This specific complex could be removed (lanes 10, 17, and 24) or supershifted (SS, lane 3) when incubated with an antibody to FLAG. Furthermore, the addition of unlabeled WT probe significantly decreased complex formation (lanes 6, 13, 20, and 27), whereas competition with an unlabeled, mutated probe (MUT, ATTA sequence mutated to GCCG) had no effect on complex formation (lanes 7, 14, 21, and 28). To show binding of endogenous Six6, EMSAs were also performed using nuclear extracts from GT1-7 cells and the −41 bp probe. This probe was selected because it shows 100% conservation of the ATTA sequence across various species analyzed (Fig. 7*A*). A specific complex formed on the −41 bp probe (Fig. 7*C*, gray arrow, lane 2) and was competed away by addition of excess WT probe (lane 3), but not excess mutant probe (MUT, lane 4). As expected, this complex migrated slightly further on the gel than the complex formed by a FLAG-tagged Six6 protein overexpressed in Cos cells (black arrow, lane 1). Several other higher molecular weight complexes can also be seen (Fig. 7*C*, a–g) and likely correspond to other homeodomain containing factors, such as Msx and Dlx (Givens et al., 2005).

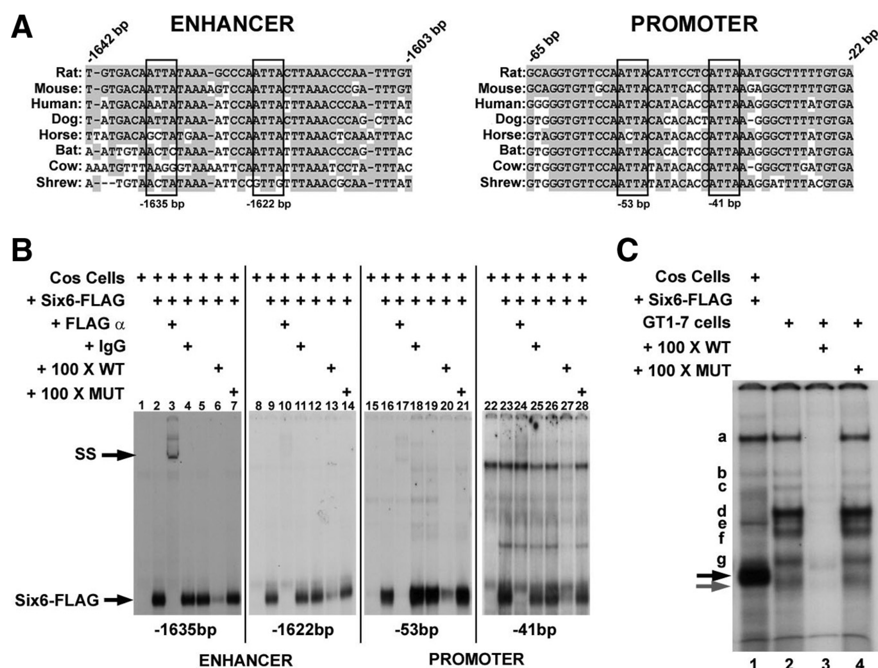

Figure 7. Six6 binds to ATTA sites within the rat GnRH promoter. ***A***, The rat GnRH promoter sequence between −22 and −65 bp and enhancer sequence between −1603 and −1642 bp were aligned with sequences from the mouse, human, dog, horse, bat, cow, and shrew GnRH regulatory regions using the ClustalW program. Previously identified ATTA binding sites are enclosed in boxes. Nucleotides that are conserved between all the species are shaded gray. ***B***, EMSA was performed with nuclear extracts from Cos cells transfected with either pCMV-FLAG (lanes 1, 8, 15, and 22) or pCMV-Six6-FLAG (all other lanes). The DNA probes consisted of radiolabeled oligonucleotides containing the ATTA sites identified at −41, −53, −1622, and −1635 bp of the rat GnRH regulatory region. A 500-fold excess of unlabeled WT or mutant (MUT) probe and an antibody against the FLAG tag (FLAG α) were included in the reaction mixtures as indicated. FLAG antibody was able to shift (SS) or remove a complex that formed on all probes after incubation with extracts from Cos cells transfected with pCMV-Six6-Flag. ***C***, EMSA was performed with nuclear extracts from Cos cells transfected with pCMV-Six6-FLAG (lane 1) or GT1-7 cells (all other lanes). The DNA probe consisted of radiolabeled oligonucleotide containing the ATTA site identified at −41 bp of the rat GnRH regulatory region. A 500-fold excess of unlabeled WT or mutant (MUT) probe were included in the reaction mixtures as indicated. A complex corresponding to FLAG-tagged Six6 was observed in lane 1 (black arrow). A complex corresponding to endogenous Six6 protein was observed in lanes 2 and 4 (gray arrow). Various other complexes were also observed (a–g) and likely correspond to other homeodomain containing proteins.

Six6 regulates expression of GnRH via ATTA sites in the promoter

To verify the functional interaction of Six6 and the ATTA sites in the regulation of GnRH transcription, we performed transient transfection assays in both GnRH neuronal cell lines using a re-

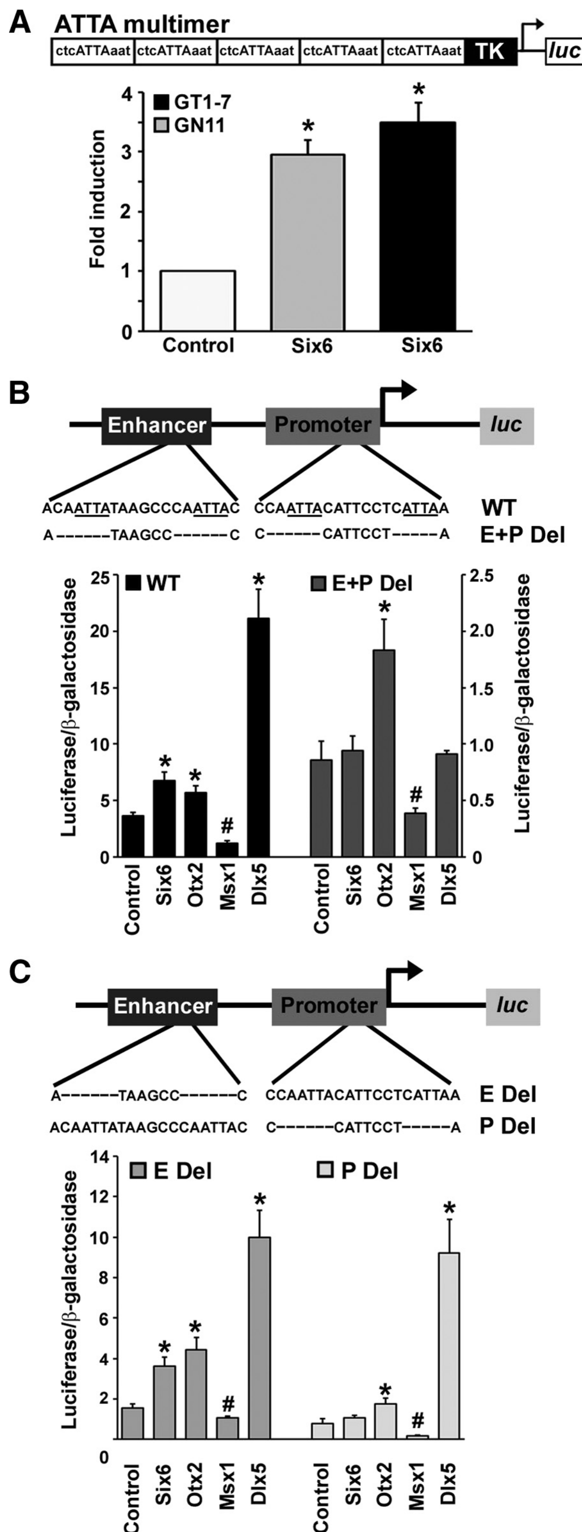

Figure 8. The primary site of Six6 activation is via promoter ATTA sites. ***A***, Effect of overexpression of Six6 on ATTA-multimer in GN11 and GT1-7 cells. TK, Thymidine kinase; luc, luciferase. Fold induction compared with empty vector alone (control) is indicated, corrected for β-galactosidase, which was used as an internal control. Overexpression of Six6 resulted in significant fold induction of an ATTA-multimer in both cell lines. All experiments were performed in triplicate and repeated three times. Results shown are average ± SEM. One-way ANOVA established statistical significance of Six6 expression plasmid versus empty expression plasmid (control) as $^*p < 0.05$. ***B***, Six6 expression plasmid was cotransfected into GT1-7 cells with either a wild-type GnRHe/p reporter (WT) or a mutant GnRHe/p reporter (E+P Del) where all four ATTA sites (underlined in WT) have been deleted ("−" in mutant). The left axis shows luc/βgal values for the WT reporter, and the right axis shows the luc/βgal values for the E+P del reporter. ***C***, Six6 expression plasmid was cotransfected into GT1-7 cells with mutant GnRHe/p reporters where either the two enhancer ATTA sites (E Del) or the two promoter ATTA sites (P Del) are deleted. Results shown are average ± SEM. All experiments were performed in triplicate and repeated three times. One-way ANOVA with *post hoc* Tukey's test established statistical significance as $p < 0.05$. *Significant activation versus empty vector control. #Significant repression versus empty vector control.

porter plasmid containing five copies of the sequence from −44 to −35 bp of the rat GnRH regulatory region (ctcATTAaat). In this context, Six6 significantly activated the ATTA multimer by approximately threefold in both cell lines (Fig. 8*A*). To determine whether induction of the GnRH promoter by Six6 was dependent on the presence of intact ATTA sites, we deleted all four sites from the GnRHe/p luciferase reporter and analyzed the effects of Six6 on this mutated reporter (Fig. 8*B*). As previously reported (Givens et al., 2005), deletion of these four sites significantly reduced the luciferase activity of the mutant reporter (E+P Del) compared with wild type (Fig. 8*B*), indicating that these sites are important for basal activity of the GnRH promoter in GT1-7 cells. As expected, the WT GnRHe/p reporter was significantly activated in response to Six6, Otx2, and Dlx5. However, neither Six6 nor Dlx5 were able to activate the mutant reporter (E+P Del), indicating that both factors function through these four homeodomain binding sites to regulate GnRH promoter activity. Importantly, deletion of the ATTA sites had no effect on the ability of Otx2 to significantly induce reporter activity because its primary site of action is via a bicoid-like target sequence within the GnRH promoter at −152 bp (Kelley et al., 2000; Larder and Mellon, 2009). Interestingly, the ability of Msx1 to significantly repress GnRH promoter activity was not compromised by the mutation of these four ATTA sites, likely because of the fact that Msx1 can regulate GnRH expression via interactions with other modulators of GnRH transcription such as Oct-1 (Givens et al., 2005).

To determine whether the activity of Six6 was dependent on the presence of all four ATTA sites, or just those in either the promoter or enhancer, deletions in either the two promoter sites (P Del) or the two enhancer sites (E Del) were generated and the transient transfection assays repeated. Dlx5 was capable of activating both the E Del and P Del mutant reporters, indicating that all four ATTA sites are required for maximal induction of the GnRH promoter by this transcription factor (Fig. 8*C*). Interestingly, Six6 was only able to significantly activate the enhancer-deleted reporter demonstrating that the primary site of Six6 action is via the ATTA sites situated within the conserved, proximal promoter.

Discussion

As the key regulator of reproduction, correct expression of GnRH is crucial to achieving appropriate reproductive function. Understanding the factors and mechanisms that control the synthesis and release of GnRH are vital to understanding fertility. Herein, we identify a member of the Six family of transcription factors as an important, novel regulator of mammalian fertility. Our data show that loss of Six6 expression leads to significantly decreased fertility in both male and female mice, because of disrupted regulation of GnRH expression.

Although progress has been made in identifying gene targets of Six1–5 (Spitz et al., 1998; Harris et al., 2000; Laclef et al., 2003; Brodbeck et al., 2004; Ozaki et al., 2004), little is known about the function of Six6 other than its role as a stimulator of progenitor

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cell proliferation in the developing pituitary and eye (Li et al., 2002). Therefore, we were intrigued to identify Six6 as one of the most differentially regulated transcripts from a microarray comparison of immature (GN11) and mature (GT1-7) GnRH neuronal cell lines (Miller et al., 2009) (R. Larder, N. L. G. Miller, and P. L. Mellon, unpublished data). The observed ~180-fold increase in Six6 expression correlated with a ~130-fold increase in GnRH expression implicating Six6 as an important regulator of GnRH gene expression. This novel role for Six6 was confirmed by experiments demonstrating that overexpression of Six6 in two GnRH neuronal cell lines significantly increased GnRH transcription and this increase was mediated via Six6 binding to ATTA sites within the GnRH proximal promoter. We previously reported the importance of these sites in the regulation of GnRH expression and showed that they can bind other homeodomain-containing transcription factors such as Msx1/2 and Dlx1/2/5 (Givens et al., 2005). Our data suggest that, although Dlx5-mediated activation of GnRH transcription can occur via all four ATTA sites, the primary sites of Six6 action are the ATTA elements located within the rat GnRH proximal promoter at −41 and −53 bp. Although Six6 is capable of binding to all four ATTA sites, only deletions involving the two promoter sites result in loss of GnRH induction in response to Six6. This suggests that cooperation between Six6 and other factors that bind at, or near, the promoter ATTA sites, may play a role in allowing maximal activation of GnRH transcription. Six3 has been shown to function as a context-dependent activator (Liu et al., 2006) or repressor (Zhu et al., 2002; Lagutin et al., 2003) in both the developing eye and forebrain, suggesting that its function is dependent on its interaction with specific cofactors. To our knowledge, this is the first report to detail activation of gene transcription by Six6. Previous studies using yeast two-hybrid screens, with Six6 as the bait, yielded members of the Groucho family of corepressors as potential Six6 interacting partners (López-Ríos et al., 1999); however, no coactivators have been identified by this screening method. Additional investigation is warranted to establish whether Six6 acts alone, or in cooperation with other factor(s), to induce GnRH gene expression.

Based on the expression profile of Six6 in the GnRH neuronal cell lines, and its ability to activate GnRH transcription, we sought to determine the reproductive phenotype of mice lacking expression of Six6. Both male and female Six6-null mice had significantly reduced fertility, reinforcing our hypothesis that Six6 is important for proper neuroendocrine control of reproduction in mammals. The presence of antral follicles in KO ovaries, along with the absence of CL, strongly implicates LH deficiency as the primary cause of infertility in female animals. Immunohistochemical analysis of pituitaries from these mice revealed comparable numbers of LHβ-expressing gonadotrope cells, suggesting that gonadotrope function is not altered, but rather that the hypothalamic signal to the pituitary is disrupted. This hypothesis was corroborated by the observation that Six6-null females have significantly lower levels of GnRH mRNA and 89% fewer GnRH neurons. Therefore, it is likely that the anovulatory phenotype seen in Six6-null females is because the decreased numbers of GnRH neurons are insufficient to generate a GnRH/LH surge. Indeed, previous studies have shown that female mice require 12–34% of the normal GnRH neuronal population to ensure the generation of an effective GnRH/LH surge (Herbison et al., 2008). Characterization of the reproductive phenotype of Six6-null males reveals a similar 84% decrease in GnRH neuron numbers and confirms previous studies showing that pulsatile secretion of LH in male mice is achievable with only a few GnRH neurons. Furthermore, our results support data that demonstrate a differential effect of reduced GnRH neuron numbers on plasma gonadotropin levels in males (Gibson et al., 1997; Herbison et al., 2008). Six6 KO males have a much greater reduction in serum FSH concentrations (~75%) than either LH or T (~35%), suggesting that a larger cohort of GnRH neurons is required to maintain normal levels of FSH in males.

At E13.5, Six6-null mice have comparable numbers of GnRH neurons to their WT littermates. Nevertheless, GnRH neuron numbers are decreased at birth mirroring a similar phenotype to that seen in NSCL-2-null mice (Krüger et al., 2004). Given that vaginal opening is not delayed in Six6-null females, this signifies that, as observed in GNR23 mutant mice, typical pubertal advancement can occur even with only a handful of GnRH neurons (Gamble et al., 2005; Herbison et al., 2008). However, normal development of the GnRH neurons in Six6-null mice is perhaps not surprising given the very low levels of Six6 expression in immature GnRH neuronal cells. Assuming Six6 expression is low in immature GnRH neurons *in vivo*, then loss of Six6 expression during embryonic development would likely have a negligible impact on GnRH neuronal migration. Moreover, expression of Six3 during development may compensate for the loss of Six6 at early stages of GnRH neuronal biology as both factors induce GnRH promoter activity in an immature GnRH neuronal cell line. However, additional investigation of the role of Six3 during GnRH development is required to confirm this hypothesis.

Unlike Six6-null mice, Six3 knock-outs die at birth (Lagutin et al., 2003), so it is not possible to assess the reproductive phenotype of these animals. Although both subgroup family members activate GnRH promoter activity in immature GnRH neurons, they have differential actions on GnRH transcription in a mature GnRH neuronal cell line. Six6 induces GnRH transcription in GT1-7 cells, whereas Six3 represses it. However, the repressive effects of Six3 are reversed by titrating in increasing amounts of Six6. Quantitative analysis of Six3 and Six6 expression levels reveals that Six3 is expressed similarly in both cell models, whereas Six6 expression is increased ~200-fold as the GnRH neuron matures, resulting in 10 times the amount of Six6 as Six3 in the GT1-7 cells. Therefore, our titration experiment mirrors the changes seen in the relative expression levels of the two factors and suggests that once a critical level of Six6 expression is achieved then activation of the GnRH promoter will be favored. We hypothesize that the substantial decrease in GnRH mRNA and protein levels observed in Six6-null mice may be attributable to the lack of Six6 expression not only affecting induction of GnRH transcription but also allowing the repressive actions of Six3 on the GnRH promoter to be fully realized.

Both Six6 and Six3 have also been shown to be expressed in the developing (Oliver et al., 1995; Jean et al., 1999) and adult pituitary (Aijaz et al., 2005), and, although it is unknown whether both factors are expressed in all six anterior pituitary cell types, our unpublished observations indicate both are expressed in two immortalized gonadotrope cell lines (LβT2 and αT3–1 cells). However, GnRH challenge experiments performed in male Six6-null mice reveal that KO pituitaries respond to GnRH in an identical manner to WT pituitaries, suggesting that loss of Six6 expression does not affect the ability of the pituitary to respond to GnRH, but rather that the fertility defects seen in these animals are attributable to disruption at the apex of the HPG axis. Our data reveal that the hypothalamic effects of deleting Six6 are not confined to the GnRH neuronal population. Disruption of kisspeptin neurons also causes infertility (Clarkson et al., 2008); however, the reduction in GnRH neurons in Six6-null mice is

likely to be the main cause of infertility. Detailed characterization of a GnRH neuron-specific knock-out of Six6 would confirm this and determine the reproductive contribution of Six6 expression in other neuronal populations. Defects in GnRH cell fate specification, differentiation, migration, axonal elongation, and targeting to the median eminence can all contribute to deficiencies in GnRH secretion, which results in the clinical syndromes known as idiopathic hypogonadotropic hypogonadism (IHH) and Kallmann's syndrome (Bianco and Kaiser, 2009). Given that loss of Six6 expression has such a dramatic effect on GnRH expression in mice, and that genetic mutations have only been identified in ~30% of IHH patients (Pitteloud et al., 2007), it will be important to determine whether *SIX6* mutations are observed in cases of IHH of unknown genetic etiology.

In conclusion, we have identified Six6 as an important hypothalamic regulator of fertility in mice. The lack of Six6 results in a decrease in GnRH gene expression, reduced numbers of GnRH neurons, and decreased targeting of GnRH neurons to the median eminence, leading to infertility in adult mice. We, therefore, hypothesize that mutations in *SIX6* may contribute to cases of hypogonadotropic hypogonadism and infertility in humans.

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