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Initiation and completion of **female reproductive maturation in the absence of kisspeptin/GPR54
signaling**

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ABSTRACT

Puberty onset is initiated in the brain by activating gonadotropin-releasing hormone (GnRH) neurosecretion. Different permissive signals must be integrated for the initiation of reproductive maturation, however the neural circuits controlling timely awakening of the reproductive axis are not understood. The identification of the neuropeptide kisspeptin as a potent activator of GnRH neuronal activity suggests that kisspeptin-releasing neurons might coordinate puberty onset. To test this hypothesis we generated mice specifically lacking kisspeptin [cells](#). Unexpectedly, puberty onset in females was unaffected by kisspeptin neuron ablation. Furthermore, the animals were fertile, albeit had smaller ovaries. Consistent with this, female mice lacking neurons that express the kisspeptin receptor GPR54 were also fertile. Acute ablation of kisspeptin neurons in adult animals inhibited fertility suggesting compensation for the loss of kisspeptin neurons early during development. Our data demonstrate that the initiation and completion of reproductive maturation can take place in the absence of kisspeptin/GPR54 signaling.

Keywords: Puberty, kisspeptin, *Kiss1*, *GPR54*, genetic ablation, diphtheria toxin, Cre recombinase, gene targeting

INTRODUCTION

Puberty -in humans as well as in mice- is a transition period of gonadal and behavioral maturation that occurs between juvenile stages and adulthood¹. Puberty onset is initiated in the central nervous system by an activation of gonadotropin-releasing hormone (GnRH) neurons. Upon activation, GnRH neurons release high-frequency pulses of GnRH neuropeptide into the hypophysial portal vessels; this leads to stimulation of gonadotropin secretion from the anterior pituitary and thus activation of gonadal function¹⁻⁴. How is the GnRH pulse generator turned on? Although it has long been known that different permissive signals such as metabolic, environmental and even social cues are all critical determinants for the initiation of reproductive maturation, little is known about how these inputs are integrated in the brain. Specifically the neural circuits mediating coordinated control of puberty onset and sexual development have not been identified. The identification of kisspeptins, neuropeptides expressed by the *Kiss1* gene, and their receptor, GPR54, has provided an entry towards understanding the physiological mechanisms mediating the timing of puberty⁵⁻⁸. Inactivating mutations of the human *GPR54* gene are linked to hypogonadotropic hypogonadism and an absence of puberty^{9,10}. Knockout of the *Kiss1*^{11,12} or the *GPR54*^{12,13} gene in mice leads to hypogonadism and infertility. Kisspeptin administration triggers increased GnRH secretion and advances puberty onset¹⁴⁻¹⁷. In contrast, administration of GPR54 antagonists delays puberty onset¹⁸. These data raise the possibility that kisspeptin neurons are needed to turn on the GnRH pulse generator, which culminates in awakening of the hypothalamus-pituitary-gonadal (hpg) axis marking the onset of puberty. To test this hypothesis we generated mice specifically lacking [kisspeptin-expressing and –in a complementary genetic approach- GPR54-expressing cells](#) and analyzed the tempo and completeness of their reproductive maturation.

RESULTS

Genetic ablation of kisspeptin neurons

To ablate kisspeptin neurons in mice, we expressed the diphtheria toxin A (DTA) chain specifically in these cells. To do this, we bred kisspeptin-IRES-Cre (KissIC) mice to ROSA26-DT-A (R26-DTA) mice. KissIC mice carry a targeted insertion of an internal ribosome entry site (IRES) followed by a Cre recombinase cDNA downstream of the kisspeptin coding sequence¹⁹. Transcription of the recombinant *KissI* allele yields a bicistronic mRNA from which kisspeptin and Cre recombinase are independently translated. Previous experiments had shown that >97% of kisspeptin neurons display Cre-mediated recombination in these animals¹⁹. The R26-DTA strain carries a transcriptionally silenced *DTA* gene in the *ROSA26* locus²⁰. Transcription of the *R26-DTA* allele is activated by Cre-mediated recombination and results in death of the Cre expressing cells²¹ (**Fig. 1a**). Therefore, Cre expression in KissIC/R26-DTA mice should lead to genetic ablation of kisspeptin neurons.

KissIC/R26-DTA mice were born with Mendelian frequencies and were viable. The body weight of KissIC/R26-DTA females was slightly but significantly increased between postnatal day (P) 31 and P46 compared to R26-DTA (control) littermates (19.6 ± 0.2 g, n=7 compared to 18.3 ± 0.4 g, n = 15 in controls at P40, $P < 0.05$; **Supplementary Fig. 1a**). Immunohistochemical analysis of coronal brain sections from control mice using antibodies against kisspeptin²² revealed kisspeptin immunoreactive perikarya and fibers in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) of the hypothalamus (**Fig. 1b** and **supplementary Fig. 1b**), consistent with the described distribution of kisspeptin neurons in the female mouse brain^{23,24}. In contrast, kisspeptin immunoreactivity was virtually absent in the ARC of P18 and adult KissIC/R26-DTA mice (**Fig. 1b** and **supplementary Fig. 1b**). No kisspeptin IR neurons were detected in the AVPV of P18 KissIC/R26-DTA (n=4) females compared to 10.5 ± 4.5 kisspeptin IR neurons in controls (n = 2). 6.5 ± 3.1 kisspeptin IR neurons were detected in the AVPV of adult KissIC/R26-DTA (n=3) females compared to 239.3 ± 10.99 in controls (n = 4) (**Fig. 1c**), suggesting efficient ablation (> 97%) of kisspeptin neurons in these animals. Consistent with this, *KissI* mRNA levels were reduced by ~95% in P20 and by ~98% in adult KissIC/R26-DTA females (**Fig. 1d**).

Sexual maturation in the absence of kisspeptin neurons

Ovarian mass was strongly reduced in adult KissIC/R26-DTA females (n=12) compared to control (n=14) animals (2.0 ± 0.1 mg versus 5.2 ± 0.4 mg in controls, $P < 0.001$; **Fig. 2a**). This phenotype was already manifest at P20 (1.0 ± 0.1 mg, n=3, in KissIC/R26-DTA females compared to 2.1 ± 0.2 mg in controls, n=3, $P < 0.05$; **Fig. 2a**). Despite reduced ovarian mass, however, ovarian histology showed follicles at all stages of development and the presence of corpora lutea in KissIC/R26-DTA females (**Fig. 2b**), raising the possibility that these animals might be fertile. Strikingly, all tested female KissIC/R26-DTA mice (5 of 5) gave birth to normal sized litters when mated to wild-type (wt) males. These data demonstrate that female mice can become fertile in the absence of kisspeptin neurons. [Taken together, these data indicate that kisspeptin cell ablation leads to smaller sized ovaries that otherwise appear functionally normal.](#)

Kisspeptin secretion is thought to be necessary for timely onset of puberty^{5,6,8,25}. Consistent with this, kisspeptin knockout mice fail to undergo sexual maturation and show delayed onset of puberty^{11,12} while kisspeptin administration in juvenile animals can advance puberty onset^{16,17}. We next asked whether ablation of kisspeptin neurons affects the timing of puberty onset and compared markers of pubertal maturation and serum hormone levels in KissIC/R26-DTA females to those in control mice. Unexpectedly, the day of vaginal opening (VO), an external marker of puberty onset, was similar in KissIC/R26-DTA females (postnatal day (P) 33.0 ± 1.2 d) (n=7) compared to control mice (33.3 ± 0.7 d) (n=18) (**Fig. 2c**). These data demonstrate that kisspeptin neurons are not essential for the timing of puberty onset in female mice. In addition they suggest that increased GnRH neurosecretion leading to awakening of the hpg axis can be initiated in the absence of kisspeptin neurons. We then compared luteinizing hormone (LH) levels in mutant and control animals. Serum LH levels remained low (< 0.2 ng/ml) in both KissIC/R26-DTA (n=7) and control (n=3) mice at P20, and rose to mean levels in adult KissIC/R26-DTA females which were not significantly different to controls (0.45 ± 0.09 ng/ml in KissIC/R26-DTA (n=16) vs. 0.33 ± 0.04 ng/ml in controls (n=25)) (**Fig. 2d**). Subsequent stages of female pubertal maturation are characterized by initiation of ovulatory cyclicity. Daily inspection of vaginal cytology revealed estrous cyclicity in KissIC/R26-DTA females (**Fig. 2e**), despite somewhat prolonged periods of persistent vaginal

cornification. These experiments suggest that kisspeptin neurons are not essential to progress through pubertal maturation and attain ovulatory cyclicality.

Genetic ablation of GPR54 neurons

Based on our results thus far, we reasoned that if kisspeptin neurons are not essential for puberty onset and fertility, then kisspeptin target cells might be dispensable as well. We therefore performed the complementary genetic experiment and generated mice, which lack cells that express the kisspeptin receptor GPR54. First, we used gene targeting in embryonic stem cells to insert an IRES sequence followed by a *Cre recombinase* cDNA downstream of the coding region of the *GPR54* gene (**Fig. 3a–c**). The resulting GPR54-IRES-Cre knock-in (GPIC) mice were viable and showed uncompromised fertility. To monitor Cre expression in GPIC mice, we bred these animals to the enhanced ROSA26- τ GFP (eR26- τ GFP) reporter line²⁶. Hypothalamic sections prepared from female GPIC/eR26- τ GFP mice contained brightly fluorescent cells in the medial preoptic area (MPA; **Fig. 3d**) and fluorescent fiber terminals at the median eminence (not shown). Immunofluorescence analysis on sections through the preoptic area of GPIC/eR26- τ GFP mice using antibodies against GnRH showed that ~99% ($99 \pm 1\%$; n=5) of GnRH neurons of the hypothalamus are labeled by τ GFP fluorescence and ~96% ($96 \pm 3\%$; n=5) of τ GFP+ neurons express GnRH (**Fig. 3d**), demonstrating faithful activation of the τ GFP reporter gene in GnRH/GPR54 neurons. We also found τ GFP+ neurons in other brain areas outside of the hypothalamus such as the hippocampus of female GPIC/eR26- τ GFP mice (not shown), consistent with the reported distribution of GPR54 neurons²⁷.

To systemically ablate GPR54 neurons, we bred GPIC mice to R26-DTA mice. The number of GnRH neurons in the hypothalamus of female GPIC/R26-DTA mice was reduced to $9.3\% \pm 0.6\%$ (n=4) compared to R26-DTA control mice, coincident with a reduction of *GnRH* mRNA to 6.7 % in the mutant animals (**Fig. 3f**). To analyze whether the remaining GnRH neurons express GPR54, we compared coronal sections through the entire brain of triple mutant mice harboring one *KissIC*, one *R26-YFP*²⁸ and one *R26-DTA* allele (KissIC/R26-YFP/R26-DTA mice) with sections from KissIC/R26-YFP animals (**Supplementary Fig. 2a**). Triple mutant mice showed complete absence of YFP+ cells and fibers in presence of the *R26-DTA* allele (**Supplementary Fig. 2a**), demonstrating efficient ablation of all GPR54

neurons. Consistent with this, *GPR54* mRNA levels were reduced below detection threshold in female GPIC/R26-DTA mice (**Fig. 4g**).

Sexual maturation in the absence of GPR54 neurons

GPIC/R26-DTA females displayed significantly reduced ovarian masses ($3.9 \text{ mg} \pm 0.4$ in GPIC/R26-DTA mice ($n=11$) versus $5.2 \text{ mg} \pm 0.4$ in controls ($n=14$)) (**Fig. 4a**). This difference became even more distinct when ovarian masses were corrected for body weight and cycle stage (**Supplementary Fig. 2c**). These data suggest, that the absence of GnRH/GPR54 neurons –and thus kisspeptin/GPR54-signaling- does affect ovarian maturation. Strikingly however, all female GPIC/R26-DTA mice tested had offspring when mated to wt males (8/8 animals). Consistent with this, GPIC/R26-DTA females displayed estrous cyclicity despite slightly prolonged phases of cornification (**Fig. 4b**). These results demonstrate, that reproductive maturation can take place in the absence of GnRH/GPR54 neurons and suggest that kisspeptin/GPR54-signaling is not essential for fertility. Furthermore, these data suggest that -in the absence of kisspeptin/GPR54-signaling- a small number of GnRH neurons suffices to become fertile.

Puberty onset in GPIC/R26-DTA females (VO at $P32.8 \pm 1.6 \text{ d}$, $n=5$) was not found to be significantly different from that in control animals (**Fig. 4c**). LH levels seemed somewhat lower in adult GPIC/R26-DTA females ($0.23 \pm 0.02 \text{ ng/ml}$, $n=15$) than those in controls ($0.33 \pm 0.04 \text{ ng/ml}$, $n=25$), however this difference was insignificant ($P=0.4$). Taken together, these data indicate that -in the absence of kisspeptin/GPR54 signaling- ~10% of the wt GnRH neuron repertoire is sufficient to develop a functional hpg axis.

Induced ablation of kisspeptin and GPR54 neurons

Finally we asked whether uncompromised fertility and normal timing of puberty onset in KissIC/R26-DTA and GPIC/R26-DTA females were due to compensatory mechanisms during development. To address this question, we conditionally ablated kisspeptin and GPR54 neurons, respectively, in adult female mice. Mice were made susceptible to the action of diphtheria toxin (DT) by expressing a human DT receptor (DTR) transgene. We bred KissIC and GPIC mice to the ROSA26-iDTR (R26-iDTR) strain

in which a transcriptionally silenced *DTR* gene is inserted into the *ROSA26* locus and can be activated by Cre-mediated recombination²⁹. KissIC/R26-iDTR and GPIC/R26-iDTR develop normally and progress through pubertal maturation in the absence of DT (not shown). To ablate kisspeptin and GPR54 neurons, we injected 20 weeks old adult female KissIC/R26-iDTR, GPIC/R26-iDTR (**Fig. 5**) and control mice (R26-iDTR littermates not bearing a *Cre* allele) twice intraperitoneally with 50 ng DT per g body weight. One week after the second DT injection, kisspeptin IR was drastically reduced in KissIC/R26-iDTR animals (**Supplementary Fig. 3a**). Importantly, other neuropeptide expressing neuronal populations in the hypothalamus seemed to be unaffected by kisspeptin neuron ablation (**Supplementary Fig. 4a** and **Fig. 5c**). In GPIC/R26-iDTR mice, GnRH IR was strongly reduced after DT administration (**Supplementary Fig. 3b**). Taken together, these data suggest efficient ablation of kisspeptin and GPR54 neurons after DT injection.

We then asked whether kisspeptin or GPR54 neuron ablation would have an acute effect on estrous cyclicity. Strikingly, -in contrast to KissIC/R26-DTA animals- KissIC/R26-iDTR females did not exhibit normal estrous cyclicity after DT administration but instead vaginal lavages collected from these animals only rarely contained cells (**Fig. 5a**). These experiments suggest that adult females cannot compensate for the acute loss of kisspeptin neurons. The importance of kisspeptin neurons for ovulatory cyclicity does not however seem to depend on kisspeptin/GPR54 signaling itself because GPIC/R26-iDTR remained cyclic after DT injections (**Fig. 5b**). Taken together, these data indicate that kisspeptin/GPR54 signaling but not kisspeptin neurons are dispensable for estrous cyclicity in adult mice. After monitoring vaginal cytology for one month, DT-injected KissIC/R26-iDTR and GPIC/R26-iDTR females were subsequently mated to wt males. Whereas none of the KissIC/R26-iDTR females (n=6) had offspring, 50% (3 of 6) of GPIC/R26-iDTR females had offspring after 8 weeks of mating. After 8 weeks of mating, animals were sacrificed and ablation efficiency was investigated. No kisspeptin IR neurons were detected in KissIC/R26-iDTR animals (n=3). In GPIC/R26-iDTR mice, GnRH neuron numbers were reduced by ~93% (**Fig. 5c** and **supplementary Fig. 2c** and **3b**). These data suggest that as little as ~7% of the GnRH neuron repertoire found in wt mice is sufficient to maintain cyclic activity of the hpg axis.

To determine when female mice become sensitive to kisspeptin neuron ablation, we injected KissIC/R26-iDTR animals with DT at P20. Because only few neurons express kisspeptin in the AVPV at P20¹⁹, DT injection at this age allows us to mainly affect the ARC kisspeptin neuronal populations. Starting at P40 we monitored estrous cyclicity in these animals. In contrast to R26-iDTR control littermates, KissIC/R26-iDTR animals did not exhibit normal estrous cyclicity but showed prolonged periods of persistent diestrus after DT injection at P20 (**Fig. 5d**). Consistent with this, none of the KissIC/R26-iDTR females (n=3) injected at P20 bore offspring when mated to wt males for 8 weeks after vaginal cytology inspection. These data suggest, that compensation in KissIC/R26-DTA females occurs before P20 and thus mainly affects the kisspeptin neuronal population in the ARC.

DISCUSSION

To determine whether kisspeptin neurons are needed to turn on the GnRH pulse generator at puberty onset we used a binary genetic strategy and systemically ablated kisspeptin cells in mice. In a complementary genetic approach we also generated animals lacking GPR54-expressing kisspeptin target cells and analyzed their reproductive maturation. Several important findings emerge from this study. (1) Female mice lacking kisspeptin or GPR54 cells have significantly smaller ovaries in comparison to littermate controls, indicating an essential role of kisspeptin/GPR54-signaling in gonadal maturation. (2) Neither kisspeptin nor GPR54 neuron ablation however, does affect the timing of puberty onset in female mice, suggesting that the GnRH pulse generator can be turned on independent of kisspeptin neurons and kisspeptin/GPR54-signaling. (3) Furthermore, these animals are fertile, indicating that kisspeptin neurons and kisspeptin/GPR54-signaling are even dispensable for coarse reproductive development. (4) Fertility despite drastically reduced numbers of GnRH neurons after GPR54 neuron ablation also demonstrates that ~10% of the GnRH neuron repertoire found in wt animals is sufficient for reproductive development. (5) Acute kisspeptin but not GPR54 neuron ablation in adult animals results in acyclicity and infertility, demonstrating an essential role of kisspeptin neurons -but not kisspeptin/GPR54 signaling- in maintaining ovulatory cyclicity in adults. (6) Acute kisspeptin neuron ablation at P20 also results in acyclicity and infertility, suggesting developmental compensation for ARC kisspeptin neurons in KissIC/R26-DTA animals before P20.

We adopted a 'toxin strategy' to specifically ablate kisspeptin and GPR54 cells in mice. With this strategy, any kisspeptin or GPR54 expressing cell in the body would be ablated. Along the hpg axis, kisspeptin and GPR54 expression has been reported in the anterior pituitary gland and in the ovaries^{30,31}. Consistent with this, some τ GFP+ cells were detected in the ovaries of KissIC/eR26- τ GFP, but not of GPIC/eR26- τ GFP animals (**Supplementary Fig. 5a**). Within the ovary, Cre-mediated recombination seemed to be restricted to a small subset of large luteal cells within a few corpora lutea (**Supplementary Fig. 5a**). Ablation of these cells in KissIC/R26-DTA mice did apparently not preclude reproductive maturation and fertility. Cre-mediated recombination was also evident in the anterior pituitary of female KissIC/eR26- τ GFP and

GPIC/R26-YFP mice, respectively, however these fluorescent cells did not express gonadotropins (**Supplementary Fig. 5b**).

Cell ablation is fundamentally different to a knockout, which assesses the function of a particular gene. Instead of removing one particular type of molecule from a cell, the toxin ablation approach eliminates the whole cell itself. In case of neurons, a genetically defined type of neuron is removed from a neural circuit. Toxin-based genetic cell ablation therefore assesses the role of a particular cell in a physiological process and can result in phenotypes different from the complementary gene knockout approaches³²⁻³⁴.

Previous studies had shown that knockout of the *Kiss1* and *GPR54* genes results in delayed puberty onset¹⁰⁻¹³. Furthermore, while some residual reproductive activity has been documented in these animals³⁵, they are infertile. In contrast, KissIC/R26-DTA and GPIC/R26-DTA females showed normal timing of puberty onset, attained ovulatory cyclicity and were fertile. **The knockout and ablation mouse models differ in their genetic background, which might contribute to some of the observed phenotypic differences.** It may seem counterintuitive that the effects of gene deletion be more deleterious than those of neuron ablation, however one possible explanation for uncompromised fertility in the DTA animals could be developmental compensation. Other neurons upstream of GnRH neurons³⁶ might functionally take over in response to cell ablation in DTA but not in knockout mice (**Fig. 6c**). Our data suggest that developmental compensation is occurring only when kisspeptin cells are ablated early in development (i.e. before P20). Kisspeptin cell ablation in adulthood after normal development seems to preclude activation or formation of these alternative reproductive circuits. **Only few neurons express kisspeptin in the AVPV before P20¹⁹, suggesting that developmental compensation mainly affects the kisspeptin neuronal population in the ARC.** Interestingly, developmental compensation has also been demonstrated for other neuronal populations in the ARC³²⁻³⁴.

Although we currently do not have insight into the molecular mechanisms underlying compensation in the DTA animals, one possibility might be that kisspeptin neurons co-express some other neuropeptide or neurotransmitter, which normally inhibits the development of other compensatory reproductive neural

circuits. While kisspeptin neuron ablation would remove this factor and thus lead to the development of the normally-quiescent compensatory circuits, *Kiss1* knockout mice would still have the inhibitory factor suppressing compensatory pathways from development. Consistent with this, most kisspeptin neurons express GABA³⁷, the major inhibitory neurotransmitter in the brain. Removal of the inhibitory factor might lead to compensatory upregulation of other neuropeptides known to modulate reproductive function. Dynorphin and neurokinin B are neuropeptides that are co-expressed in kisspeptin neurons but also expressed by other neurons in the hypothalamus³⁸. However, we found *dynorphin* and *neurokinin B* mRNA levels reduced by ~80% in KissIC/R26-DTA but not in GPIC/R26-DTA females compared to control animals (Supplementary Fig. 4b), suggesting that these two neuropeptides are not upregulated in response to kisspeptin neuron ablation. The strong reduction of *dynorphin* mRNA levels in KissIC/R26-DTA animals is surprising as dynorphin is widely expressed in the hypothalamus and will be addressed in future studies.

An alternative explanation for the more deleterious phenotype in the knockout mice might be that dysregulation of kisspeptin or GPR54/GnRH neurons within a neural circuit is more detrimental to reproductive function than complete removal of the neuron, which in principle allows for compensation. Potential dysregulation of kisspeptin neurons by removing either kisspeptin or estrogen receptor α (ER α) from these cells, can have opposite effects on puberty onset in female mice. While a knockout of ER α in kisspeptin neurons results in advanced puberty onset due to precocious awakening of the hpg axis¹⁹, knockout of kisspeptin leads to delayed onset of puberty^{11,12}. Despite these opposite effects, dysregulation of kisspeptin neurons may preclude complete pubertal maturation and thus result in infertility in both animal models. While kisspeptin neurons in the knockout mice might still release other neurotransmitters³⁷ potentially disturbing downstream neurons in the absence of kisspeptin or ER α signaling, these would be removed together with the kisspeptin neuron in the DTA animals. In GPR54 knockout mice, the GnRH neuronal network might be dysregulated due to absence of the GPR54 receptor on the cell surface. In contrast, the remaining GnRH neurons in GPIC/R26-DTA females (which do not express GPR54) are not dysregulated and are apparently sufficient for reproductive development. Consistent with this hypothesis, previous studies demonstrated substantial redundancy in the GnRH neuronal population^{39,40}. Acute

GPR54 neuron ablation in adults does lead to prolonged phases of cornification, consistent with previous studies demonstrating direct effects of kisspeptin/GPR54 signaling on GnRH/LH secretion^{14,41-43}. However, acute ablation of GPR54 neurons in adults does not inhibit ovulatory cyclicity and fertility, demonstrating that kisspeptin/GPR54 signaling is not essential for reproductive function in adult females.

One potential caveat that we must consider is the following: could it be that due to the binary genetic strategy used to ablate kisspeptin neurons (i.e. DTA expression is indirectly coupled to the kisspeptin promoter via Cre-mediated recombination) a small amount of kisspeptin is produced and secreted while the neurons are dying and that this is sufficient to activate GnRH neurosecretion? Our data suggest that this is unlikely the explanation for the reproductive phenotype of KissIC/R26-DTA animals. The initial activation of the GnRH pulse generator is most likely mediated by the arcuate nucleus population of kisspeptin neurons, because of the differential onset of kisspeptin expression in the [AVPV](#) and [ARC](#)¹⁹. In the majority of ARC kisspeptin neurons, Cre-mediated recombination is already evident at birth, i.e. several weeks before vaginal opening. Virtually no kisspeptin IR was detected in the ARC of P18 KissIC/R26-DTA females, suggesting efficient ablation of kisspeptin neurons at this timepoint.

The consequences of puberty, such as the defense of territory or mate, pregnancy and care of young, are energetically expensive¹. For this reason, the timing of puberty is critical. Our data show that puberty onset is precisely timed in absence of kisspeptin/GPR54 signaling. If the GnRH pulse generator can be activated in the absence of kisspeptin and GPR54 neurons, what is the physiological function of kisspeptin neurons and GPR54 receptors on GnRH neurons? Based on comparison of the different mouse strains presented in this study with the complementary knockout mice, we propose that kisspeptin neurons integrate various cues and relay this information to GnRH neurons via kisspeptin/GPR54-signaling to optimize reproductive success in wt mice (**Supplementary Fig. 6**). Kisspeptin/GPR54-signaling however is neither essential to activate GnRH neurosecretion at puberty onset nor to become fertile.

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AUTHOR CONTRIBUTIONS

C.M. and U.B. designed the experiments. C.M. carried out most of the experiments. C.M. and U.B. analyzed the data. U.B. conceived the project and wrote the paper.

FIGURE LEGENDS

Figure 1 Targeted ablation of kisspeptin neurons. **(a)** Genetic strategy to activate DTA expression specifically in kisspeptin cells. KissIC mice carry a recombinant *Kiss1* allele from which a bicistronic mRNA is transcribed. R26-DTA mice carry a transcriptionally silenced *DTA* gene, which is activated upon Cre-mediated recombination in KissIC/R26-DTA mice. **(b)** Immunohistochemical analysis of coronal brain sections from P18 (left) and adult (right) KissIC/R26-DTA (bottom) and KissIC control mice (top) using antibodies against kisspeptin. Kisspeptin immunoreactivity (IR) was found in the AVPV (top) and ARC (bottom) of the hypothalamus in control mice. Note the dramatically reduced kisspeptin IR in KissIC/R26-DTA mice, suggesting efficient ablation of kisspeptin neurons in both AVPV and ARC. Scale bar, 200 μ m. **(c)** No kisspeptin IR neurons were detected in the AVPV of P18 KissIC/R26-DTA (n=4) females compared to 10.5 ± 4.5 kisspeptin IR neurons in controls (n = 2). 6.5 ± 3.1 kisspeptin IR neurons were detected in the AVPV of adult KissIC/R26-DTA (n=3) females compared to 239.3 ± 10.99 in controls (n = 4). **(d)** *Kiss1* mRNA levels were reduced by ~95% in P20 KissIC/R26-DTA (n=2) females compared to control (n=8) animals and by ~98% in adult KissIC/R26-DTA (n=8) females compared to controls (n=4). Error bars represent s.e.m.

Figure 2 Sexual maturation in female mice lacking kisspeptin neurons. **(a)** Ovarian mass of female KissIC/R26-DTA mice was strongly reduced at P20 and in adults. *P < 0.05, **P < 0.001 **(b)** Representative ovary section from a KissIC/R26-DTA mouse showed follicles at all stages of development. Scale bars, 500 μ m. **(c)** Day of vaginal opening (VO) was not significantly different in female KissIC/R26-DTA mice compared to control animals, suggesting normal timing of puberty onset in the absence of kisspeptin neurons. **(d)** Mean serum LH levels were not significantly different in P20 and adult KissIC/R26-DTA females compared to controls. ns, not significant. **(e)** Vaginal cytology of KissIC/R26-DTA mice (right) showed all stages of estrous cyclicity despite somewhat prolonged periods of vaginal cornification compared to R26-DTA control animals (left). C, cornified (estrous); N, nucleated (proestrous); L, leukocytic (metestrous and diestrous); CL, corpora lutea.

Figure 3 Genetic ablation of GPR54 neurons. **(a)** Targeting strategy to express Cre recombinase under control of the *GPR54* promoter. The inserted cassette is composed of an internal ribosomal entry site (IRES) followed by the coding sequence for Cre recombinase (Cre), and a *phosphoglycerate kinase* promoter driven neomycin resistance flanked by Flp recombinase recognition sites (FRT). **(b)** Southern blot analysis of *SacI*-digested ES-cell DNA. The expected fragment sizes are indicated (wt, 8.8 kb; mutant, 7.5 kb). Clone 2 carries the recombinant *GPR54-IC* allele (*GPIC^{neo+}*). Uncropped blot images are shown in **Supplementary Fig. 7**. **(c)** Southern blot analysis of *SacI*-digested DNA from a homozygous mutant mouse after neomycin selection cassette removal. The expected fragment size (*GPIC^{neo-}*) is 10.5 kb. **(d)** Faithful activation of a Cre-dependent *YFP* reporter gene in GnRH neurons. Immunofluorescence analysis of sections through the medial preoptic area (MPA) of the hypothalamus prepared from *GPIC/R26-YFP* mice using antibodies against GnRH. All GnRH neurons (red) express YFP (green). **(e)** Efficient ablation of GnRH neurons in *GPIC/R26-DTA* mice. Immunofluorescence analysis of MPA sections from *GPIC/R26-DTA* and *GPIC* control mice using antibodies against GnRH. **(f)** The number of GnRH neurons (top) was reduced by ~90% in *GPIC/R26-DTA* (n=6) but not in *KissIC/R26-DTA* (n=3) mice compared to *R26-DTA* control animals (n=4). *GnRH* mRNA levels (bottom) were reduced by ~93% in *GPIC/R26-DTA* mice (n=3) compared to *KissIC/R26-DTA* (n=4) and control (n=3) animals. **(g)** *GPR54* mRNA levels were below detection threshold in *GPIC/R26-DTA* females (n=2) compared to controls (n=5). Error bars represent s.e.m.

Figure 4 Unaltered timing of puberty onset in female mice lacking GPR54 neurons. **(a)** Genetic ablation of GPR54 neurons significantly reduced ovarian mass ($P < 0.05$). **(b)** Vaginal cytology of *GPIC/R26-DTA* mice showed all stages of estrous cyclicity despite somewhat prolonged periods of vaginal cornification. **(c)**. GPR54 neuron ablation did not affect the day of vaginal opening. ns, not significant **(d)** Mean serum LH levels in *GPIC/R26-DTA* females were not significantly different compared to levels in controls. **(e)** Representative ovarian section showed follicles at all stages of maturation and corpora lutea (CL) in *GPIC/R26-DTA* animals. Scale bar, 500 μ m.

Figure 5 Acyclicity after DT-mediated ablation of kisspeptin neurons in adult KissIC/R26-iDTR mice. **(a)** Genetic strategy to conditionally ablate kisspeptin neurons via DT administration. KissIC/R26-iDTR animals did not exhibit normal estrous cyclicity but showed prolonged periods of persistent diestrous or estrous after DT injection. On days without dots, no cells were detected in the vaginal lavage. **(b)** In contrast, vaginal cytology of GPIC/R26-iDTR mice showed all stages of estrous cyclicity after DT injection. **(c)** GnRH neuron numbers were reduced by ~97% in GPIC/R26-iDTR mice (n=3) compared to R26-iDTR control mice (n=3) after DT injection. In contrast, GnRH neuron numbers were unchanged after conditional ablation of kisspeptin neurons via DT administration in KissIC/R26-iDTR mice (n=4). ***P < 0.0001. **(d)** Vaginal cytology of P40 R26-iDTR mice (left) showed all stages of estrous cyclicity after DT injection at P20. In contrast, P40 KissIC/R26-iDTR animals (right) did not exhibit normal estrous cyclicity but showed prolonged periods of persistent diestrous after DT injection at P20. C, cornified (estrous); N, nucleated (proestrous); L, leukocytic (metestrous and diestrous). Error bars represent s.e.m.

ONLINE METHODS

Generation of GPR54-IRES-Cre (GPIC) mice

4 kb of genomic DNA containing exon 5 of the *GPR54* gene (Fig. 3a) were PCR-amplified from bacterial artificial chromosome (BAC) RP23-11H21 DNA (Wellcome Trust Sanger Institute) and subcloned into the pKO-V901 plasmid (Lexicon Genetics) containing a *phosphoglycerate kinase* (*pgk*) promoter-driven diphtheria toxin A cassette. Accuracy of PCR-products was confirmed by sequencing. A unique *AscI* site was created 3' to the stop codon of the *GPR54* gene to insert an IRES-Cre-FRT-NEO-FRT⁴⁴ cassette containing a *pgk* promoter-driven neomycin resistance cassette flanked by FRT sites. The targeting construct was electroporated into IDG3.2 mouse embryonic stem cells (kindly provided by Dr. Ralf Kühn, GSF Munich, Germany) and resistant clones were analyzed by Southern blotting using *SacI* and an external 800 bp probe (Fig. 3a). Correctly targeted ES cells were injected into C57BL/6J blastocysts to generate chimeras that were backcrossed to C57BL/6J animals to give *GPR54*^{neo+} mice (Fig. 3b). For genotyping, genomic DNA from tail biopsies was prepared by proteinase K (Roche) digestion and subsequent purification with DNA Isolation Reagent for genomic DNA (AppliChem).

Mice

Animal care and experimental procedures were performed in accordance with the guidelines established by the animal welfare committee of the University of Hamburg. Mice were kept under standard light/dark cycle with food and water *ad libitum*. To remove the FRT flanked neomycin resistance cassette, *GPR54*^{neo+} animals were bred to Flp recombinase deleter mice⁴⁵ to result in *GPR54*^{neo-} (GPIC) mice. Flp mediated excision of the selection cassette was verified by Southern blot analysis (Fig. 3c). Both GPIC and KissIC mice were kept in a mixed (129/SvJ and C57BL/6J) background. R26-YFP²⁸, eR26- τ GFP²⁶, R26-DTA²⁰, and R26-iDTR²⁹ (kindly provided by Dr. Ari Waisman, University of Mainz, Germany) mice were kept in a mixed (129/SvJ and C57BL/6) background. All KissIC/R26-DTA, GPIC/R26-DTA, KissIC/R26-iDTR, GPIC/R26-iDTR animals analyzed in this study were heterozygous for the respective *Cre* and *R26* alleles. Littermates carrying one *KissIC*, *GPIC*, *R26-DTA*, or *R26-iDTR* allele, respectively, were used as controls.

Immunofluorescence

Mice were perfused transcardially with 4% paraformaldehyde (fixative) under ketamin/xylazine (Bayer) anaesthesia. Tissues were removed and soaked in fixative for 2h, in 30% sucrose for 48h, and then frozen in tissue freezing medium OCT (Leica) and cut into 14 μm sections with a cryostat³⁶. Coronal brain sections were blocked in 1X PBS, 0.025% TX-100, 5% horse serum, and then treated either with rabbit anti-GnRH (1:1000; Affinity Bioreagents) or with rabbit anti-kisspeptin (1:500; Millipore) antiserum overnight at 4°C, followed by Cy3-donkey anti-rabbit IgG (1:500; Jackson Labs) for 1h at room temperature. Pituitary sections were stained with rabbit anti-LH β (1:2000) and rabbit anti-FSH β (1:1000; (both from National Institute of Diabetes and Digestive and Kidney Diseases, NIDDK), followed by Cy3-donkey anti-rabbit IgG. For nuclear staining, sections were incubated with 5 $\mu\text{g}/\text{ml}$ Hoechst 33258 dye (Sigma) in 0.1 M PBS for 10 min and mounted with Fluoromount-G (Southern Biotech). Appropriate controls omitting primary antibodies were performed and did not yield any staining.

Ovarian histology

For ovarian histology, anesthetized mice were perfused with PBS, killed and ovaries were dissected. 14 μm cryosections were fixed in 4% paraformaldehyde for 10 min at room temperature, stained with hematoxylin and eosin, dehydrated and mounted with DPX mounting medium (Sigma).

Immunohistochemistry

For immunohistochemistry mice were perfused and 14 μm brain sections were prepared as described above. After blocking and quenching using peroxidase, sections were incubated over night at 4°C with rabbit anti-kisspeptin 10 antiserum at 1:5000 (a kind gift from A. Caraty, Tours, France), followed by biotinylated anti-rabbit antibodies (Vectorlabs) at 1:1000 for 2h at room temperature. Sections were then treated according to manufacturer's instructions with components of the ABC and DAB (Vectorlabs) kits. Sections were dehydrated and coverslipped with DPX mounting medium (Sigma).

Quantitation of kisspeptin and GnRH neurons

Neurons were manually counted in every fifth section extending from 0.4 mm anterior to bregma to 0.7 posterior to bregma (kisspeptin) and from 0.76 mm anterior to bregma to 0.5 posterior to bregma (GnRH)⁴⁶.

qPCR

To extract RNA from different brain areas, mice were perfused with ice-cold ACSF (125 mM NaCl, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 0.05 mM CaCl₂, 6 mM MgCl₂, 2.5 mM glucose, 50 mM sucrose) under ketamin/xylazine (Bayer) anaesthesia and decapitated. The brain was quickly removed and coronal slices were cut on a Leica VT 1200S vibratom. The hypothalamic area between bregma 0.8 and -2.0 was punched out and RNA was extracted using the RNeasy mini kit (Qiagen). For cDNA synthesis, superscript II (Invitrogen) was used following the manufacturer's instructions. mRNA expression was analyzed using quantitative RT-PCR (qPCR). cDNA was amplified using TaqMan Universal PCR Master Mix with TaqMan Assay-on-Demand kits for GnRH (GnRH1: Mm01315605_m1), kisspeptin (Kiss1: Mm03058560_m1), GPR54 (Kiss1r: Mm00475048_g1), neurokinin B (Tac2: Mm01160362_m1), dynorphin (Pdyn: Mm00457573_m1) and Gusb to measure reference gene levels (Gusb: Mm00446956_m1) (all from Applied Biosystems). Each reaction was run in triplicate. qPCR was performed on an ABI Prism 7700 sequence detector (Applied Biosystems). mRNA levels were calculated by a comparative method $\Delta(\Delta Ct)$, where $\Delta Ct = Ct(\text{gene of interest}) - Ct(\text{Gusb})$ and $\Delta(\Delta Ct) = \Delta Ct(\text{mutant}) - \text{mean } \Delta Ct(\text{control})$. Results are expressed as PCR-cycle differences in relative gene expression with respect to control.

DT injections

Adult (20 weeks old) and P20 KissIC/R26-iDTR, GPIC/R26-iDTR and control (R26-iDTR littermates) mice were injected intraperitoneally twice with 50 ng diphtheria toxin (Calbiochem; dissolved in H₂O) per g body weight on day one and three of the experiment. Vaginal lavages from adult animals were analyzed starting one week after the first DT injection to ensure efficient ablation. Vaginal lavages from animals injected at P20 were analyzed starting at P40. After monitoring vaginal cytology for one month, DT-

injected KissIC/R26-iDTR and GPIC/R26-iDTR females were subsequently mated to wt males. After 8 weeks of mating, animals were sacrificed and ablation efficiency was investigated.

Vaginal Cytology

To examine estrous cyclicity, vaginal lavages from adult females were collected on glass slides and viewed under a microscope daily (1000–1100 h) for 25–30 d. A normal estrous cycle was defined as exhibiting vaginal cytology that was leukocytic for 2 d followed by 1 d of nucleated and 1–2 d of cornified vaginal cytology.

Statistical methods

All data are presented as the mean \pm s.e.m. One-way analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test was used to determine statistical significance for ovarian mass in adult KissIC/R26-DTA, GPIC/R26-DTA and control mice. Two-tailed unpaired student's T-tests were used to determine statistical significance in the remaining experiments. Differences were considered significant when $P < 0.05$.

Hormonal assays

Animals were anesthetized with ketamine/xylazine i.p., and blood was withdrawn by cardiac puncture (0900–1100 h). Serum was assayed for LH using RIA reagents obtained from the National Institute of Diabetes and Digestive and Kidney Diseases, including the LH reference (RP-3) and S-11 antibody. The assay had a lower limit of detection of 0.2 ng/ml. The intraassay and interassay coefficients of variance (CVs) were 4.1 and 7.9 %, respectively.

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1 Increased body weight in female KissIC/R26-DTA mice. **(a)** Increased body weight between P31 and P46 in female KissIC/R26-DTA (red), but not in GPIC/R26-DTA (green) mice compared to control animals (black). $P < 0.05$ **(b)** Immunofluorescence analysis of coronal brain sections from KissIC/R26-DTA (right) and KissIC control mice (left) using antibodies against kisspeptin. Kisspeptin immunoreactivity (red) was found in the AVPV (top) and ARC (bottom) of the hypothalamus in control mice. Note the dramatically reduced kisspeptin IR in KissIC/R26-DTA mice, suggesting efficient ablation of kisspeptin neurons in both AVPV and ARC. Scale bars, 250 μm . Nuclei were stained with Hoechst 33258 (blue).

Supplementary figure 2 Targeted ablation of GPR54 neurons. **(a)** Breeding strategy to visualize complete ablation of GPR54 neurons (left). Coronal sections (right) through the preoptic area of the hypothalamus prepared from triple mutant (KissIC/R26-YFP/R26-DTA) mice (bottom) and double mutant (KissIC/R26-YFP) mice (top). Note the complete absence of YFP fluorescence in the triple mutant mice, demonstrating efficient ablation of GPR54 neurons in the presence of the *R26-DTA* allele. Scale bars, 100 μm . **(b)** GnRH neuron numbers in different mouse lines. GnRH neurons were dramatically reduced in GPIC/R26-DTA (16.5 ± 1.1 , $n=4$) and GPIC/R26-iDTR (11.0 ± 1.7 , $n=3$) but not in KissIC/R26-DTA (169.3 ± 6.6 , $n=3$) and KissIC/R26-iDTR (169.2 ± 4.8 , $n=5$) females compared to GPIC/eR26- τGFP (176.3 ± 1.2 , $n=3$), R26-DTA (176.8 ± 4.6 , $n=4$) and R26-iDTR controls (164.0 ± 5.6 , $n=3$). GnRH neurons were manually counted in every fifth section extending from 0.76 mm anterior to bregma to 0.5 posterior to bregma. **(c)** Ovarian masses in KissIC/R26-DTA, GPIC/R26-DTA and control animals corrected for body weight and cycle stage (diestrous). $**P < 0.001$, $***P < 0.0001$.

Supplementary figure 3 Efficient depletion of GPR54 and kisspeptin neurons after DT injection. Sections through the medial preoptic area (left), AVPV (middle) and ARC (right) of the hypothalamus prepared from GPIC/R26-iDTR and KissIC/R26-iDTR animals stained with antibodies against GnRH **(b)**

and kisspeptin **(a)**. Note the massively reduced IR (bottom) compared to R26-iDTR control mice (top), suggesting efficient depletion of GPR54 and kisspeptin neurons, respectively. Scale bars, 100 μ m.

Supplementary figure 4 Genetic ablation of kisspeptin neurons does not affect other neuropeptide expressing neuronal populations in the arcuate nucleus of the hypothalamus. **(a)** Immunofluorescence analysis (red) of sections through the arcuate nucleus using antibodies against neuropeptide Y (NPY, left) and cocaine- and amphetamine- regulated transcript (CART, right) shows a normal distribution of CART and NPY neurons in KissIC/R26-DTA and KissIC/R26-iDTR mice compared to R26-DTA and R26-iDTR control animals. Scale bar, 200 μ m. *Dynorphin* (*Pdyn*) **(b)** and *neurokinin B* (*Tac2*) **(c)** mRNA levels were reduced by ~80% in KissIC/R26-DTA (n=4) but not in GPIC/R26-DTA (n=2) females compared to control (n=8) animals.

Supplementary figure 5 Kisspeptin and GPR54 expression in the anterior pituitary gland and in the ovaries. **(a)** τ GFP⁺ cells (green) were detected in the ovaries of KissIC/eR26- τ GFP, but not GPIC/eR26- τ GFP animals. **(b)** Immunofluorescence for LH β and FSH β (red) in pituitary sections from female KissIC/eR26- τ GFP and GPIC/eR26-YFP mice. Only few YFP⁺ cells (arrowhead) were detected in the pituitary of GPIC/R26-YFP mice. None of the τ GFP/YFP⁺ cells (green) expressed gonadotropins. Nuclei were stained with Hoechst 33258 (blue). Scale bars, 100 μ m.

Supplementary figure 6 Proposed neural circuits underlying central control of sexual maturation and reproduction in female mice. **(a)** Kisspeptin neurons integrate various cues and relay this information to GnRH neurons via kisspeptin/GPR54-signaling to optimize reproductive success in wt mice. **(b)** The kisspeptin neuronal population is dysregulated in *Kiss1* knockout ($K^{-/-}$) mice, thereby delaying puberty onset and precluding completion of reproductive maturation. **(c)** In KissIC/R26-DTA (K-DTA) mice, developmental compensation rescues reproductive development. **(d)** Kisspeptin cell ablation in adulthood in KissIC/R26-iDTR (K-iDTR) mice precludes formation of compensatory circuits during development. **(e)** In the absence of kisspeptin/GPR54 signaling, ~10% of the wt GnRH neuron repertoire is sufficient to

develop a functional hpg axis in GPIC/R26-DTA (G-DTA) and GPIC/R26-iDTR (G-iDTR) mice. ov, ovaries; pit, anterior pituitary.

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Corresponding Author:	Ulrich Boehm

Supplementary Item & Number	Title or Caption
Supplementary Figure 1	Increased body weight in female KissIC/R26-DTA mice.
Supplementary Figure 2	Targeted ablation of GPR54 neurons.
Supplementary Figure 3	Efficient depletion of GPR54 and kisspeptin neurons after DT injection.
Supplementary Figure 4	Genetic ablation of kisspeptin neurons does not affect other neuropeptide expressing neuronal populations in the arcuate nucleus of the hypothalamus.
Supplementary Figure 5	Kisspeptin and GPR54 expression in the anterior pituitary gland and in the ovaries.
Supplementary Figure 6	Proposed neural circuits underlying central control of sexual maturation and reproduction in female mice.
Supplementary Figure 7	Uncropped images of blots.

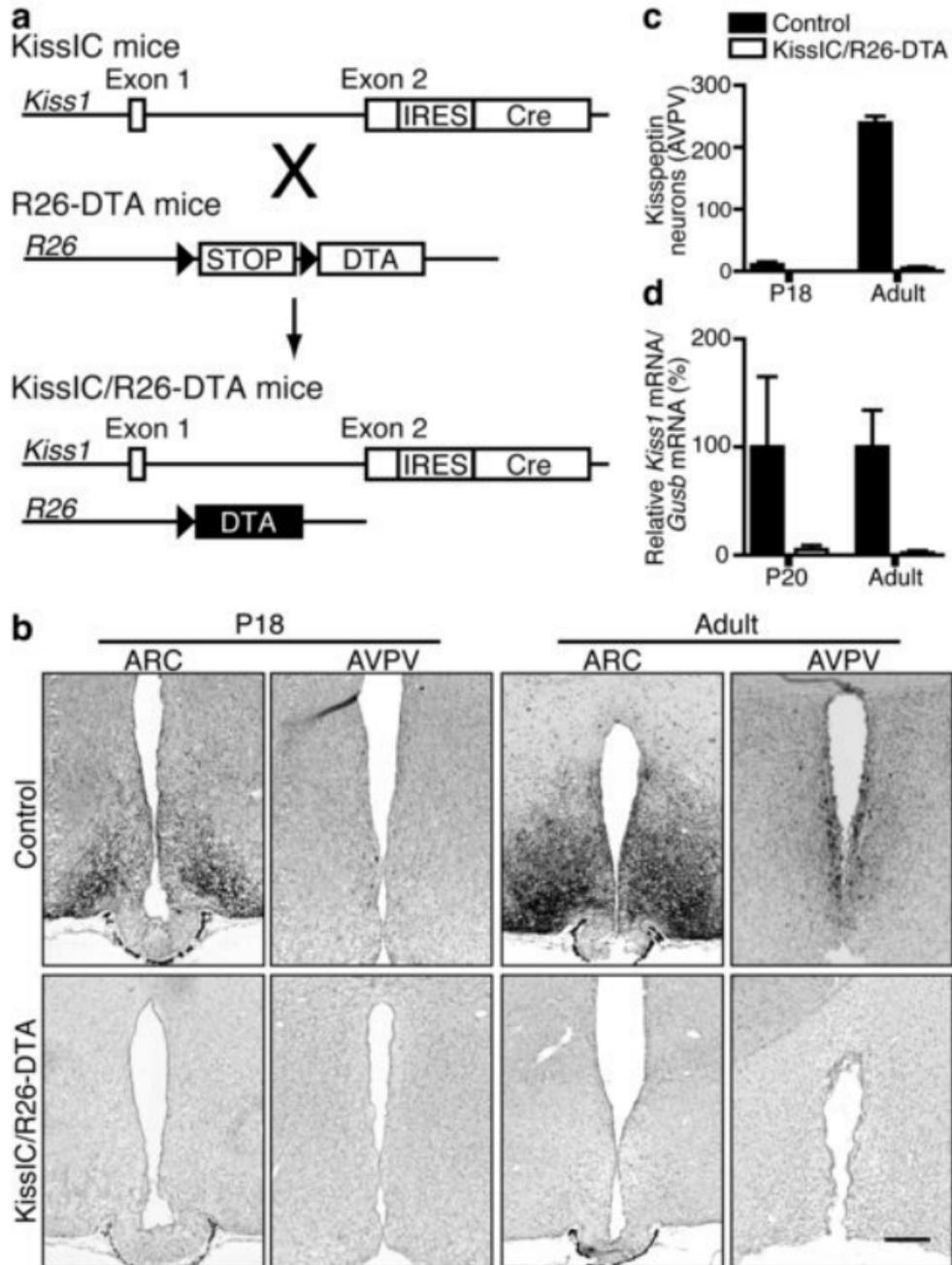


Figure-1 (Boehm)

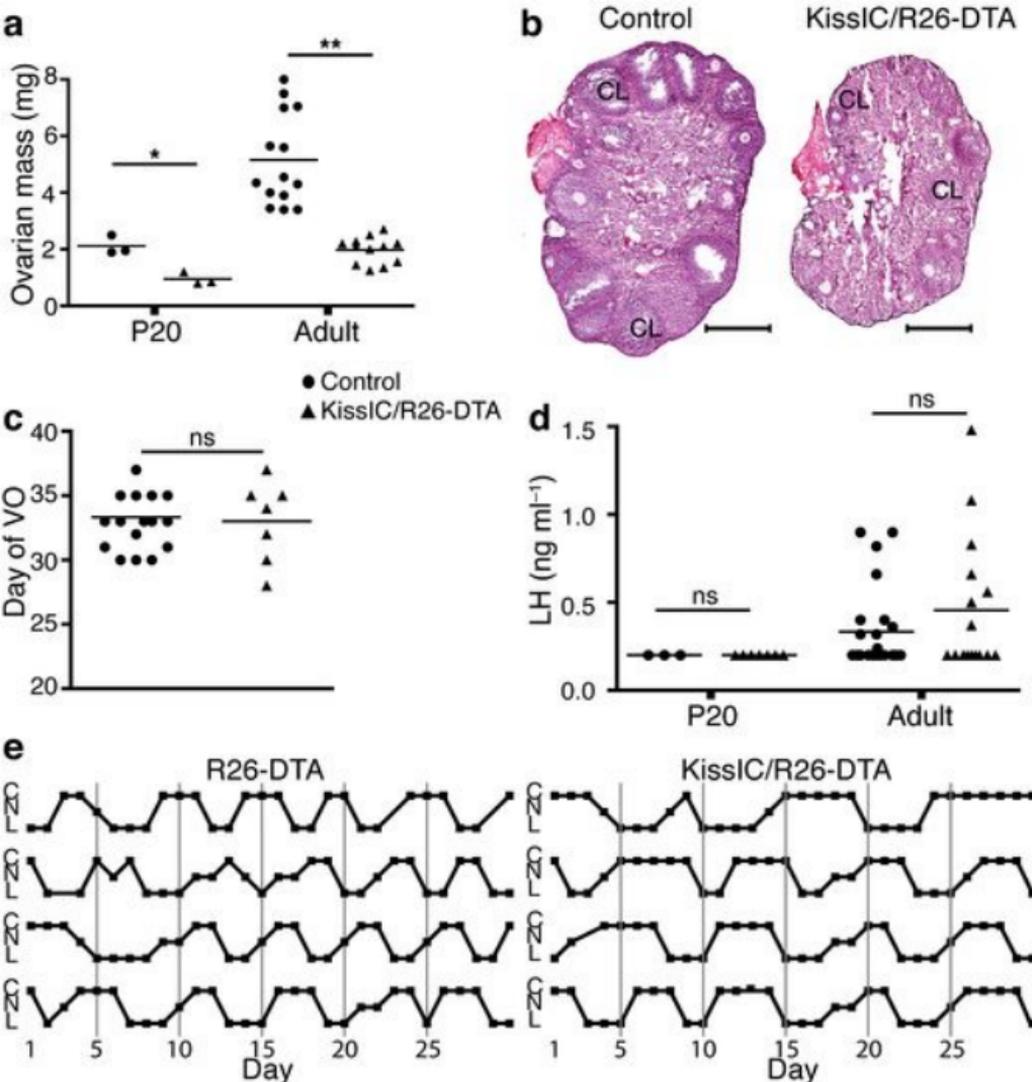


Figure-2 (Boehm)

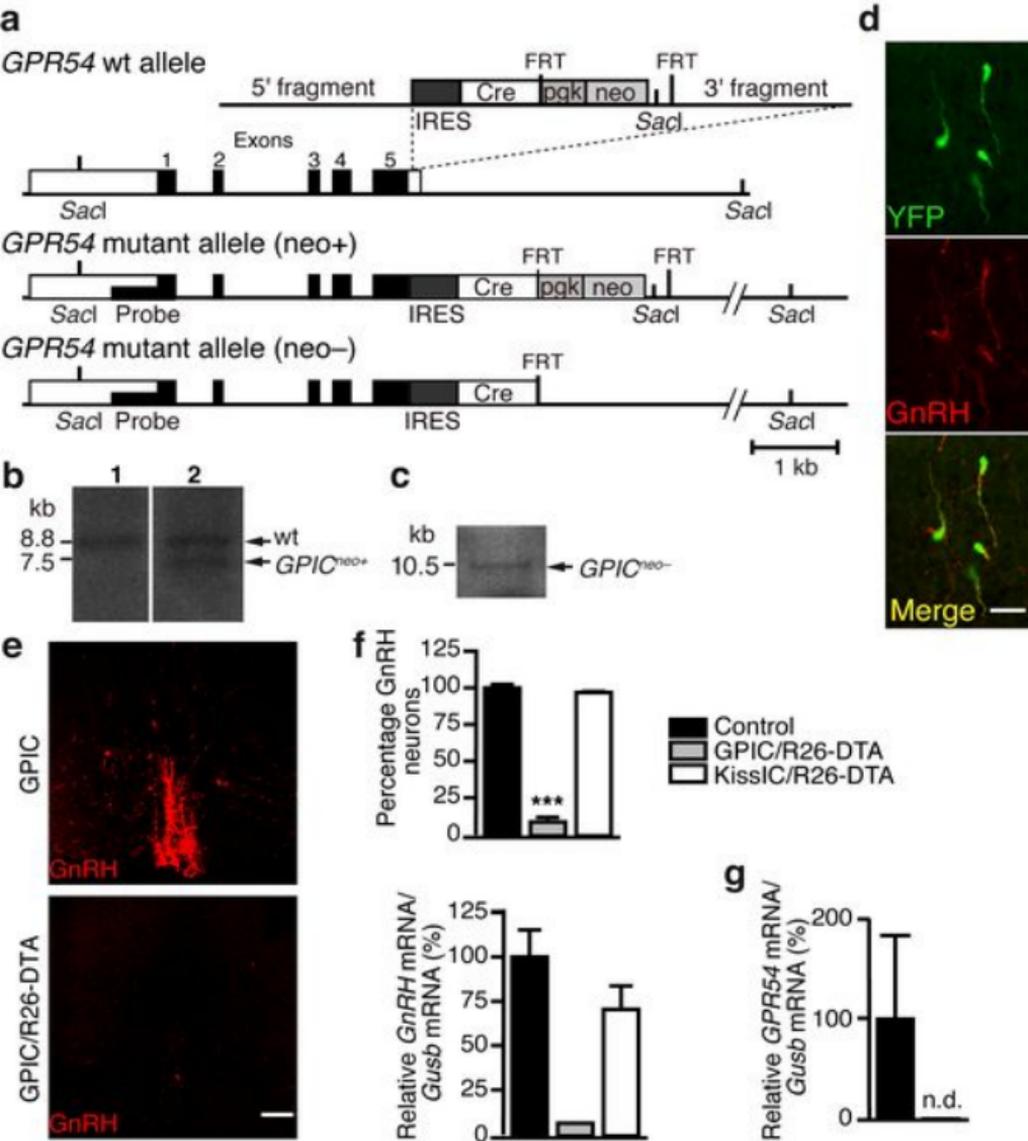


Figure-3 (Boehm)

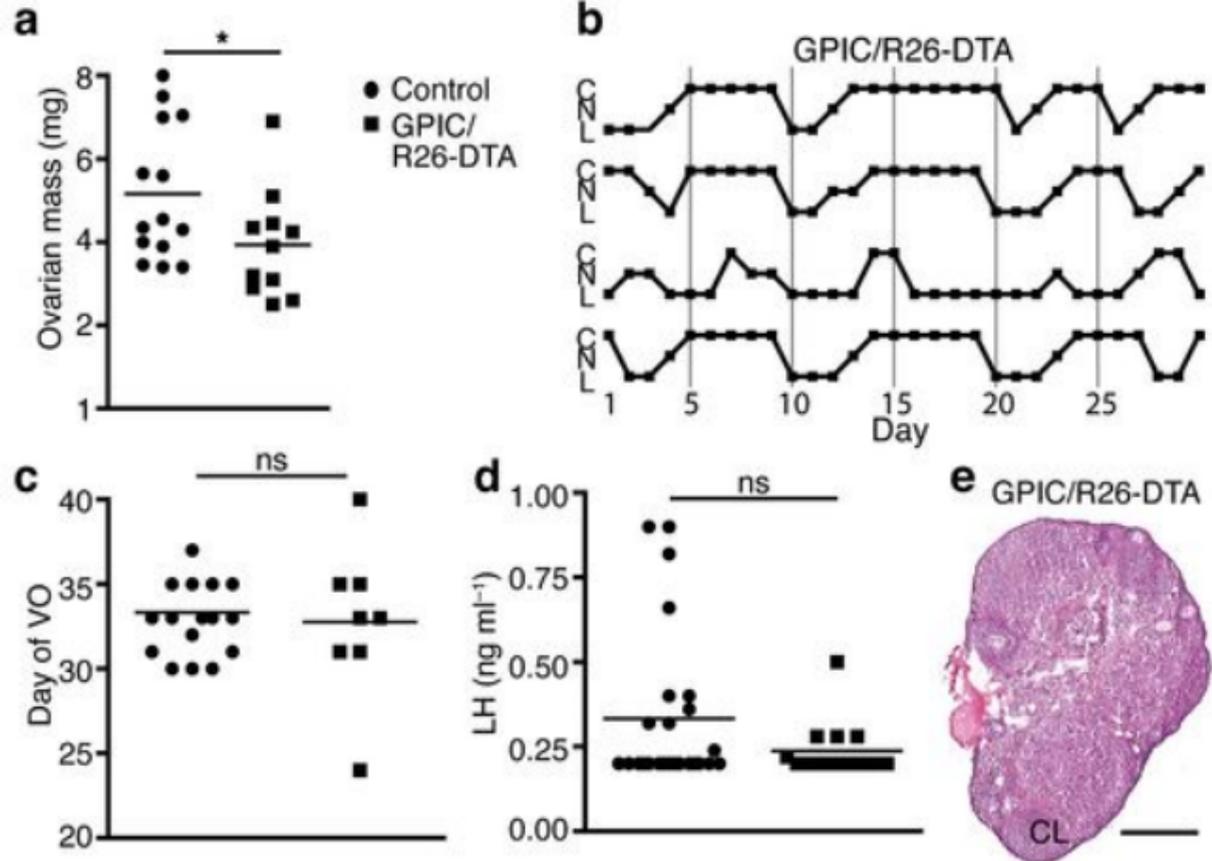


Figure-4 (Boehm)

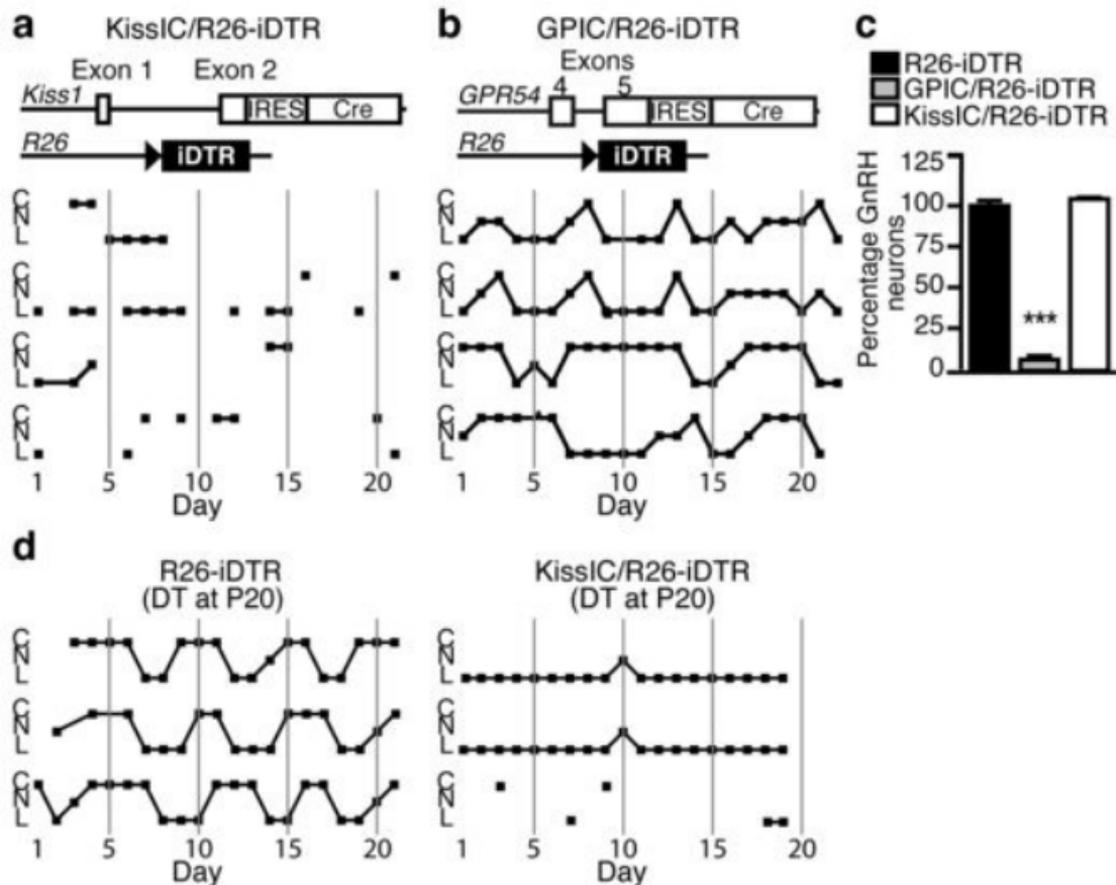


Figure-5 (Boehm)