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Jak2 is Necessary for Neuroendocrine Control of Female Reproduction

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Abstract

GnRH neurons represent the final common output of signals from the brain that regulates reproductive function. A wide range of environmental factors impact GnRH neuron activity including disease, stress, nutrition, and seasonal cues, as well as gonadal steroid hormones. The CNS response is thought to be mediated, at least in part, through intermediate signaling molecules that affect GnRH neuronal activity. *In vitro*, GnRH neuronal cell lines respond to a variety of ligands which activate the Jak/STAT intracellular signaling pathway. In order to determine its biological function in reproduction, we used Cre/LoxP technology to generate GnRH neuron specific Jak2 conditional knockout (Jak2 G−/−) mice. GnRH mRNA levels were reduced in Jak2 G−/− mice when compared to controls, while the number of GnRH neurons was equivalent, indicating a reduction in GnRH gene expression. Secretion of GnRH is also reduced as basal serum LH levels were significantly lower in female Jak2 G−/− mice while the pituitary responded normally to exogenous GnRH. Preovulatory LH surge levels were blunted in Jak2 G−/− mice, which was correlated with reduced GnRH neuronal activation as assessed by c-Fos. However the activation of GnRH neurons following release from estrogen negative feedback is retained. Female Jak2 G−/− mice exhibited significantly delayed puberty and first estrus, abnormal estrous cyclicity and impaired fertility. These results demonstrate an essential role for Jak2 signaling in GnRH neurons for normal reproductive development and fertility in female mice.

Keywords

GnRH; Jak2; Cre/LoxP; fertility; estrous cycle; ovary

Introduction

Gonadotropin releasing hormone (GnRH) is the key regulator of reproduction and sexual behavior. GnRH travels via the portal vasculature to the anterior pituitary stimulating the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the gonadotroph. LH and FSH are released into the circulation and stimulate the maturation and development of the gonads and the synthesis and secretion of the gonadal steroid hormones. GnRH neurons integrate signals that regulate reproduction; for example

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nutrition, stress, developmental cues, and seasonal and circadian information which in turn regulate the expression and secretion of GnRH (Porkka-Heiskanen et al., 1997;Bucholtz et al., 2000;Nagatani et al., 1996;Pitteloud et al., 2007;Popa et al., 2008;Smith and Grove, 2002). The cytokine family of hormones, growth factors and chemokines interact with the cytokine class of receptors and have been implicated in the central regulation of reproduction (Gao et al., 2004;Bates et al., 2003;Stanley et al., 2000;Dozio et al., 2005;Watanobe and Yoneda, 2001;Argetsinger et al., 1993;Danilovich et al., 1999;Bartke et al., 1999;Shillingford et al., 2002), although the precise mechanisms of action are still being explored. Jak2, STAT3 and STAT5 are expressed in models of GnRH neurons, such as the GN11 and GT1-7 cells (Argetsinger et al., 1993;Magni et al., 2007), and activation of Jak2 has been suggested to mediate the effects of cytokines such as LIF and CNTF through their receptors on GnRH neurons (Magni et al., 2007;Dozio et al., 2005).

The cytokine receptors lack intrinsic kinase activity and require interaction with members of the Janus-activated-kinase (Jak) family of intracellular signaling molecules. Jak2 belongs to the non-receptor tyrosine kinase family (Ihle, 1995). It is the predominant Jak kinase mediating the responses of single chain cytokine receptors and plays an important role in signaling via the gp130 receptor family or the Class II cytokine receptors (Kisseleva et al., 2002). Receptor activation causes Jak2 autophosphorylation which phosphorylates its associated receptor. The phosphorylated receptor provides multiple docking sites that recruit Signal Transducers and Activator of Transcription (STAT) proteins of which there are six family members (STAT1-6) (Pellegrini and Dusanter-Fourt, 1997). STAT is phosphorylated by Jak2 resulting in dimerization and translocation to the nucleus where it acts as a transcription factor to regulate gene expression (Ihle, 1995;Parganas et al., 1998). A complete KO of the Jak2 gene *in vivo* has been produced and results in embryonic lethality (Parganas et al., 1998).

Since in vitro models provide evidence that Jak/STAT signaling may mediated important signals to the GnRH neurons, but *in vivo* evidence for a specific role for GnRH neuronal Jak2 is lacking, therefore, we generated mice lacking Jak2 in GnRH neurons using the Cre/LoxP binary recombination system (Singh et al., 2009;Hamilton and Abremski, 1984) and report here a role for the Jak2 signaling molecule in the regulation of reproductive development and function in female mice.

Materials and Methods

Generation of GnRH neuron specific Jak2 conditional knockout (Jak2G−/−) mice

GnRH promoter driven Cre recombinase (GnRH-Cre) transgenic mice were created in our laboratory on a CD1 background (Wolfe et al., 2008). Jak2 $^{fl/fl}$ mice were produced as previously described and were in a 129SvJ strain (Krempler et al., 2004). Exon 2 of Jak2 is flanked by loxP sites. Excision of exon 2 results in complete disruption of Jak2 expression (Krempler et al., 2004). The GnRH-Cre; Jak2$^{fl/fl}$ conditional knockout (KO) mice, designated as Jak2 G−/−, were generated by first mating female (GnRH-Cre^{+}) with male (Jak2$^{fl/fl}$) and then crossing a heterozygous (GnRH-Cre^{+}; Jak2$^{fl/wt}$) female with a heterozygous (GnRH-Cre^{-}; Jak2$^{fl/wt}$) male to generate 6 genotype combinations. GnRH-Cre^{+}; Jak2$^{fl/fl}$ mice represent the homozygous conditional KO mice. GnRH-Cre^{-}; Jak2$^{fl/fl}$ littermates were used as controls for all studies except for the mating and puberty assessments, which also used GnRH-Cre^{-}; Jak2$^{wt/wt}$, GnRH-Cre^{-}; Jak2$^{fl/wt}$, and GnRH-Cre^{+}; Jak2$^{wt/wt}$ littermates due to limited litter sizes of our early matings. No difference was observed among these genotypes.

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Animal Housing

Mice were maintained with food and water *ad libitum* in a 14:10hr light:dark cycle at 24°C in the Broadway Research Building animal facility at the Johns Hopkins University School of Medicine. All procedures were approved by the Johns Hopkins University Animal Care and Use Committee.

Genotyping and DNA extraction

For genotyping, two pairs of primers were used: GnRH-Cre specific primers: sense 5′-GGTAGCTTCAGCTGTGAAAG-3′; antisense 5′-CATCTTCAGGTTCTGCGGGAAACC-3′, and Jak2$^{fl/fl}$ primers: sense 5′-ATTCTGAGATTCAGGTCTGAGC-3′; antisense 5′-CTCACAACCATCTGTATCTCAC-3′

To obtain genomic DNA of pups, a clipping from the ear or tail was collected from the mice and put into 10% chelex-100 resin (Bio-Rad, Hercules, CA) with 0.1% tween-20 and 0.15mg/mL proteinase K. Samples were incubated at 50°C for 90 min, proteinase K was inactivated at 95°C for 20 min, and the solution was cooled to 10°C. One μL of supernatant was removed for PCR. The PCR reaction was as follows: 94°C for 3 min, 35 cycles with 94°C for 30sec, 58°C for 30sec, 72°C for 30sec and last cycle at 72°C for 7 min. For identifying tissue-specific Jak2 allele recomination, different tissues were collected including hypothalamus, cerebellum, cortex, pituitary, liver, heart, ovary and testes. DNA was obtained from these tissues by phenol-chloroform extraction and isopropanol precipitation. Primers used for detection of Jak2 G−/− : sense 5′-GTCTATACACCACCACTCCTG-3′ and antisense 5′-CGAGCTGGAAAGATAGGTCAGC-3′. The sequences of the primers related to Jak2 are the same as in (Krempler et al., 2004). The locations of primers are labelled in Fig. 1A.

Puberty and fertility examination

Female vaginal opening was assessed daily after 21days of life. Following vaginal opening, daily vaginal cytology was performed in the morning by collecting vaginal cells using 0.9% saline lavage. Cells were dried on slides, fixed in ethanol and stained with Diff-Quik staining kit (IMEB INC, California). Estrous cycle staging was assessed using the method previously described (Nelson et al., 1982). Proestrus was assigned as predominantly basal and cornified nucleated cells, estrus was assigned as predominantly cornified epithelial cells, metestrus was assigned as mixed cornified epithelial cells and leukocytes, and diestrus was assigned as predominantly leukocytes.

Adult female (2.5 months old) were mated with proven fertile adult male mice for a period of 90 days. Time to each litter and litter size for each pair was recorded.

Total RNA extraction, Reverse transcription and Real Time PCR

Hypothalamic RNA was extracted by Trizol (Invitrogen) according to the manufactor's protocol. One μg of total RNA was reverse transcribed (iScript cDNA Synthesis Kit, BioRad, Hercules, CA) to cDNA. For GnRH, Taqman quantitative PCR (Bioresearch Technologies, Novato, CA) was performed and GAPDH was used as the internal control. Primers for GnRH: sense 5′-CCAACGGAAGCTCGAGATCC-3′, and antisense 5′-TGCCGGCCATCAGTTTGAG-3′ with the probe 5′TGACTTTCACATCCAAACAGAGTGGACA-3′ labeled with FAM and BHQ-1. Primers for GAPDH: sense 5′-GGGCATCTTGGGCTACACT-3′ and antisense 5′-GGCATCGAAGGTGGAAGAGT-3′ with the probe 5′-AGGACCAGGTTGTCTCCTGCGA-3′ labeled with Cal fluoro red-610 and BHQ-2.

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Reactions were performed using an iCycler iQ5 real time PCR machine (BioRad, CA). PCR conditions were optimized to generate >95% PCR efficiency and only those reactions between 95% and 105% efficiency were included in subsequent analysis. Cycle threshold (*C*t) was obtained for each sample. A corrected *C*t (delta *C*t) was calculated by subtracting the GAPDH *C*t from the unknown sample *C*t for each sample. Relative differences from the control sample were then calculated by using the formula: fold change=2^(control delta *C*t minus sample delta *C*t).

Hormonal assay

Blood from female mice was obtained by mandibular vein puncture in the morning and vaginal lavage was performed at the same time for cytological assessment. Blood was centrifuged at 4000×g for 15 min at 4°C and serum was collected and stored at −80°C until needed. LH and FSH were measured using a Milliplex MAP immunoassay (Rat Pituitary panel; Millipore, Billerica, MA) in the Luminex200 (Austin, Texas). Analysis was performed using the XPonent 3.0 software program with Logistic 5D Weighted analyses. Further analysis details are described in (Singh et al., 2009). Estradiol was measured with an estradiol enzyme immunoassay kit (Cayman Chemical co. Ann Arbor, MI) according to the manufacturer's directions.

The mouse surge protocol was adapted from (Christian et al., 2005) with modifications. Mice were ovariectomized (OVX) in the morning (day 0) and diluted 17-β estradiol (Cayman Chemical Company) filled alzet micro-osmotic pumps (DURECT™, model 1007D) were inserted under the skin dorsally at the neck. 17-β estradiol was dissolved in ethanol at a concentration of 750ng/μl. This solution was further diluted with PBS to a final concentration of 7.5ng/μl and injected into the mini-pump. Estradiol is released at 90ng/ml per day by the pump, with a 0.5μl per hour rate. On day 2, blood was taken (~50μl) from each individual OVX mouse by mandibular vein puncture at 10:00, 16:00 and one hour before lights off at 20:00. Intact control mice and their Jak2 G−/− litter mates were bled at 20:00 for 7 consecutive days and vaginal lavage for estrous cycle assessment was performed at the same time.

Another group of mice treated as above were perfused at 20:00 and brains were preserved for colocalization of GnRH and c-Fos.

Histology

Ovaries were collected at metestrus or diestrus and fixed in 10% buffered formalin phosphate (Fisher Scientific) solution and stored at 4°C. Paraffin embedded ovaries were sectioned at 7 microns thickness (Phenotype Core Facility, Johns Hopkins University School of Medicine). Ovarian sections were stained with hematoxylin and eosin, examined with a Zeiss microscope and photographed with an AxioCamICc1 camera and exported to AxioVision Software.

Perfusion and immunostaining

Mice were anaesthetized with ketamine and xylazine, and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 1xPBS. Brains were post fixed in 4% paraformaldehyde overnight and dehydrated in 30% sucrose for 24h or until they sank to the bottom. Brains were then embedded in OCT at −20°C and sections were cut coronally using a cryostat (MICROM, HM550) beginning rostrally at the olfactory bulb and ending caudally at the median eminence.

For the purpose of counting GnRH neurons, GnRH promoter driven GFP (GnRH-GFP) mice obtained from Dan Spergel (University of Chicago, (Spergel et al., 1999)) were used. Jak2 G

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−/− mice were produced on the background of the GnRH-GFP mice. Forty micron sections were mounted onto slides with Vectashield (Vector laboratories). All GnRH neurons were counted directly by GFP fluorescence by analyzing sequential section from the rostral olfactory tissue through the median eminence using a Zeiss microscope with X-Cite 120 fluorescence Illumination System and photographed with an AxioCamMR camera and exported to AxioVision Software. All images were exposed for 1sec with the same brightness and contrast parameters.

For the purpose of examining colocalization of GnRH and Jak2, brains of GnRH-GFP mice were coronally cut at 10 microns, mounted onto slides and stored at −20°C until needed. Slides were thawed at room temperature for 30 min and sections were washed 3 times with 1xPBS for 5 min each. Sections were then permeablized with 0.25% Triton-100 in 1xPBS for 6 min and washed 2 times with 1xPBS quickly and blocked with 8% goat serum in 1xPBS for 1h at room temperature. Rabbit-anti Jak2 C-terminal polyclonal antibody (Santa Cruz Biotechnology, CA) was diluted 1:100 in 0.02% Tween-20 of 1% BSA PBS. Sections were incubated with primary antibodies for 48h and washed 4–5 times with PBS for 10 min each. Then sections were incubated with goat anti-rabbit IgG Alexa fluro-594 (Invitrogen) at 1:400 dilution in 0.02% Tween-20 of 1% BSA PBS for 1.5 h. Sections were then washed 5 times with 1xPBS for 10 min per wash. Sections were mounted with Vectashield and imaged as for GnRH-GFP neuronal counts.

For colocalizing c-Fos and GnRH, sections were cut at 30 microns and stored in cryoprotectant antifreeze until use. Staining for c-Fos and GnRH used the same protocol and antibodies described in (Hoffman et al., 2005). Stained sections were photographed with an AxioCamICc1 camera and exported to AxioVision Software.

Statistical analysis

All data was analyzed by unpaired student's t test using GraphPad Prism, (GraphPad Software, Inc. San Diego, California) and expressed as means ± S.E.M. $P<0.05$ were defined as statistically significant.

Results

Jak2 specific conditional knockout in GnRH neurons (Jak2 G−/−)

Floxed Jak2 mice with *LoxP* sites flanking exon 2 (Fig. 1A) were mated with GnRH-Cre mice which express the Cre recombinase gene in GnRH neurons. As expected, the KO allele (400bp) was only found in the hypothalamus and not in other tissues of Jak2 G−/− mice (Fig. 1B). The $Jak2^{fl/fl}$ allele (320bp) was identified in all collected tissues from the same genomic DNA pool that was used for the Jak2 G−/− PCR reaction (Fig. 1B). To confirm cell-specific deletion of Jak2 in GnRH neurons, immunostaining for Jak2 was performed on 10μm brain sections. Fig. 1C shows GnRH expressing neurons localized in the vertical limb diagonal band (VDB) in green, reflecting the expression of GFP under the direction of the murine GnRH promoter (top panel). Binding of the Jak2 antibody is visualized by red fluorescence (Alexa594; middle panel). Overlaying the images shows Jak2 colocalization in hypothalamic GnRH neurons of control GFP mice (bottom left panel) but no colocalization in GnRH neurons of Jak2 G−/− GFP mice (bottom right panel). About 35% of the GnRH cell bodies colocalized with Jak2 in control mice examined from the rostral olfactory bulb through the median eminence. We found no regional differences in colocalization within the hypothalamus.

To investigate whether the absence of Jak2 signaling in GnRH neurons affects GnRH neuronal development and migration, the number of GnRH neurons and their distribution were examined. There were a similar number of GnRH cell bodies (602.0±13.0 (control) vs

611.7±11.3 (Jak2 G−/−); Fig. 2A)) counted across the entire basal forebrain, and no significant difference in the number of GnRH cell bodies was observed in each successively counted region across the hypothalamus (Fig. 2B). A normal distribution of GnRH neurons was observed between Jak2 G−/− and control groups (representative regions of the hypothalamus are shown in Figs. 2C-F for control and H-K for Jak2 G−/−). GnRH terminal distribution at the median eminence was examined to determine whether Jak2 signaling contributed to the axonal migration, a critical step in GnRH neuronal development, and was not found to differ between control and Jak2 G−/− mice (Figs 2G and L respectively). Interestingly, results from quantitative real-time PCR showed that hypothalamic GnRH mRNA was reduced by more than 50% of controls (P<0.001) (Fig. 2M).

Jak2 G−/− mice have reduced serum LH

Basal serum LH levels from female Jak2 G−/− mice were significantly reduced when compared to controls (0.10 ng/mL ±0.02, n=16 (Jak2 G−/−) vs 0.25 ng/mL ±0.06, n=15 (control), P<0.05; Fig. 3A). In contrast, female basal FSH levels were similar between Jak2 G−/− mice and control mice (26.34 ng/mL ±5.07, n=16 (Jak2 G−/−) vs 30.83 ng/mL ±5.17, n=15 (control); Fig. 3B). To confirm that this difference in serum LH levels was not due to changes in pituitary responsiveness, a GnRH stimulation test was performed. The pituitaries from control and Jak2G−/− mice exhibited equal responses to 100ng/kg body weight GnRH (Fig. 3A, B). No increase in FSH was observed at 10 minutes following GnRH stimulation in either Jak2 G−/− or control mice.

Hypothalamic-pituitary gonadotropic activity is influenced by estrogen negative feedback during most of the estrous cycle. When LH and FSH levels were measured following removal of negative feedback by OVX there was no difference between Jak2 G−/− and control litter mates (Fig. 3A, B).

Jak2 G−/− mice exhibit delayed puberty

Vaginal opening has been shown to be an estrogen-dependent process and is used as an indicator of the onset of puberty (Safranski et al., 1993). The age of vaginal opening was significantly delayed in the Jak2 G−/− mice compared to control litter mates (29 day of life (dol) ± 1.11, n=14 (Jak2 G−/−) vs 24 dol ±1.9, n=20 (control), P<0.001; Fig. 4). The day of first estrus implies progressive sexual maturation and is widely used as an indicator of progression through puberty (Safranski et al., 1993). Daily vaginal smears were obtained to identify the age of first estrus. Jak2 G−/− mice exhibited a significant delay of first estrus compared to control litter mates (39.3 dol±1.6, n=7 (Jak2 G−/−) vs 33dol±1.1, n=7 (control), P<0.01; Fig. 4). However, these data do not include the Jak2 G−/− mice that had no first estrus when evaluated for up to 50 dol (2 of 9 examined).

Jak2 G−/− mice have abnormal estrous cycles and impaired ability to generate an LH surge

Vaginal cytology was analyzed from adult female mice for 15 consecutive days and demonstrated that Jak2 G−/− mice (n=8) exhibited irregular estrous cycles when compared to their control litter mates (n=17) (Fig. 5A-C). During the 15 days of analysis only 50% of Jak2 G−/− mice had one complete cycle, compared to 95% in the control group. Furthermore, only 2 of 8 Jak2 G−/− mice (25%) had two complete cycles in the 15 day period, whereas 82% of the control litter mates exhibited two complete cycles (Fig. 5A). The duration spent in the proestrus phase in Jak2 G−/− mice was significantly shortened compared to control litter mates (Fig. 5B). Acyclic mice mainly remained predominantly in persistent metestrus/diestrus (Fig. 5C).

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The gonadotropic LH surge stimulates ovulation in female mice which is an essential process in fertility and is induced by positive feedback of estradiol on the hypothalamus and pituitary. To explore whether positive feedback regulation is disrupted in Jak2 G−/− mice, a surge induction paradigm was used (Christian et al., 2005) that produces LH surge generation within an hour before lights off on day 2 of the paradigm. Chronic treatment with estradiol in OVX females results in levels of LH that were near the limit of detection in both Jak2 G−/− and control females at 10:00 and were elevated but not significantly different at 16:00 (Data not shown). Surge LH levels in control mice at 20:00 on day 2 in our study were nearly identical to the levels observed by Christian et al. (Christian et al., 2005). However, LH levels were significantly blunted in Jak2 G−/− mice (Fig. 5D). The estrogen levels after OVX+E replacement in all genotypes were found to be 29.5±7.9pg/ml n=7, which is similar to estrogen levels in proestrus (Wu et al., 2010;Christian et al., 2005;Nelson et al., 1982) and is nearly three-fold higher than the basal morning circulating levels in intact female mice (around 10pg/ml for both WT and Jak2 G−/− mice; data not shown).

While Jak2 G−/− mice frequently exhibit persistent diestrus a number of KO females went through proestrus (Fig. 5A-C). To explore the magnitude of the LH surge in cycling females, evening blood samples were obtained in control and Jak2 G−/− mice during the estrous cycle and proestrus LH levels were found to be more than 6-fold lower in Jak2 G−/− mice compared to WT mice (Data not shown).

Jak2 G−/− mice demonstrated attenuated GnRH neuronal activation during the surge

In order to determine whether changes in GnRH neuronal activation correlated with the reduced LH levels during the surge, c-Fos immunostaining in GnRH neurons was assessed by dual labeling immunohistochemistry after mice underwent the surge induction paradigm. All sections with immunostained GnRH neurons were counted and colocalization was documented in two regions as has been described previously (Herbison et al., 2010;d'Anglemont, X et al., 2010). In both medial septum/diagonal band of Broca (MS/DBB) and organum vasculosum of the lamina terminalis/rostral preoptic area (OVLT/rPOA) regions we observed a significantly higher percentage of GnRH neurons with c-Fos in control mice than Jak2 G−/− mice (104% and 115% more dual labeled neurons for the MS/DBB and VOLT/rPOA, respectively; Fig. 6A-C).

Jak2 G−/− mice exhibit impaired fertility

Fertility was also examined in control and Jak2 G−/− mice in a continuous mating protocol. Jak2 G−/− female mice demonstrated an impaired ability to produce offspring, as shown in the mating paradigm in Fig. 7A. The birth of each litter is represented by a black dot and litter sizes are indicated by numbers above each line for every successful mating event. Jak2 G−/− mice exhibited decreased fertility compared to control littermates (Fig. 7A). None of the 8 Jak2 G−/− females had 4 litters during the 90 days mating period where 4 of the 6 control mice had 4 litters in this period. Quantification of these data indicate that female Jak2 G−/− mice bore their first litter significantly later after introduction to males than control female mice (33.63 days ±6.71 (control), n=8 vs 22.00 days ±0.47, n=14 (Jak2 G−/−), P<0.05; Fig. 7B). During 90 days of mating, female Jak2 G−/− mice had a significantly fewer number of litters per female (2.5 ±0.33, n=8 (Jak2 G−/−) vs 3.67 ±0.21, n=6 (control), P<0.05) and a reduced number of pups per litter (5.6 ±0.6 (Jak2 G−/−), n=20 vs 12.0 ±1.76, n=27 (control), P<0.01) compared to control females (Fig. 7C and D).

Since impaired neuroendocrine function is expected to result in fewer ovulations, we next counted corpora lutea from ovaries obtained from 4 month old mice. A dramatic decrease in the number of corpora lutea from Jak2 G−/− mice compared to controls (7.25±1.3 (Jak2 G−/−), n=4 vs 17±2.3, n=4 (control), P<0.01; Fig. 8A, B) directly reflects a reduction in the

number of follicles that have ovulated. The ovarian weights were also found to be significantly reduced in Jak2 G−/− mice compared to control mice in diestrus (7.68 mg ±0.89, n=6 (Jak2 G−/−) vs 11.79 mg ±0.10, n=8 (control), P<0.01; Fig. 8C) and is likely due to the reduced number of corpora lutea.

Discussion

The regulation of reproductive function by the brain is mediated by the pulsatile secretion of GnRH from the hypothalamus. Numerous signals are integrated by the brain and ultimately result in changes in GnRH neuronal activity which may activate or inhibit reproductive function. The signaling pathways in GnRH neurons that mediate these cues, including regulation by cytokines such as LIF and CNTF, have been characterized to some extent using *in vitro* cell culture models of transformed GnRH neurons (Dozio et al., 2005; Magni et al., 2007). Here we show that the Jak2 signaling molecule within the GnRH neuron plays an important role in the regulation of puberty and reproduction *in vivo*.

Few *in vivo* studies have explored the role of Jak2 in reproductive neuroendocrine function although recent work has shown that specific disruption of STAT3 in the neurons of the central nervous system ($STAT3^{N-/-}$) mimics leptin receptor disrupted (db/db) and leptin deficient (ob/ob) mice in the regulation of energy homeostasis and reproduction (Gao et al., 2004). However, the role of neuronal STAT3 signaling on reproduction is leptin receptor independent (Bates et al., 2003), therefore STAT3 is mediating non-leptinergic signals in the brain to impact reproductive function. The present studies were undertaken to determine whether GnRH neurons are a locus in which disrupted Jak2/STAT3 signaling may contribute to the infertility observed in the $STAT3^{N-/-}$ mouse. A central nervous system STAT5 KO mouse has also been produced (Lee et al., 2008) and the mice are fertile although specific parameters of reproductive function were not analyzed (Personal communication with Martin Myers). While the phenotypic analyses of the STAT KO mice provides important information about the role of STAT in the regulation of energy balance and reproduction we sought to specifically explore the role of Jak2 in GnRH neuronal function.

We used Cre-LoxP technology to generate mice lacking Jak2 in GnRH neurons without interfering with peripheral Jak2 function (Jak2 G−/− mice). The GnRH-CRE mouse (Wolfe et al., 2008) has been validated to target CRE expression to GnRH neurons in the hypothalamus and was crossed with a floxed Jak2 mouse which has been shown to be viable and fertile and to effectively exhibit recombination in cells expressing CRE (Krempler et al., 2004;Wagner et al., 2004). We demonstrate that CRE recombinase expression in the GnRH neurons of the hypothalamus results in ablation of Jak2 expression (Fig. 1). Hypothalamus-specific deletion of Jak2 was demonstrated by PCR analysis of genomic DNA from peripheral tissues such as heart, liver, pituitary, ovary, and non-hypothalamic brain structures such as cerebellum and cortex (Fig. 1B) and GnRH neuron-specific deletion was confirmed by immunohistochemical analysis of the brain (Fig. 1C).

Phenotypic analysis of the Jak2 G−/− mice indicated that there was a significant delay in puberty in females (Fig. 4). Onset of puberty can be changed by genetic and external factors and various markers can be used such as vaginal opening, first estrus and cyclicity. Vaginal opening is steroid dependent and therefore indirectly assays the age of GnRH neuronal activation that underlies puberty. While the age of puberty varies widely in different mouse strains (Pinter et al., 2007), the examination of litter mates in these analyses revealed a significant delay in puberty in female Jak2G−/− mice (Fig. 4). The delay in puberty is not the result of reduced numbers of GnRH cell bodies or terminals (Fig. 2A-L) as has been shown in another mouse model demonstrating delayed puberty (Herbison et al., 2008),

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suggesting that an intrinsic change in function of the GnRH neuron underlies the delay in puberty. A number of factors have been proposed to regulate the elaboration of puberty including, leptin (Ahima et al., 1997), kisspeptin (Seminara et al., 2003) and IGF-1 (Divall et al., 2010;Hiney et al., 1991). Although leptin and its receptor have been reported to influence puberty, this is most likely not at the level of the GnRH neuron as we (data not shown) and others (Quennell et al., 2009) do not detect the functional form of leptin receptor mRNA (long form). Moreover, the leptin receptor has been conditionally deleted in GnRH neurons and shown to have no effect on female puberty (Quennell et al., 2009). Kisspeptin (Kiss-1) has recently been implicated as playing an essential role in controlling puberty in mice (Seminara et al., 2003). The Kiss-1 receptor (Kiss-1R) has not been found to signal via Jak/STAT, so the delay in puberty observed in the Jak2 G−/− mice suggests that overlapping signaling mechanisms exist. The contribution of Jak2 mediated signaling to pubertal development may explain the low level activation of the reproductive axis observed in the Kiss-1 and Kiss-1R KO mice (Chan et al., 2009) which exhibit less severe reproductive impairment when compared to mice lacking GnRH signaling (Mason et al., 1986;Wu et al., 2010). IGF-1 contributes to the elaboration of puberty in rats (Hiney et al., 1991;Hiney et al., 1996) and mice (Divall et al., 2010) and, interestingly, has been shown to activate Jak/STAT signaling in 293T cells (Zong et al., 2000). While the Jak2 G−/− mouse recapitulates the phenotype of the GnRH-IGF1RKO mouse with regard to a delay in puberty, the Jak2 G−/− exhibits significantly more severe reproductive defects. This supports a model in which Jak/STAT is a common downstream pathway for multiple ligands.

Female Jak2 G−/− mice exhibited impaired reproductive axis function and fertility. GnRH mRNA and LH hormone levels were significantly decreased in female Jak2 G−/− mice suggesting that the reproductive dysfunction is due to attenuated GnRH function. The decrease in GnRH mRNA suggests that Jak/STAT signaling regulates GnRH gene expression. The decreased serum LH observed in the Jak2 G−/− mice is likely due to a decrease in GnRH secretion and/or pulsatility. There were no recombination events detected in the pituitary (Fig 1B) and GnRH stimulation testing revealed similar LH levels in the Jak2 G −/− mice and control mice (Figure 3A), implying intact pituitary function. Reduced activation of GnRH neurons, as assessed by c-Fos labeling (Hoffman et al., 2005), was observed in Jak2 G−/− mice during the LH surge (Fig. 6) further demonstrating the dysfunction of the GnRH neuron.

Estrogen exerts both negative and positive feedback regulation of the hypothalamic-pituitary gonadotropic axis. Release from negative feedback results in stimulation of GnRH neuronal activity. Elevated GnRH secretion, coupled with reduced negative feedback at the level of the pituitary (Singh et al., 2009) produces an increase in LH secretion. We find no impairment in this process in the Jak2 G−/− females as LH levels are equivalent to control mice (Fig. 3A, B). Thus, while there is reduced baseline secretion of GnRH in Jak2 G−/− mice, this is not observed following release of negative feedback. It has been shown that kisspeptin likely mediates the stimulation of GnRH neuronal activity when estrogen negative feedback is removed (Kauffman et al., 2007) and our results suggest that Jak/STAT mediated signaling may not be contributing to this process or that kisspeptin stimulation of the GnRH neurons can compensate for disrupted Jak/STAT signaling in the complete absence of estradiol. Morning LH levels in estradiol replaced OVX females were near the limit of detection of the assay in both Jak2 G−/− and control mice (Fig. 5D) resulting from circulating estradiol levels that were designed to mimic proestrus levels.

Positive feedback by estradiol is essential for generating the preovulatory LH surge. We observed a significant attenuation in the LH surge in Jak2 G−/− mice both in an induced surge paradigm (Fig. 5D) and in cycling females, suggesting that Jak2 mediated signaling

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pathways contribute to the development of the LH surge. Further evidence for Jak/STAT mediated activation of GnRH neurons during the surge is demonstrated by reduced levels of c-Fos observed in GnRH neurons in Jak2 G−/− mice when compared to controls (Fig. 6). Kisspeptin is proposed to play an important role in estrogen positive feedback effects on GnRH neuron function (Clarkson et al., 2008), although non-kisspeptin mediated activation may also contribute to the generation of the LH surge (Dungan et al., 2007). Thus, multiple signaling pathways, including the Jak/STAT pathway, may contribute to the generation of the preovulatory surge.

Female Jak2 G−/− mice also had a reduced number of litters and additionally exhibited a reduced number of pups per litter (Fig. 7C and D). LH levels influence follicle maturation, luteinization and ovulation in female ovaries. Attenuated LH surge levels (Fig 5D) result in smaller litters in Jak2 G−/− female mice and contribute to reduced ovarian weights and disrupted estrous cyclicity. The reduced ovarian weight is correlated with a significant reduction in numbers of corpora lutea (Fig. 8) which serve as a confirmation that there are reduced numbers of ovulations.

In conclusion, we report the development of a novel mouse model of Jak2 ablation in GnRH neurons. A role for Jak2 signaling in puberty and in the expression of the preovulatory gonadotropin surge has been demonstrated while Jak2 signaling does not appear to play a role in the regulation of estradiol negative feedback. This work demonstrates the critical role of GnRH Jak2 in the integration of cellular signaling pathways that control mammalian reproduction.

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Fig. 1. Generation of GnRH neuron specific Jak2 knockout mouse

A, Exon 2 of Jak2 is flanked by two *LoxP* sites and Cre recombinase expression was regulated by the mouse GnRH promoter. Primers used in the PCR reactions are marked by arrows. B, PCR of DNA isolated from various tissues of control and Jak2 G−/− mice. C, Immunostaining of Jak2 in GnRH-GFP mice. Brain was sectioned at 10 μm thick from control (left) and Jak2 G−/− mice (right). GnRH neurons shown here are localized in the vertical limb of the diagonal band. Red is Jak2 immunostaining. Green is GnRH neuron. Yellow is colocalization (arrow) of Jak2 and GnRH. Scale bar is 20 microns.

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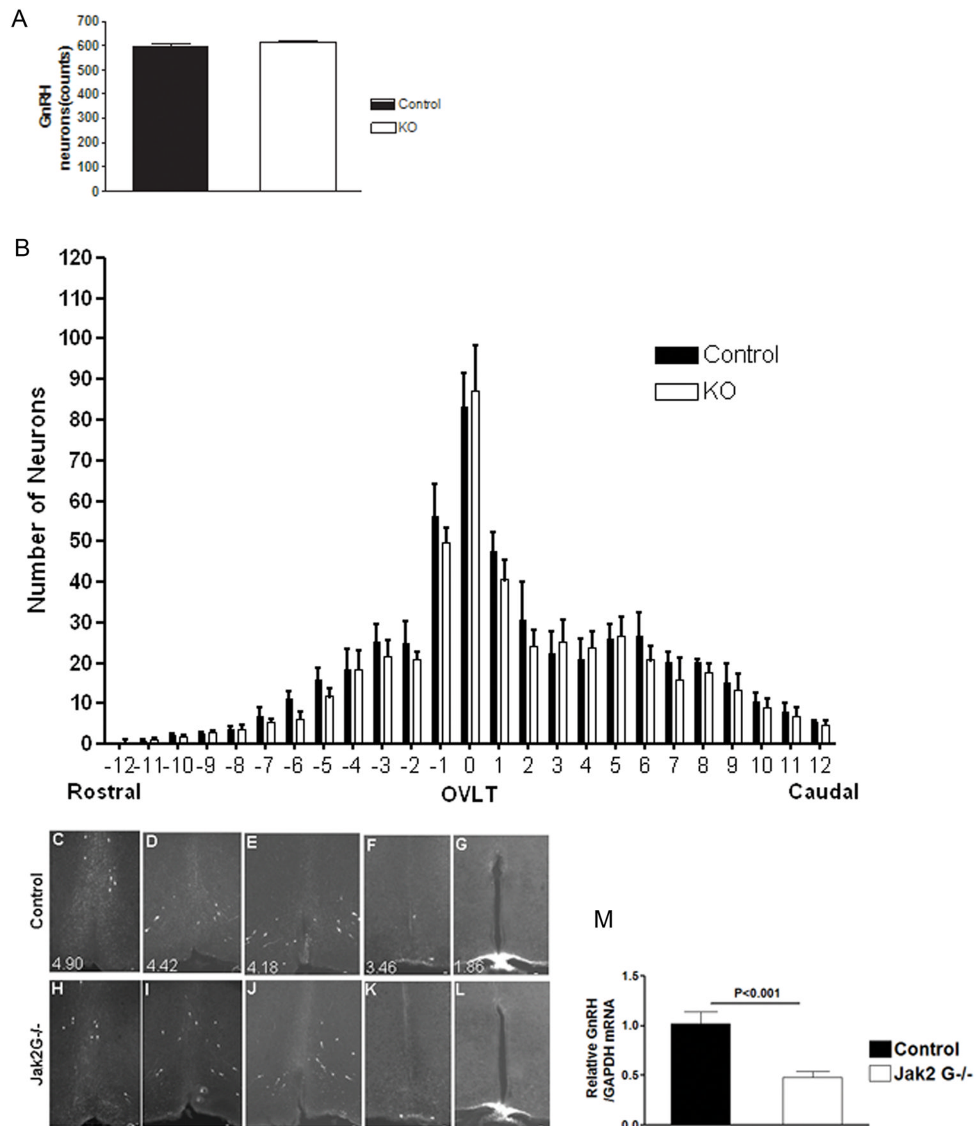

Fig. 2. GnRH neuron cell number, distribution and expression

A, Brain of GnRH-GFP mice was sectioned at a thickness of 40μm and GnRH cell bodies (green fluorescence) were counted in control (n=4) and Jak2 G−/− mice (n=4). Total GnRH neuron number is expressed as mean ± S.E.M. B, GnRH cell body distribution across the hypothalamus. OVLT is labeled as zero. Each interval counted from four successive slices (160μm). C-L, Images from control and a KO litter mate at the indicated stereotaxic position from interaural (indicated at the left bottom corner, (Franklin and Paxinos, 1997)). M, Relative GnRH mRNA levels as assessed by Taqman Real Time PCR assay for control (n=8) and Jak2 G−/− (n=10) mice expressed as fold change ± S.E.M. of normalized GnRH mRNA levels. Significance is indicated.

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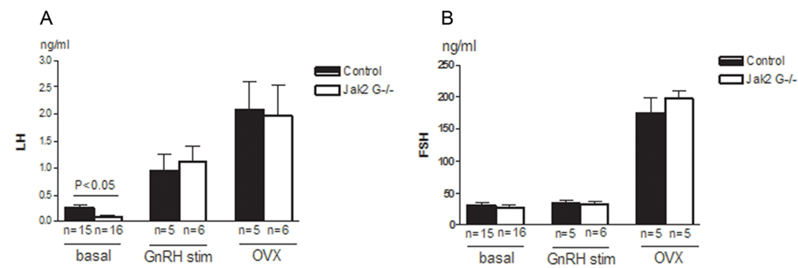

Fig. 3. Hormonal assay

A, Serum LH levels are graphed for basal, 10 minutes after GnRH stimulation or after ovariectomy (OVX). B, FSH levels for basal, 10 minutes after GnRH stimulation or after OVX. Values are mean ± S.E.M and significant differences are noted with brackets above bars.

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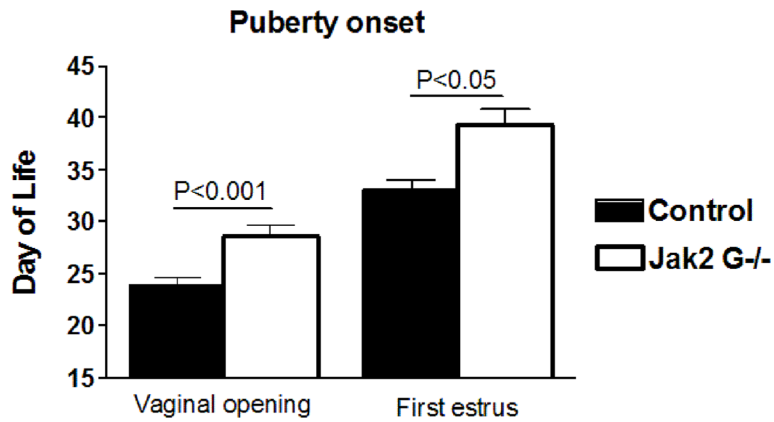

Fig. 4. Female Jak2 G−/− mice exhibit delayed puberty

Time of vaginal opening (VO) was examined daily from 21 days of life in both control (n=20) and Jak2 G−/− (n=14) litter mates. Vaginal lavage was performed following VO and the time of first estrus (defined as 100% cornified cells) was recorded (n=7 each group). Values are mean±S.E.M and significance is indicated.

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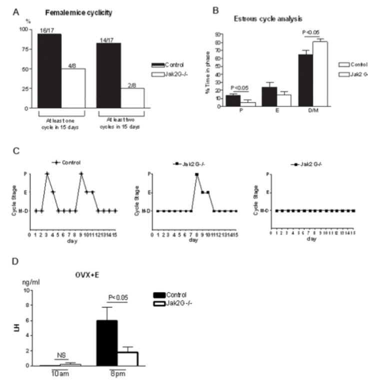

Fig. 5. Estrous cycle pattern in control and Jak2 G−/− mice

Vaginal cytology was assessed for 15 days in control and Jak2 G−/− mice. A, Plotted are the percent of females that exhibited one complete cycle during the 15 days (Left two bars) or two complete cycles during the 15 days (right two bars). Numbers of mice used to calculate the percentage are at the top of the bars. B, Time in each estrous cycle phase. E: estrus P: proestrus M/D, metestrus/diestrus. C, Representative estrous cycles of control, Jak2 G−/− with irregular cycles, and Jak2 G−/− without cycles. D, OVX mice were implanted with an estradiol pump on day 0 and bled on day 2 (control n=4 vs Jak2 G−/− n=7). Values are mean±S.E.M and significance is indicated.

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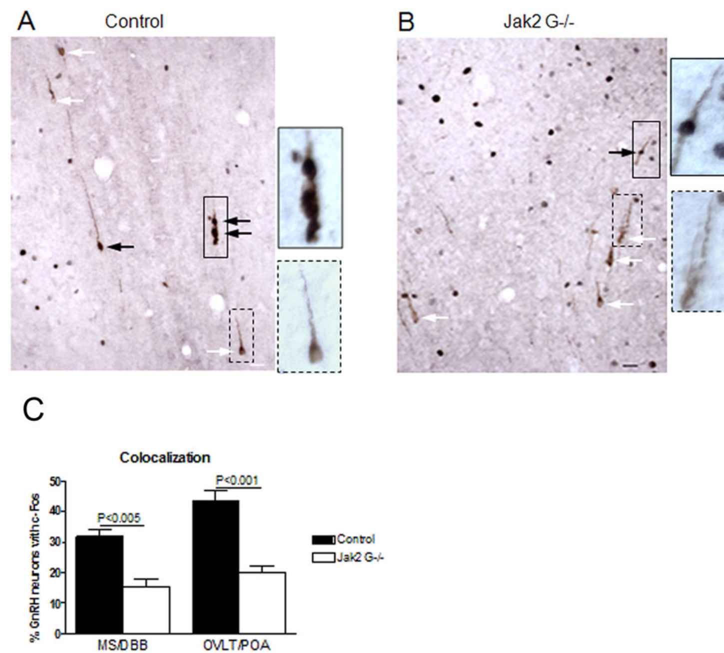

Fig. 6. Colocalization of GnRH and c-Fos after after surge generation

Images of dual labeling of GnRH and c-Fos in control (A) and Jak2 G−/− mice (B) at the level of the OVLT. Black arrows point to neurons with labeling of c-Fos (black/gray dot) and GnRH neuron (brown color). White arrows point to neurons labeled only for GnRH. A neuron with dual labeling is bordered by a solid line, with higher magnification to the right. A neuron with only GnRH staining is bordered by a dashed line, with higher magnification to the right. Scale bar: 20μm. C, Percentage of GnRH neurons with c-Fos at two different regions (n=4 per group). Values are mean ± S.E.M and significance and numbers of animals examined is indicated.

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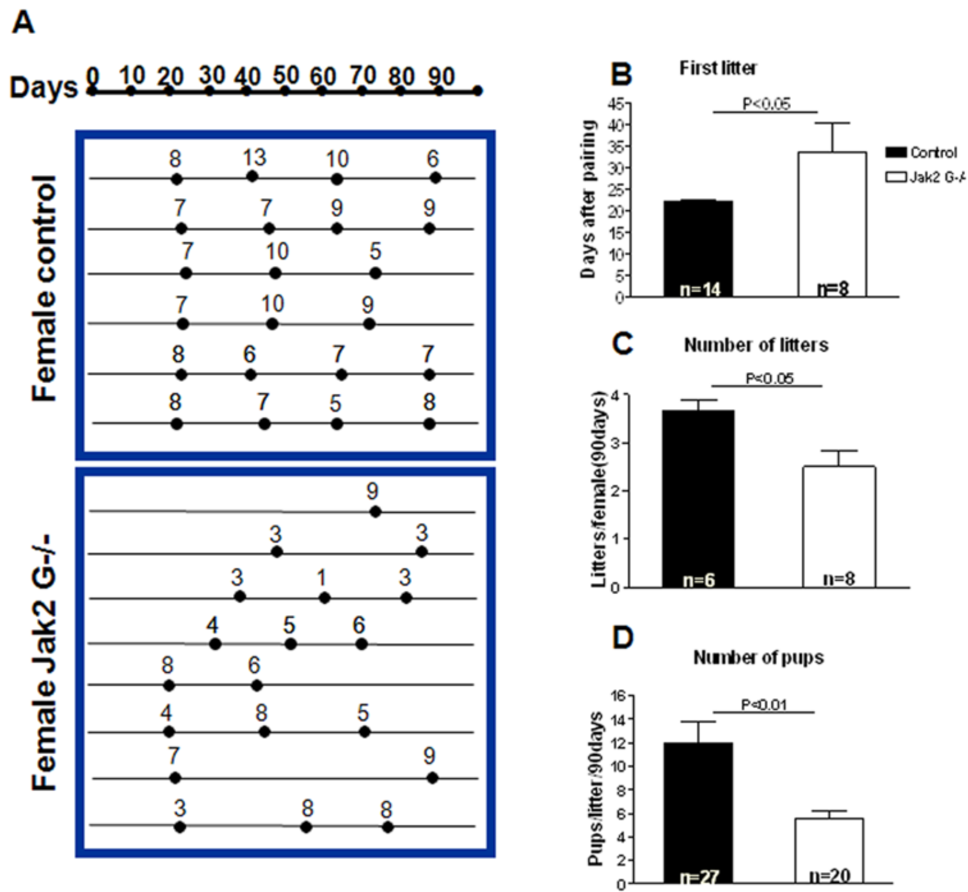

Fig. 7. Fertility in control and Jak2 G−/− female mice

A, Female mice were mated with wild type male mice for 90 days. Each line represents an individual female mouse. The black dot represents the day that each litter was born after introduction to male. Number on the top of the line represents how many pups in each litter. Top panel is female control mice. Bottom panel is Jak2G−/− female mice. Data from A, are summarized in B-D. B, After introduction with wild type male, the day of first litter was recorded in both groups. C, Total numbers of litters per female was significantly reduced in Jak2 G−/− mice compared to control mice during the 90 days. D, Number of pups per litter was significantly reduced in Jak2 G−/− mice compared to controls. For B-D, Values are mean ± S.E.M and significance and numbers of animals examined is indicated.

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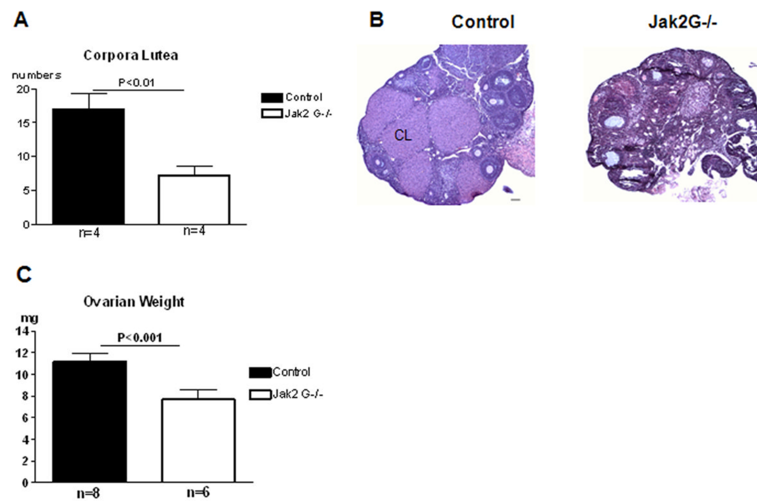

Fig. 8. Ovarian morphology

A, Ovary was sectioned at 7μm and corpora lutea (CL) were counted every 10th section per ovary (n=4 per group). B, 7 μm ovary sections with H&E staining. CL was labeled in the control ovary. C, Ovarian weights (mg) of control and Jak2 G−/− mice (n=8, control; n=6, Jak2 G−/−). Values are mean ± S.E.M and significant differences are noted with brackets above bars.