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Differential CRE Expression in Lhrh-cre and GnRH-cre Alleles and the Impact on Fertility in Otx2-Flox Mice

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Abstract

There is an increasing trend in studies utilizing cell-specific deletion of genes through conditional gene deletion by CRE recombination. Despite numerous advantages, this strategy also has limitations such as ectopic CRE-expression and germline recombination. Two commonly used gonadotropin-releasing hormone (*Gnrh*)-driven CRE-expressing mice both target GnRH neurons. However, a direct comparison of the cells targeted and their phenotypic outcome have not yet been presented. To compare where recombination takes place, we crossed the *Gnrh-cre* and *Lhrh-cre* lines with the *Rosa26-LacZ* reporter mouse. *Lhrh-cre* allowed recombination of the *Rosa26-LacZ* gene in ~700 cells, which is comparable to the GnRH neuronal population. Surprisingly, there were >20 times more LacZ expressing cells in the adult *Gnrh-cre:Rosa26-LacZ* than the *Lhrh-cre:Rosa26-LacZ* brain. The greatest differences in targeting of the *Gnrh-cre* and *Lhrh-cre* lines were found in the septum, the suprachiasmatic nucleus, and the septohypothalamic area. This difference in cells targeted was present from embryonic day 12. A prior study using the *Gnrh-cre* to delete the transcription factor *Otx2* found fewer GnRH neurons, leading to male and female sub-fertility. To recapitulate this study, we performed a fertility assay in *Otx2:Lhrh-cre* mice. We confirmed the requirement for *Otx2* in GnRH neuron development, fertility and correct gonadotropin hormone release in *Otx2:Lhrh-cre* males, but the subfertility was more modest than in *Otx2:Gnrh-cre* and absent in female *Otx2:Lhrh-cre.* This suggests that ectopic expression of *Gnrh-cre* contributes to the reproductive phenotype observed. Finally, the *Cre* alleles caused germline recombination of the *flox* allele when transmitted from either parent, generating embryonic lethal knock-out offspring, producing smaller live litters.

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Authors Contribution

H.M.H., R.L., and P.L.M. wrote the manuscript. H.M.H., R.L., D.D.C., and P.L.M. designed the experiments, analyzed the data and discussed the results. H.M.H., R.L., D.D.C., J.S.L., R.J.H., C.T., and B.M.D. performed the experiments. All authors read and approved of the final version of the manuscript.

Ethics Statement

Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body.

Disclosure Statement

The authors have no conflicts of interest to declare.

Keywords

Lhrh; Gonadotropin-releasing hormone; Otx2; Fertility; Cre-LoxP

Introduction

Conditional gene deletion in mice by the use of the cre-loxP system is a powerful tool to address the specific role of genes in cellular function and disease [1]. To obtain a conditional knock-out mouse, 2 strains are crossed; the *"flox"* strain carries two 34 bp *loxP* sequence elements flanking the gene region to be excised from the DNA, and the *"Cre"* strain expresses the bacteriophage-derived CRE recombinase under the control of a specific regulatory region. As with most techniques, the cre-lox system comes with certain pitfalls and limits, which, if not considered, can lead to erroneous conclusions [2–4]. These limits include the possible cytotoxicity of *Cre* expression [5], germline recombination [6], inefficient gene deletion [1], as well as low or non-specific *Cre* expression [7]. To determine the specific cells or tissues expressing *Cre*, successful recombination can be evaluated by PCR, southern blot, or by identifying the expression of a reporter gene such as a ROSA26-driven reporter [8–12]. Reporter mice permit determination of the approximate time at which recombination takes place. However, the sensitivity of the ROSA26 allele, containing the *loxP*-flanked STOP cassette, might differ from the sensitivity of the *loxP* site of the experimental gene. In addition, both the site of *Cre* transgene insertion into the DNA, as well as the DNA region used to drive transgene expression impact the fidelity of *Cre* expression [13–15].

The role of well-defined transcriptional regulatory regions, which allow specific expression of a peptide to a limited cell population, are well studied for gonadotropin-releasing hormone (GnRH or LHRH) [13–18], a neuropeptide required for pubertal onset and fertility [11, 19–22]. Despite the careful characterization of the regulatory region of *Gnrh1*, it has proven difficult to create a *Cre* mouse that recapitulates the onset of GnRH expression and specifically targets this small neuronal population compromised of a little under 1,000 neurons in the adult mouse. These challenges are likely associated with the unique origin of GnRH neurons in the vomeronasal organ [23]. After GnRH neurons arise around embryonic day 10 (e10), they initiate their migration into the ventral forebrain, and localize throughout the anterior hypothalamic area where most GnRH neurons are found at ~e18–e19. Once at their final location, GnRH neurons project to the median eminence (ME) and release GnRH in a pulsatile pattern promoting luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release from the anterior pituitary. The scattered location of GnRH neurons in the anterior hypothalamic area makes it challenging to specifically manipulate gene expression in these neurons by viral injections [24, 25], and has led to the generation of at least 4 different *Gnrh*-driven CRE expressing mice: the Tg(Gnrh1-cre)35Awo [26], the Tg(Gnrh1-cre)1Dlc [27], the Tg(Gnrh1-cre)1Gsc [28] and the Tg(Gnrh1-cre)1Rsp [29]. The *Gnrh-cre* (Tg[Gnrh1-cre]35Awo) mouse was generated by inserting a transgene that utilizes the mouse 3.4 Kb promoter to drive *Cre* recombinase expression in all GnRH neurons, in addition to some ectopic expression [26]. The *Lhrh-cre* (Tg[Gnrh1-cre]1Dlc) mouse, in which a mouse bacterial artificial chromosome containing the *Gnrh1* gene driving *Cre* expression is inserted

as a transgene into the genome, leads to *Cre* expression in ~96% of the GnRH neurons, with very limited off-target expression [16, 27].

Due to the known widespread ectopic expression of the *Gnrh-cre* (Tg[Gnrh1-cre]35Awo), we checked the extent to which this would be physiologically relevant. We hypothesized that the deletion of the developmental homeodomain transcription factor *Otx2* using the *Lhrh-cre* (Tg[Gnrh1-cre]1Dlc, *Otx2:Lhrh-cre*), might lead to a less severe reproductive phenotype than *Otx2* deletion using the *Gnrh-cre* **(***Otx2:Gnrh-cre***)** [30].

Materials and Methods

Mouse Breeding

Mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. *Otx2-flox* [31], *Six6-flox* (Mellon laboratory, unpublished), *Lhrh-cre* (Tg[Gnrh1-cre]1Dlc) [27], *Gnrh-cre* (Tg[Gnrh1-cre]35Awo) [26], *Rosa26-LacZ* (JAX #003309), and *Rosa26-TdTo- mato* (JAX #007909) mice were maintained on a C57BL/6J genetic background, and housed under a 12 h light-dark cycle, with food and water ad libitum. Mice were sacrificed by isoflurane or CO_2 overdose, followed by cervical dislocation. All mice were systematically genotyped for germline recombination of the Floxed allele, and only mice without germline recombination were included in our study.

Fertility Assessments, Pubertal Onset, and Embryo Collection

Procedures for pubertal onset, embryo collection and fertility assessment were performed as described in detail previously [19, 32, 33]. For fertility assays, 8-week-old *Otx2:Lhrh-cre* mice were housed with *Otx2-flox/flox* mice. The number of litters born and the number of pups per litter were recorded over 180 days. Inspection of pubertal onset was initiated after weaning (20 days of age). Mice were inspected daily for vaginal opening in the females and preputial separation in the males. The day of preputial separation and vaginal opening was recorded. To generate embryos (e) of the desired age, timed-mated females were sacrificed at gestation day 11.5 (e11.5), 12.5 (e12.5), 13.5 (e13.5), and 17.5 (e17.5). Embryos were fixed in a solution composed of 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) and processed for immunohistochemistry.

Hormone Measurements

Blood was collected by cardiac puncture. Serum was separated by centrifugation (RT, 2,300 *g* for 15 min), and serum stored at −20 ° C till RIA analysis of LH, FSH, and testosterone at the Ligand Assay and Analysis Core in the Center for Research in Reproduction at University of Virginia.

Protein and Transcript Detection

Single immunohistochemistry and quantitative RT-PCR were performed as previously described [11, 19]. For double immuno-histochemistry, the same protocol as for single

immunohisto-chemistry was used, with the modification that after developing and imagine the anti-CRE (1/1,000) staining using Impact DAB (brown), the slides were washed in PBS-Tween 0.02%, followed by 5 min in denaturing solution (Biocare #DNS001), after which the sections were blocked in 5% goat serum and Avidin-Biotin solutions (Vector Labs), and the anti-LHRH (1/1,000) antibody was applied O/N following the regular immunohistochemistry protocol, using Impact VIP (purple) to visualize GnRH expressing cells. All antibodies were validated using negative controls such as cre^{-} tissue for the anti-CRE antibody, and Cre^{-}:ROSA26-$LacZ^{+}$ tissue for the anti-LacZ antibody. We have previously validated the GnRH and LacZ antibodies [11, 19, 32]. The primary antibodies used for single immunohistochemistry are rabbit anti-GnRH (Thermo Scientific #PA1–121, dilution 1/1,000, RRID:AB_325077), chicken anti-beta galactosidase (Abcam #AB9361, dilution 1/1,000, RRID:AB_307210), rabbit anti-CRE recombinase (Biolegend #908001, dilution 1/500, RRID: AB_2565079), and rabbit anti-RFP (Abcam #AB62341, dilution 1/500, RRID:AB_945213). For double immunohistochemistry, we used rabbit anti-CRE recombinase (Biolegend #908001, dilution 1/1,000, RRID: AB_2565079), and rabbit anti-LHRH antibody (Immunostar #20075, dilution 1/1,000, RRID: AB_572248). Primary antibodies were detected by secondary biotinylated antibodies (Vector Laboratories #BA-1000 and #BA-9010) and visualized using the Vectastain ABC elite kit with VIP, Impact VIP, or Impact DAB peroxidase (Vector Labs). To compare protein expression patterns between mouse lines, staining was done in parallel with identical conditions. Hypothalamic *Gnrh1* content was determined as described previously [19, 34]. The enzymatic LacZ staining was performed by incubating the tissue overnight at 37 ° C in X-gal staining buffer (1 mg/mL 4-chloro-5-bromo-3-indolyl-β-galactosidase, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$ in 100 mM PBS, pH 7.4).

Statistical Analysis

Data were analysed by Student *t* test or two-way ANOVA using GraphPad Prism 7 (Graph Pad Software, La Jolla, CA, USA) as noted in the figure legends. Significant differences were designated as $p < 0.05$.

Results

Substantial Difference in the Number of Cells Targeted by the Gnrh-cre *versus the* Lhrh-*cre Allele*

To determine whether the *Lhrh-cre* and *Gnrh-cre* alleles allow recombination in the same number of cells in the same locations, we performed LacZ staining in adult *Lhrh-cre:Rosa26-LacZ* and *Gnrh-cre:Rosa26-LacZ* mice and counted the number of LacZ expressing cells. We found >20 times more LacZ expressing cells in the *Gnrh- cre:Rosa26-LacZ* as compared to the *Lhrh-cre:Rosa26-LacZ* mice (Fig. 1a). The additional LacZ-expressing cells in the *Gnrh-cre:Rosa26-LacZ* were restricted to the ventral forebrain and were principally present in the septum (Fig. 1b, c). To determine if the additional LacZ positive cells expressed LacZ due to early recombination, but now did not actively transcribe the *Cre* allele, we performed staining for the CRE protein, which is only present in cells that are actively transcribing *Cre*. We found the CRE expression pattern to be similar to that of LacZ in both mouse lines (Fig. 1d), although with a slight reduction in the number of cells

expressing CRE as compared to LacZ in adult *Gnrh-cre:Rosa26-LacZ* mice (Fig. 1b, c). To revalidate the targeting of GnRH neurons of the 2 studied *Cre*-alleles [26, 27], we performed double immunohisto-chemistry in adult brains of *Lhrh-cre* and *Gnrh-cre* mice. First, we validated the specificity of our antibodies performing double immunohistochemistry in a wild-type mouse, where CRE is not expressed (C57BL/6J), in a CRE-expressing mouse (*Gnrh-cre*+ mouse) and in a mouse model lacking GpRH expressing neurons [11]. We detected GnRH neurons in control mice (Fig. 1e, wild-type and *Gnrh-cre*+ mouse). This signal was specific as evidenced by the absence of GnRH staining in the negative control tissue [11] (Fig. 1e, GnRH lacking mouse). The anti-CRE antibody specifically detected CRE expressing cells (Fig. 1e, compare wild-type to *Gnrh-cre*+ mouse). The anti-CRE and anti-LHRH antibodies did not cross-react as evidenced by the distinction of cells expressing GnRH (purple) and CRE (brown) in the *Gnrh-cre* mouse (Fig. 1f). In agreement with previous studies [26, 27], we observed that *Lhrh-cre* targeted >95% of the GnRH expressing neurons and the *Gnrh-cre* targeted all the detected GnRH neurons in adulthood (Fig. 1f). Supporting our LacZ and CRE staining in Figure 1b and d, the *Gnrh-cre*, but not the *Lhrh-cre* allele, drove CRE expression in a high number of non-GnRH expressing cells (Fig. 1f, septum and POA). This high ectopic expression of *Gnrh-cre* was further supported by LacZ staining in *Lhrh-cre:Rosa26-LacZ* and *Gnrh-cre:Rosa26-LacZ* mice (Fig. 2), where LacZ staining reflects all living cells that have had the *flox* allele recombined prior to the time of imagine, and does not depend on active expression of the *Cre*-allele. We carefully compared the LacZ expression pattern in the adult brain of *Lhrh-cre:Rosa26-LacZ* and *Gnrh-cre:Rosa26-LacZ* mice and found major differences in the number of LacZ expressing cells, specifically we noted a significant number of LacZ expressing cells in the lateral septum (Fig. 2), preoptic area, the suprachiasmatic nucleus (SCN), the septohypothalamic nucleus, the olfactory tubercule, and the medial hypothalamus.

The Gnrh-cre Allele Targets More Cells than the Lhrh-cre Allele from e12.5 Onward

To determine when the difference in cells targeted by the 2 *Cre* alleles arose, we performed CRE staining in *Gnrh-cre* and *Lhrh-cre* embryos at embryonic day 11.5 (e11.5), e12.5, e13.5 and e17.5. We detected CRE expression as early as e11.5 in both mouse lines (Fig. 3, e11.5, nose). However, more cells expressed CRE at e12.5 in the *Gnrh-cre* than the *Lhrh-cre* embryos, a pattern we observed in all older age groups. Again, the major difference in expression was found in the developing septum, and hypothalamus, a difference that was prominent at e13.5 and e17.5 (Fig. 3).

Brain Areas with High CRE Expression in the Gnrh-cre *Mouse also Express* Otx2

Using in situ hybridization images obtained from www.brain-map.org (consulted April 2018), we mapped the *Otx2* expression pattern in the developing mouse head. As expected at e13.5 *Otx2* expression in the olfactory placode, cribriform plate, and anterior head colocalize with the expression pattern of *Gnrh1* (Fig. 4, *Gnrh1* e13.5, and *Otx2* e13.5) and CRE expression in the *Gnrh-cre* mouse (Fig. 3, e12.5 and e13.5). At e15.5, *Otx2* expression was high in the hypothalamus, a time when GnRH neurons start arriving in this brain area. At e18.5, *Otx2* was highly expressed in the septum, an area with high CRE expression in the *Gnrh-cre* mouse at e17.5 (Fig. 3, e17.5). *Otx2* expression was not observed in the hippo-campus or the SCN (Fig. 4, *Otx2* e18.5).

Otx2 Expression Is Required for GnRH Neuron Development

A prior study using the *Gnrh-cre* (Tg[Gnrh1-cre]35Awo) to delete the *Otx2-flox* allele (*Otx2:Gnrh-cre*) determined that the transcription factor *Otx2* within GnRH neurons is required for proper GnRH neuron development and fertility [30]. Based on the different expression patterns of *Gnrh-cre* and *Lhrh-cre* (Fig. 1–3), and the overlap between CRE expression in the *Gnrh-cre* (Fig. 3) and *Otx2* during development (Fig. 4), we decided to repeat this study, deleting the *Otx2-flox* allele using *Lhrh-cre* (Tg[Gnrh1-cre]1Dlc, *Otx2:Lhrh-cre*). We first asked how many GnRH neurons would be present in *Otx2:Lhrh-cre* mice at e13.5 and e17.5. As expected, in e13.5 controls, the full complement of GnRH neurons was located within the nasal and cribriform plate regions [23] (Fig. 5a, b). In contrast, at e13.5 in *Otx2:Lhrh-cre,* a reduction in the number of GnRH neurons was observed in all regions of migration (Fig. 5a, b), whereas an accumulation of GnRH neurons was found at the cribriform plate and a reduction in the hypothalamus and brain at e17.5 (Fig. 5c, d). The intensity of the GnRH staining in neuronal terminals at the ME was also substantially reduced in *Otx2:Lhrh-cre* embryos (Fig. 5c, ME). This reduction in GnRH was maintained into adulthood at the level of *Gnrh1* transcription (Fig. 5e).

Specific Deletion of Otx2 from GnRH Neurons Leads to Male Subfertility

To determine if the 56% reduction in *Gnrh1* in adult *Otx2:Lhrh-cre* mice (Fig. 5e) resulted in subfertility, we performed a fertility assay. We found that the number of litters generated by *Otx2:Lhrh-cre* males, but not *Otx2:Lhrh-cre* females, was reduced (Fig. 6a). Surprisingly, we noted a high rate of pup mortality when mating male *Otx2-flox/flox* mice to female *Otx2-flox/WT:Lhrh-cre* mice. The reduction of litter size was due to some pups being born with severe cranial abnormalities, a developmental defect of full body knock-out *Otx2* mice [35–37]. This suggests that *Lhrh-cre* transgene expression was occurring within the ovary, allowing germline recombination and transmission of a recombined null allele. Genotyping of progeny, where the *Cre* transgene was obtained from the female, demonstrated recombination within tail DNA of the offspring (Fig. 6b). Interestingly, in many cases, progeny of *Otx2:Lhrh-cre* exhibited recombination in tail DNA, independent of whether they inherited the *Cre* allele, suggesting that recombination occurred frequently during female germ cell development (Fig. 6b). To determine the frequency of germline recombination, we designed primers allowing us to analyze the percent of mono- and bi-allelic (heterozygote and homozygote recombination respectively) germline recombination of the *Six6-flox* allele, a mouse model that is fully viable as both a heterozygote and homozygote germline knockout mouse [19]. Analyzing our data from *Six6:Lhrh-cre* and *Six6:Gnrh-cre* mice, we found that both *Lhrh-cre* and *Gnrh-cre* caused germline recombination (mono- or bi-allelic) of the *Six6-flox* allele when the *Cre*-allele was transmitted from either the mother or the father (Fig. 6c). The recombination occurred more frequently when the *Cre*-allele was transmitted from the mother than from the father, and germline recombination of the *Six6-flox* allele was more frequent in the *Lhrh-cre* mice than the *Gnrh-cre* mice (Fig. 6c, Two-way ANOVA, overall effect of sex $p = 0.0053$, overall effect of mouse strain $p = 0.0022$). The germline recombination was obvious in our R26Rfp reporter mice, where *Gnrh-cre* induced R26Rfp recombination generating mice with a red-pinkish color (Fig. 6d). To determine how frequently homozygous recombination of the *Six6-flox* allele occurred, we

calculated the percentage homozygous recombination (Fig. 6e). When the *Lhrh-cre* allele was transmitted from the mother, almost 1/5 of the offspring had homozygous recombination of the *Six6-flox* allele (Fig. 6e). Homozygous recombination of the *Six6-flox* allele happened in < 5% of the pups generated from *Gnrh-cre* males, females, and *Lhrh-cre* males (Fig. 1e, Two-way ANOVA, overall effect of sex $p = 0.0206$). The high ratio of germline recombined pups from *Lhrh-cre* mothers was validated using staining of the gonads where the germline recombination would cause functional recombination of a tracer gene such as R26LacZ or R26Rfp. LacZ or RFP staining of gonads confirmed that recombination was occurring in some oocytes (Fig. 6f, arrows). We were unable to see recombination in the sperm probably due to the high compaction of the sperm head (Fig. 6f, testis). The frequent homozygous recombination in pups from female *Lhrh-cre* mice caused *Otx2:Lhrh-cre* females to produce smaller litters than controls, due to the lethality of *Otx2* knock-outs [30]. In contrast, males fathered litters the same size as controls (Fig. 6g). As the only fertility defect detected in *Otx2:Lhrh-cre* females was a decrease in litter size, which we attributed to the lethality of the offspring to germline recombination, we next focused on the *Otx2:Lhrh-cre* males.

Otx2:Lhrh-cre Males Have Decreased LH Serum Levels and Reduced Sperm Production

GnRH stimulates the release of the gonadotropins FSH and LH, which are the 2 hormones required for fertility. Circulating levels of both LH and FSH were significantly reduced in *Otx2:Lhrh-cre* males (Fig. 7a, b). This led to a slight, non-significant, reduction in testosterone (Fig. 7c). Despite a reduction in hormone levels, testes weight (Control = 6.4 ± 0.4 mg, *Otx2:Lhrh-cre* 6.5 ± 0.5 mg, $n = 5$, Student *t* test, $p > 0.05$) and seminal vesicle weights (normalized to body weight) were comparable between control and *Otx2:Lhrh-cre* males (Control = 0.0064 ± 0.0006, *Otx2:Lhrh-cre,* 0.0006 ± 0.001, $n = 5$, Student *t* test, $p > 0.05$). In contrast, the total number of sperm was significantly reduced in *Otx2:Lhrh-cre* (Fig. 7d).

Discussion

Lhrh-cre Recombination in the Ovary of Otx2:Lhrh-cre Results in Smaller Litters

The surprising difficulty in reproducing scientific findings is a major concern in research [38], and an increasing focus of the National Institutes of Health (NIH; http://grants.nih.gov/grants/RigorandReproducibilityChart508.pdf). Although very few techniques and experimental designs come without pitfalls, understanding and recognizing such limits is important to correctly interpret data. While the *Lhrh-cre* transgenic mouse line offers an excellent percentage of recombination in GnRH neurons, targeting over 96% of adult GnRH neurons [27], and efficiently deletes the floxed allele [10, 39], our study demonstrates that CRE activity is not just limited to the brain in these mice. We observed CRE recombinase activity in the female oocyte of both *Lhrh-cre* and *Gnrh-cre,* which resulted in germline recombination in progeny derived from females. Based on other reports, it is not unexpected that the GnRH promoter could drive transgene expression within the oocyte [40, 41]. Although we did not detect recombination in sperm using staining, based on our PCR results, up to 20% of the offspring generated from a Cre+ father experienced germline recombination. When the *Lhrh-cre* allele was transmitted from the mother, ~50% of pups

had the flox-allele recombined in the entire body (germline recombination), where ~1/5 of these pups experienced homozygous recombination (bi-allelic recombination), generating a full body, germline knock-out mouse. The frequency of germline recombination was significantly lower when the *Lhrh-cre* allele was transmitted from the father and the frequency of homozygous recombination when the *Lhrh-cre* allele was transmitted from the father was a rare event. In both the *Gnrh-cre* and the *Lhrh-cre* mice, the frequency of germline re-combination was higher when the *Cre*-allele was transmitted from the female, as compared to the male. Overall, germline recombination, whether mono- or bi-allelic was more frequent in *Lhrh-cre* than *Gnrh-cre* mice. Because *Otx2* knock-out is lethal [35–37], we attribute the reduced litter size of *Otx2:Lhrh-cre* females to the high frequency of homozygous germline recombination when the *Lhrh-cre* allele was transmitted from the mother, resulting in a reduction in the litter size. This study clearly illustrates that germline recombination is prominent in the 2 studied mouse lines, and if not considered, can cause wrongful conclusions.

Extensive Ectopic Expression of the Gnrh-cre Allele in the Brain

The cre-lox system is a wonderful tool to generate conditional knock-out and/or knock-in mice and allows for efficient cell-tracing. However, in some instances, lack of consideration for the limits of the cre-lox system can lead to overstated or misleading conclusions. Germline re-combination and ectopic expression of different *Gnrh*-driven cre mouse lines has previously been described in the literature. However, prior to this study, a careful side-by-side comparison of the expression pattern of the widely used *Lhrh-cre* (Tg[Gnrh1-cre]1Dlc) [10, 27] and *Gnrh-cre* (Tg[Gnrh1-cre]35Awo) [26, 42] alleles had not been performed. Our side-by-side comparison of the number of cells targeted by *Gnrh-cre* and *Lhrh-cre* confirmed previous reports of a greater number of cells targeted by the *Gnrh-cre* allele than the *Lhrh-cre* allele in the brain [26, 27]. The difference in CRE expression could be detected from e12.5 onward in the developing mouse brain. The early increase in CRE expression in non-GnRH neurons is important to consider when the studied flox-allele deletes genes important in septum and ventral forebrain development [15]. To determine the number of cells that had been targeted by both the *Lhrh-cre* and *Gnrh-cre,* we used lineage tracing, which allows the expression of a tracer gene (here LacZ) in all cells that have expressed CRE prior to the time of staining. We found >20-fold more cells expressing LacZ in the *Gnrh-cre:Rosa26-LacZ* as compared to the *Lhrh-cre:Rosa26-LacZ* brain. The additional LacZ expression in the *Gnrh-cre:Rosa26-LacZ* mouse was restricted to the ventral forebrain, and principally observed in the septum, a brain area known to be targeted by both the *Lhrh-cre* and *Gnrh-cre* promoters [13, 26, 27, 39, 43], as well as the hypothalamus, including the SCN. A part of the *Gnrh1* promoter-driven CRE expression in the lateral septum most likely reflects a neuronal population, which transiently activates the *Gnrh1* promoter [15]. To validate that no genetic derivation or mouse strain differences [44] had impacted the CRE or GnRH expression in our mice, we performed double immunohistochemistry for CRE and GnRH in adult *Gnrh-cre* and *Lhrh-cre* mice. As expected, both *Lhrh-cre* and *Gnrh-cre* targeted 95–100% of GnRH expressing neurons in adulthood. The dual immunohistochemistry confirmed that the *Gnrh-cre* allele drove CRE expression in a significant number of non-GnRH expressing cells, with the most profound expression pattern in the lateral septum and the broader hypothalamic area. In agreement

with others, we found fewer CRE, than LacZ, expressing cells in the adult septum. This transient expression of CRE has previously been noted [10, 45], and suggests some GnRH neurons might silence the *Gnrh*-promoter prior to adulthood. However, it should be noted, that once the *Cre*-allele has been expressed in a cell, allowing the recombination of the *"flox"* sequences, this DNA re-combination is permanent. Therefore, using lineage tracing to understand the number of cells targeted at any given time point prior to staining more accurately reflects the cells where the gene of interest has been deleted. Thus, to determine where the *Otx2-flox/flox* allele could have been deleted, in addition to within GnRH neurons, we compared *Otx2* in situ hybridization images from www.brain-map.org. Comparing the overlap between *Gnrh-cre* targeted areas and *Otx2* expressing areas during development, showed that *Gnrh-cre* targets areas that later express *Otx2* including the preoptic area of the hypothalamus, an area where GnRH neurons are located in adult-hood, and the lateral septum, a brain area known to be critical for sexual behavior [46, 47]. It will thus be of interest in future studies to determine if *Otx2:Gnrh-cre* mice have abnormal sexual behavior.

The Number of GnRH Neurons Required for Male and Female Fertility

Otx2 is expressed in GnRH neurons, where it regulates the expression of GnRH as well as the survival of these neurons [22, 30, 34, 36, 41, 48, 49]. Despite the increased number of cells targeted by the *Gnrh-cre* allele from as early as e12.5 (see paragraph 5.2), GnRH neuron distribution and the number of GnRH neurons was comparable between *Otx2:Lhrh-cre* (this study) and *Otx2:Gnrh-cre* [30]. The reduction of brain/hypothalamic GnRH neurons in *Otx2:Lhrh-cre* at e17.5, and the decreased targeting to the ME, was a defect also noted in *Otx2:Gnrh-cre* mice [30], supporting the validity of both mouse lines for the study of GnRH neuron development.

GnRH neuron migration is halted around birth; thus, any delay in GnRH neuron migration causes a reduction of hypothalamic GnRH neurons and this has serious consequences to GnRH neuron targeting to the ME and fertility [10, 19, 30, 32, 45]. We found an increase in the number of GnRH neurons at the cribriform plate in e17.5 old *Otx2:Lhrh-cre* embryos. This increase in GnRH neurons at the entrance to the brain (cribriform plate) was associated with a reduction of GnRH neurons in the brain, although the total number of GnRH neurons in these embryos was only decreased by 21%, a reduction not expected to impact fertility [19, 32, 36, 50, 51]. However, our study was not designed to address if the differential distribution of GnRH neurons in *Otx2:Lhrh-cre* mice was caused by GnRH neuron death, abnormal migration, or a change in proliferation. The reduction of GnRH neurons in the brain at e17.5 in *Otx2:Lhrh-cre* mice was in agreement with the overall reduction of hypothalamic *Gnrh1* gene expression in adulthood, and caused reduced circulating LH and FSH in adult males. The reduction of LH and FSH in *Otx2:Lhrh-cre* males led to subfertility, supporting previous findings using *Otx2:Gnrh-cre* animals [30]. *Otx2:Lhrh-cre* male subfertility was characterized by a decrease in the number of litters sired and fewer sperm, a phenotype recapitulated in *Otx2* heterozygote males [36]. This supports that correct dosage of *Otx2* within GnRH neurons is critical for normal reproductive function. The decreased expression of *Gnrh1* was associated with a reduction in LH and FSH, and a slight reduction in testosterone, probably causing the subfertility. Surprisingly, unlike Diaczok et al. [30], we

did not observe any fertility phenotype in the *Otx2:Lhrh-cre* females, which have normal ovaries (not shown), suggesting correct release of LH and FSH. The maintained fertility of *Otx2:Lhrh-cre* females is supported by other studies showing that fewer than 34% of GnRH neurons are required for estrous cycling and fertility [32, 50]. The greater subfertility of the *Otx2:Gnrh-cre* females [30], as compared to the *Otx2:Lhrh-cre* (this study) might be the result of germline recombination in the female *Otx2:Gnrh-cre* oocytes, incomplete recombination of the *Otx2-flox* allele when using the *Lhrh-cre* allele, or ectopic expression of the *Gnrh-cre* allele.

Conclusion

We have confirmed the importance of *Otx2* within GnRH neurons for establishing a correctly located GnRH neuronal population, and its control of male fertility. The comparison of CRE expression during development and adulthood between *Gnrh-cre* and *Lhrh-cre* mice identified a significant difference in the number of cells targeted as early as e12.5. Specifically, the *Gnrh-cre* has high ectopic expression in the preoptic area and the septum. Finally, both the *Gnrh-cre* and *Lhrh-cre* alleles can recombine in both male and female germline cells, thus allowing the generation of mosaic and full body-knock out off-spring.

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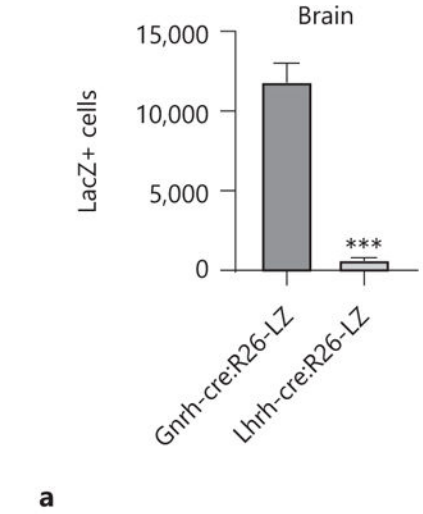

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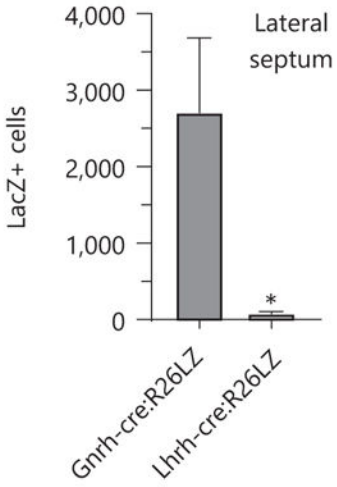

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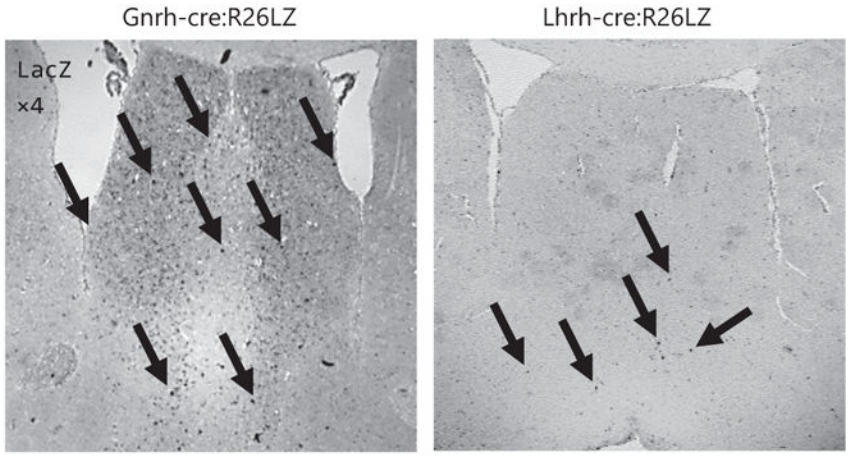

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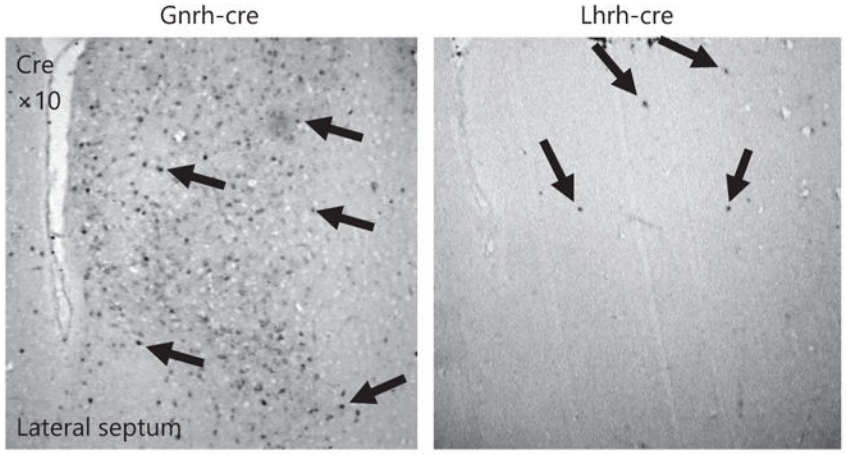

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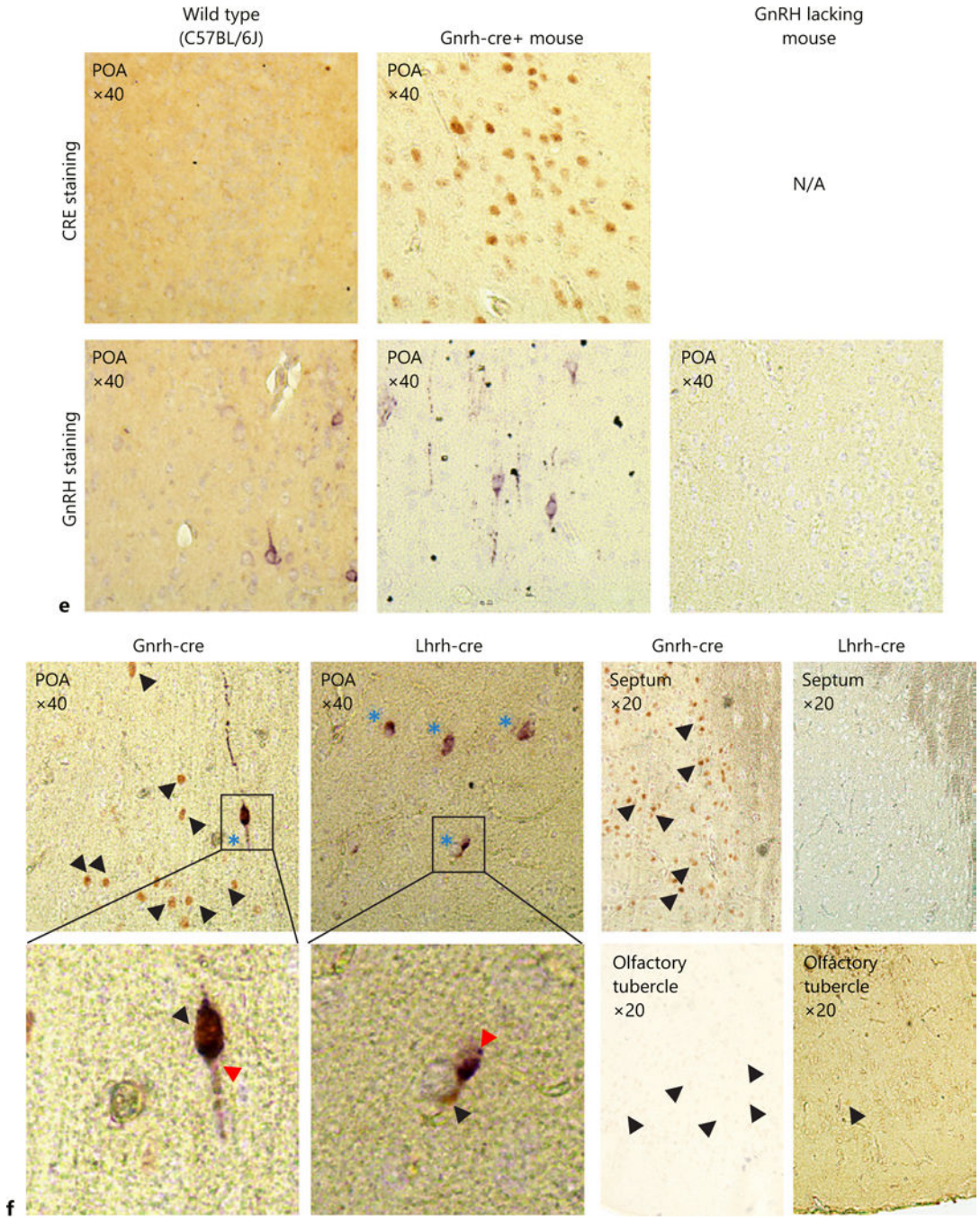

Fig. 1.

Quantification of LacZ positive (LacZ+) cells in the whole brain (**a**) and the lateral septum (**c**) from adult *Gnrh-cre:Rosa26-LacZ* and *Lhrh-cre:Rosa26-LacZ* mice, $n = 3$, Student t test; * $p < 0.05$. Immunohistochemical staining in the lateral septum for (**b**) LacZ in *Gnrh-cre:Rosa26-LacZ* and *Lhrh-cre:Rosa26-LacZ* (×4), and (**d**) CRE in *Gnrh-cre* and *Lhrh-cre* mice (×10). Arrows high-light LacZ or CRE expressing cells in coronal sections. **e** Negative controls for double immunohistochemistry, showing specificity of the anti-CRE and anti-LHRH antibodies. To assure that no cross-reaction of the antibodies occurred, the antibody

validation was done following the entire double immunohistochemistry protocol where the anti-LHRH antibody was omitted for the CRE staining, and the anti-CRE antibody omitted for the GnRH staining. The GnRH-lacking mouse is the *Gnrh-cre:Vax1-flox/flox* mouse that we previously published as lacking GnRH expressing neurons in the hypothalamus as a negative control for the anti-LHRH antibody [11]. **f** Double immunohistochemistry for GnRH (purple, GnRH staining is indicated by a red arrow head) and CRE (brown, single staining is indicated by a black arrow head). Dual staining is indicated by blue stars. $n = 3$ adult brains.

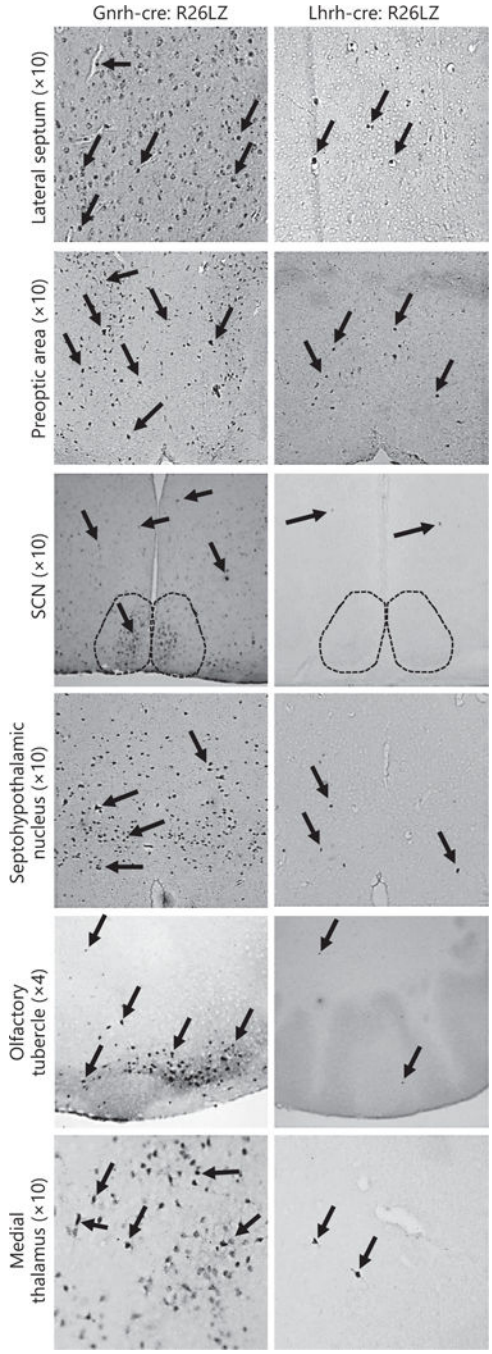

Fig. 2.

Illustrative immunohistochemical images of LacZ staining in coronal sections of adult *Gnrh-cre:Rosa26-LacZ* and *Lhrh-cre:Rosa26-LacZ* brains. Arrows highlight LacZ expressing cells. All images are ×10, except the olfactory tubercle, which is ×4. SCN, suprachiasmatic nucleus.

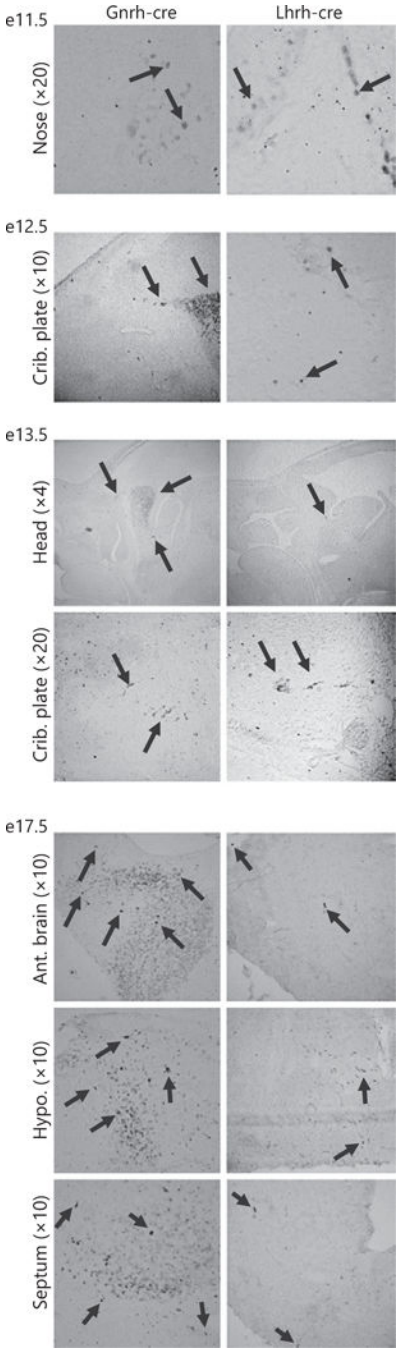

Fig. 3.

Early developmental differences in CRE expression in the *Gnrh-cre* and *Lhrh-cre* brains. Sagittal sections of *Gnrh-cre* and *Lhrh-cre* embryos were stained for CRE at embryonic day 11.5 (e11.5), e12.5, e13.5, and e17.5. Ant. brain, anterior brain; crib. plate, cribriform plate; hypo, hypothalamus. Images were taken at ×4, ×10, or ×20 as indicated in the figure.

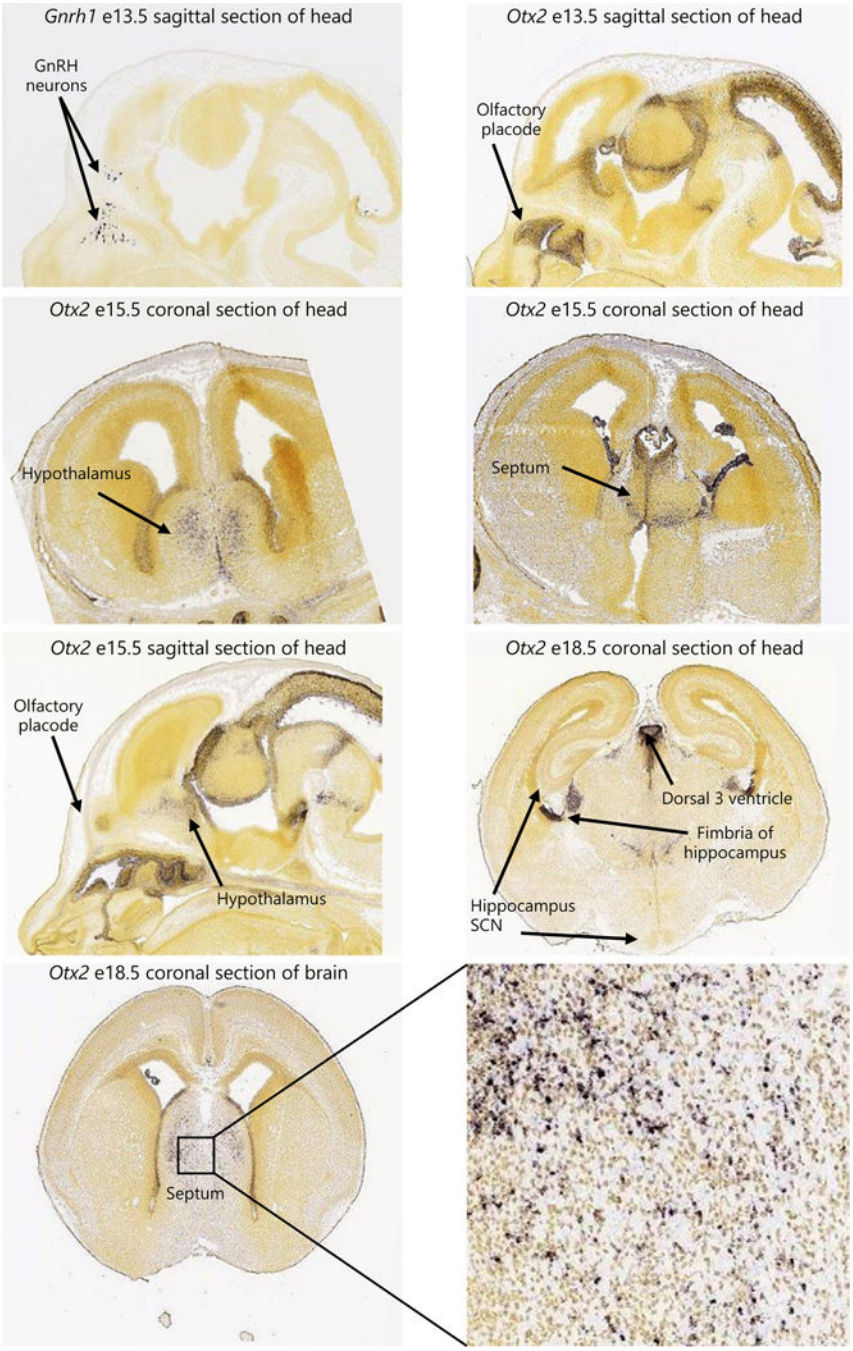

Fig. 4.

In situ hybridization images obtained from www.brain-map.org (consulted in April 2018) for *Gnrh1* and *Otx2* in brain sections from embryos (e) at the indicated ages. Positive staining is brown.

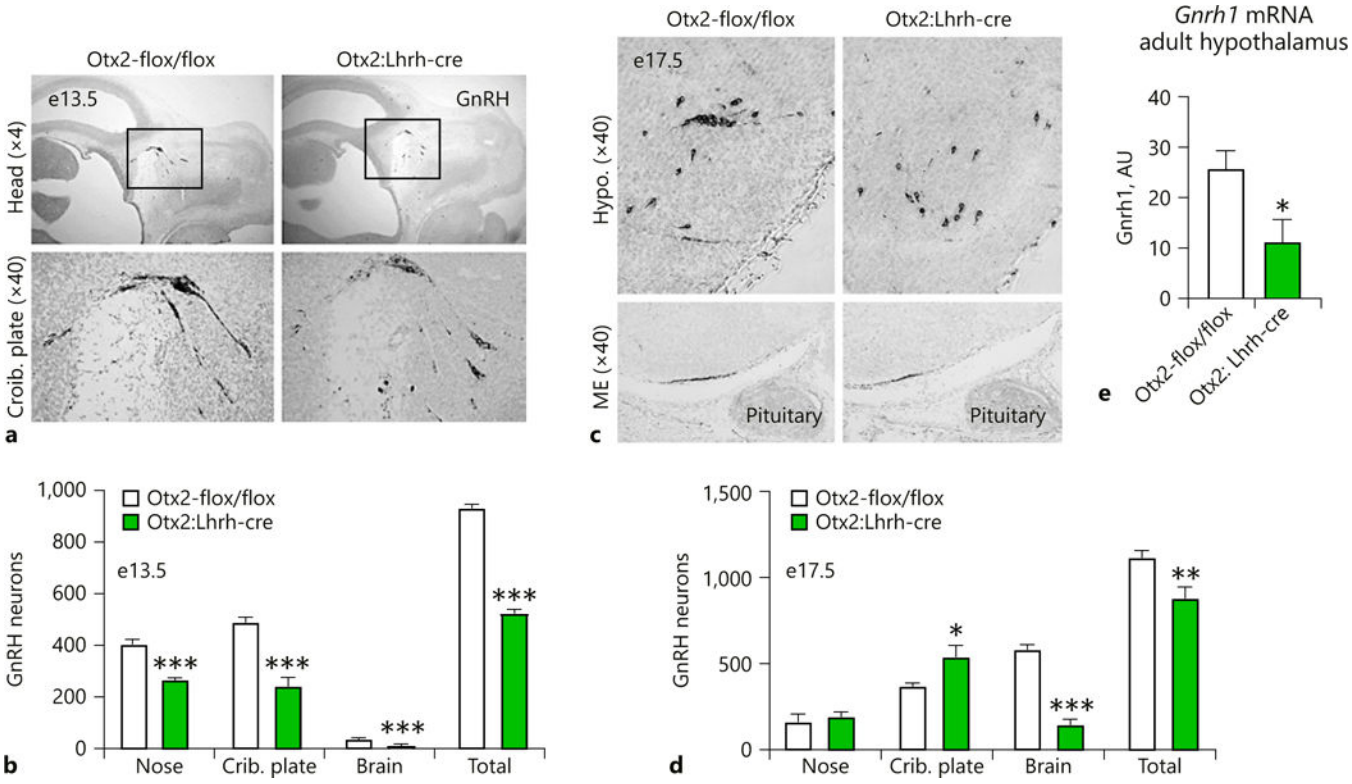

Fig. 5.

Otx2:Lhrh-cre embryos have abnormal GnRH neuron location in the head. Immunohistochemical staining and quantification of GnRH expressing cells on sagittal sections at (**a**, **b**) e13.5 and (**c**, **d**) e17.5. The black boxes in (**a**) indicate the area of the section that has been enlarged below. Crib. plate, cribriform plate; ME, median eminence. Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as compared to control mouse, $n = 3–4$. (**e**) Quantitative RT-PCR analysis of *Gnrh1* in adult male hypothalamus. Student *t* test, * $p < 0.05$, $n = 4$.

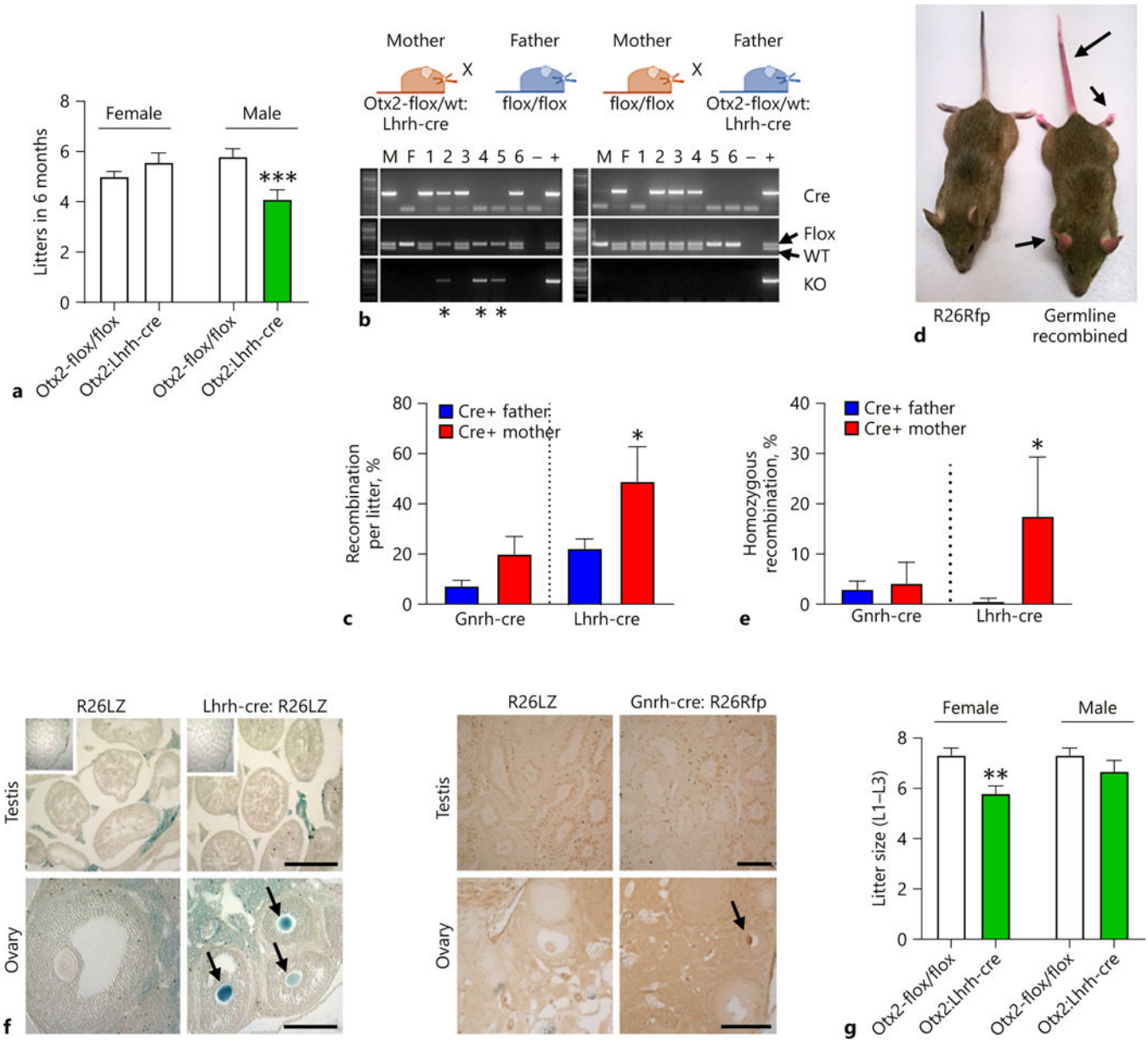

Fig. 6.

Otx2:Lhrh-cre males, but not females, are subfertile. **a** A fertility assay established the number of litters born in 6 months (n = 7–15). **b** Example of PCR showing germline recombination when *Lhrh-cre* is transmitted by the dam (*) in the *Otx2:Lhrh-cre* strain. Due to lethality of pups with homozygous recombination of the *Otx2* allele, we were unable to calculate whether the recombination was mono- or bi-allelic in this mouse strain. To establish the re-combination frequency and whether it was mono- or bi-allelic, we analyzed recombination in *Six6:Lhrh-cre* and *Six6:Gnrh-cre* mice because *Six6-flox* mice with homozygous recombination is viable. Histograms showing (**c**) the average percentage of pups with germ-line recombination of the *Six6-flox* allele when the indicated *Cre*-alleles were transmitted from the father (blue) or mother (red), and (**e**) the percentage of pups with homozygous recombination. n = 3–14 litters. Two-way ANOVA followed by a Tukey's

multiple comparison test. * $p < 0.05$ as compared to opposite sex of the same mouse strain. **d** Picture showing germline recombination when the *Gnrh-cre* was transmitted by the dam in the *Gnrh-cre:Rosa26-Td-Tomato* mouse line (arrows). **f** X-gal staining in *Lhrh-cre:Rosa26-LacZ* (left images, blue staining) and immunohistochemistry to detect the TdTomato protein in *Gnrh-cre:Rosa26-TdTomato* (right images, brown staining) revealed recombination within the female germline (arrows, ovary). The square in the top left corner of the testis shows intact testis morphology (×4). Scale bar 50 μm. **g** Average litter sizes generated of *Otx2-flox/flox* (control) and *Otx2:Lhrh-cre* mice ($n = 40–76$). M, male; F, Female; WT, wild-type allele; KO, knock-out allele; Flox, floxed allele; –, negative control; +, positive control. **a**, **g** Student *t* test compared to control of either sex, ** $p < 0.01$; *** $p < 0.001$.

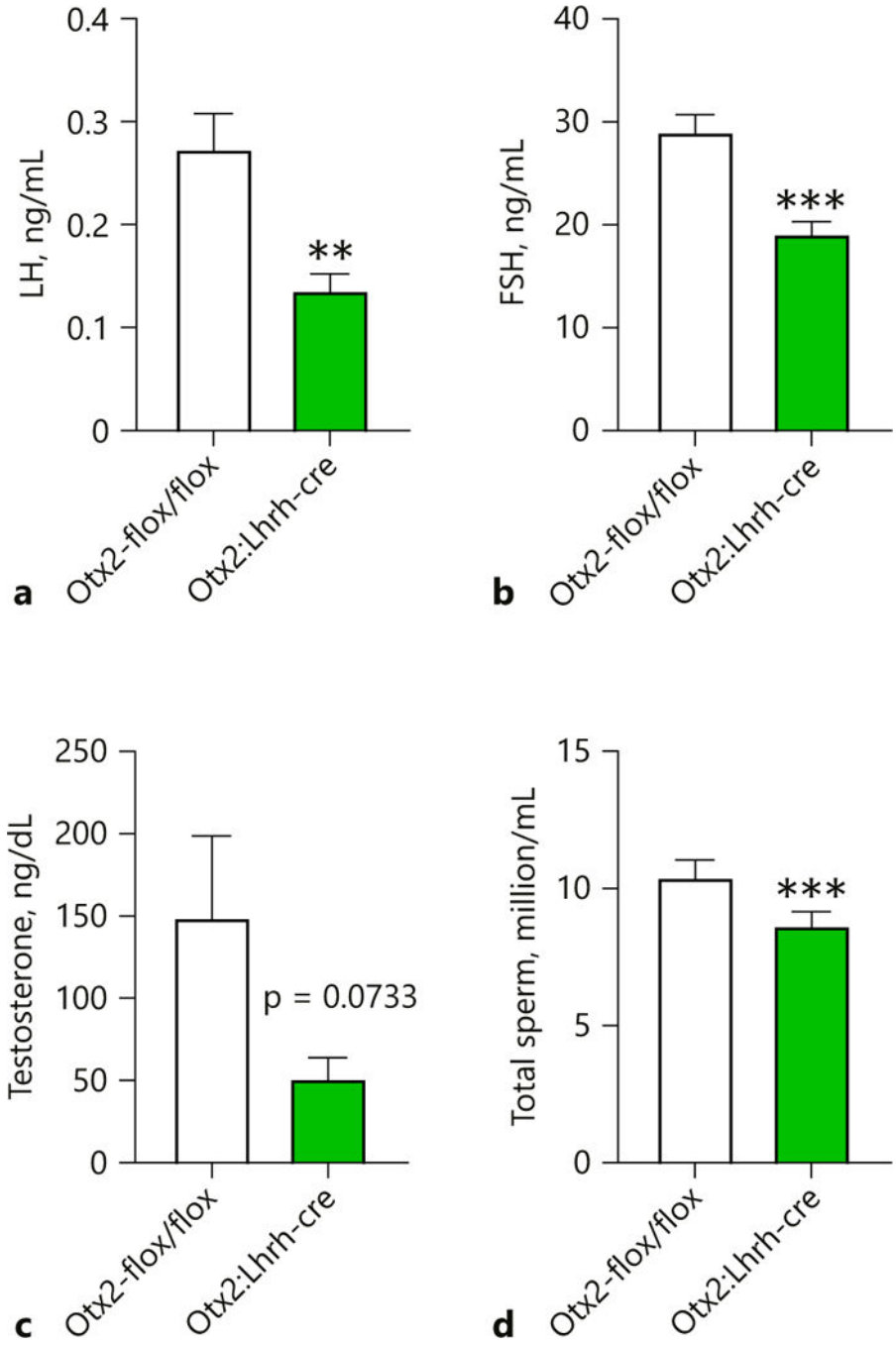

Fig. 7.

Loss of *Otx2* expression results in decreased LH serum levels and fewer sperm. Average serum (**a**) LH and (**b**) FSH levels of 6–8-month-old male mice ($n = 5–7$). **c** Average testosterone level of 6–8-month-old males ($n = 5$). **d** Average number of sperm per epididymis of 6–8-month-old males ($n = 5$). Student *t* test, ** $p < 0.01$, *** $p < 0.01$.