

Supplementary Information for

**Conditional *Oprk1*-dependent *Kiss1* deletion in kisspeptin neurons caused estrogen-dependent LH pulse disruption and LH surge attenuation in female rats**

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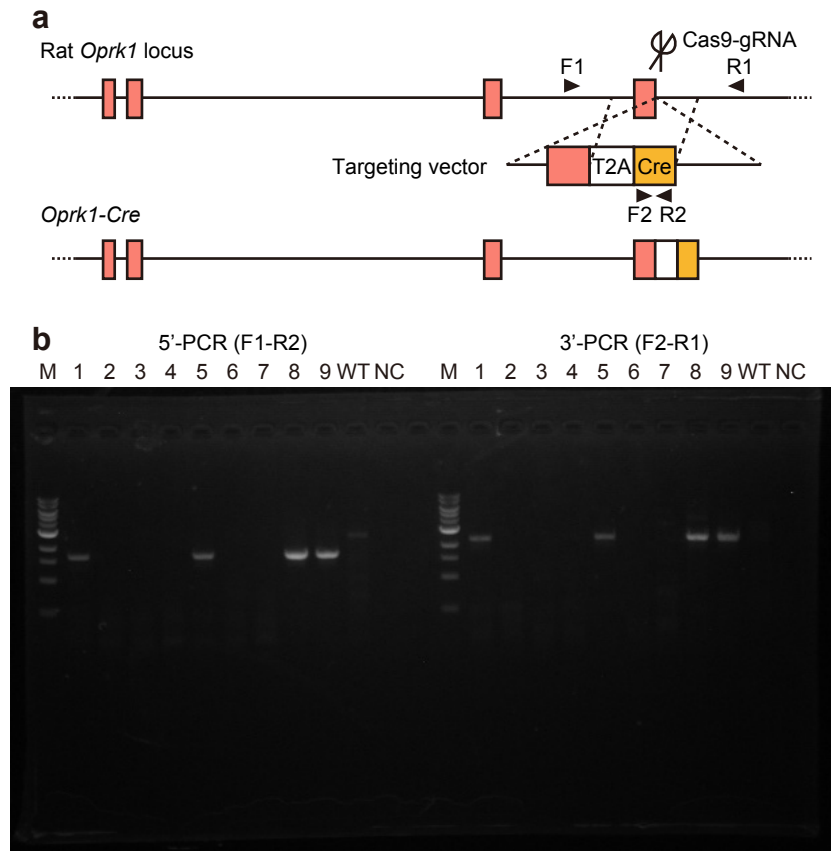
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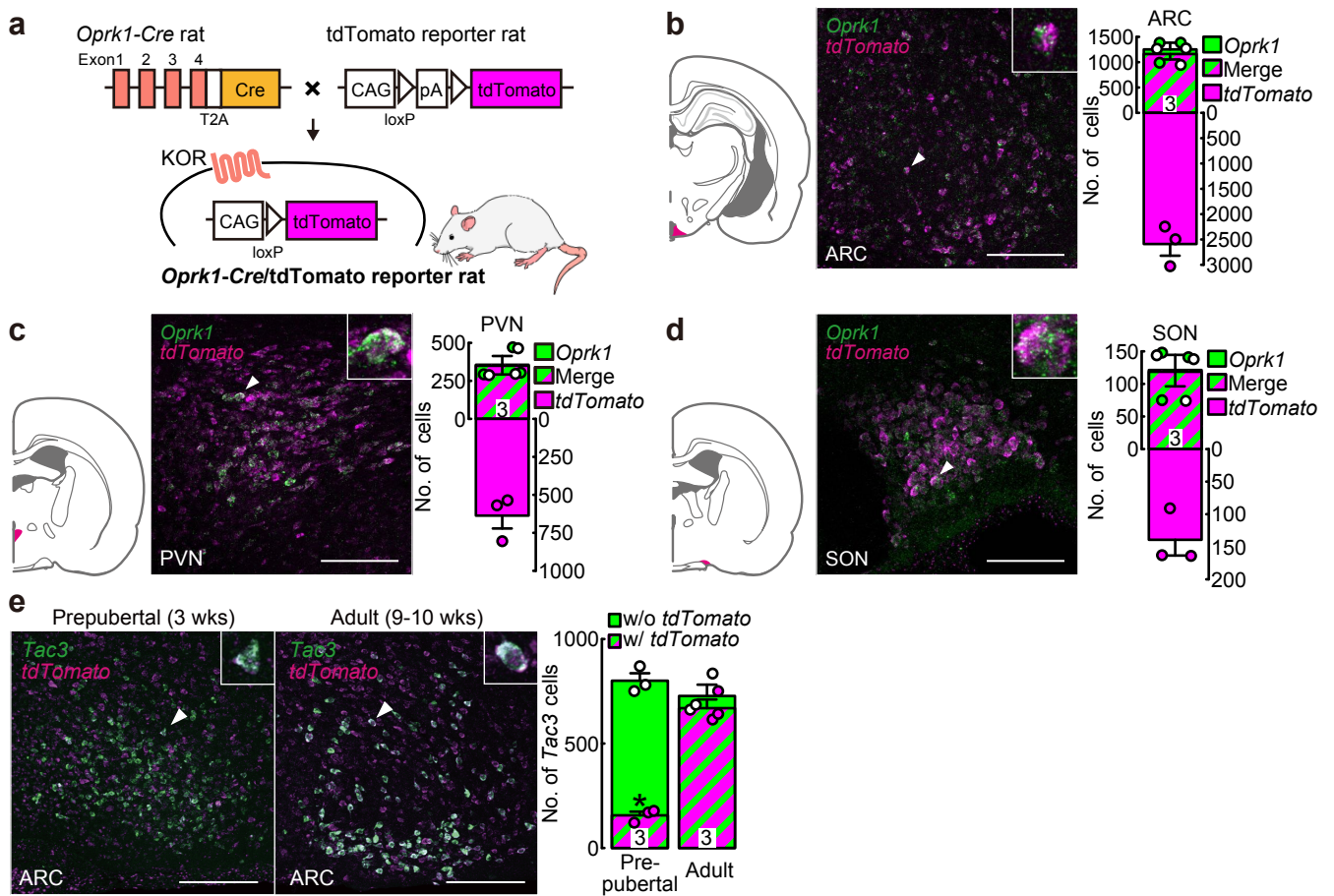
**Table S1.** Primer sequences for the genotyping of *Oprk1-Cre*, *Kiss1-Cre*, and *Kiss1*-floxed rats, screening of *Oprk1-Cre* founder rats, and for the reverse transcription-quantitative PCR (RT-qPCR) analysis for *Kiss1* and *Actb*.

| Purpose    | Targets                                   | Sequence                    |
|------------|-------------------------------------------|-----------------------------|
|            |                                           | forward<br>reverse          |
| Genotyping | <i>Oprk1-Cre</i> rats                     | 5'-gatgtcattgaatgctccttg-3' |
|            |                                           | 5'-ttggctactggcatcatctg-3'  |
|            | <i>Kiss1-Cre</i> rats                     | 5'-ccttggttggggttattcct-3'  |
|            |                                           | 5'-ttgcccctgtttcactatcc-3'  |
|            | <i>Kiss1</i> -floxed rats                 | 5'-tcctgcctgaccttaccac-3'   |
|            |                                           | 5'-agtaccgatttggcaccag-3'   |
| Screening  | <i>Oprk1-Cre</i> founder rats<br>(5'-PCR) | 5'-atgtcataccaccacccag-3'   |
|            |                                           | 5'-ccgccgcataaccagtgaac-3'  |
|            | <i>Oprk1-Cre</i> founder rats<br>(3'-PCR) | 5'-cagcaacatttggccagcta-3'  |
|            |                                           | 5'-ggcctccatctccagaaagt-3'  |
| RT-qPCR    | <i>Kiss1</i> (NM_181692.1)                | 5'-agctgctgcttctcctctgt-3'  |
|            |                                           | 5'-aggcttgctctctgcatacc-3'  |
|            | <i>Actb</i> (NM_031144.3)                 | 5'-tgtcaccaactgggacgata-3'  |
|            |                                           | 5'-gggggtgtgaaggctcaaa-3'   |
|            | <i>Gnrhr</i> (NM_031038.3)                | 5'-ccagcctcatgatggtggt-3'   |
|            |                                           | 5'-gggatgatgaacaggcagct-3'  |
|            | <i>Lhb</i> (NM_012858.2, NM_001033975.1)  | 5'-atgagttctgccagctctgc-3'  |
|            |                                           | 5'-tggggaaggtcacaggtcat-3'  |
|            | <i>Fshb</i> (NM_001007597.2)              | 5'-agctgttgacttacctggcc-3'  |
|            |                                           | 5'-gggtgttggtctagctggg-3'   |

Gene symbols and protein names are as follows: *Actb*,  $\beta$ -actin; *Fshb*, FSH  $\beta$ -subunit; *Gnrhr*, GnRH receptor; *Kiss1*, kisspeptin; *Lhb*, LH  $\beta$ -subunit; and *Oprk1*,  $\kappa$ -opioid receptor.



**Figure S1.** Targeting of the *Oprk1* locus for the generation of *Oprk1-Cre* rats. **(a)** Structure of the wild-type *Oprk1* allele (top), the *Oprk1-Cre* targeting vector (middle), and the targeted *Oprk1* allele (bottom), resulting from replacement recombination at the dotted lines by CRISPR/Cas9 system. **(b)** Screening of founder rats by PCR using primers (solid arrowheads in the panel **a**) located outside the 5' or 3' end of targeting vector and in the *Cre* gene. Primer sets of 5'-PCR (F1 and R2 primers) and 3'-PCR (F2 and R1 primers) generate 1,712 and 2,343 bp products, respectively. The sequences of the primers and guide RNA are shown in Table S1 and main text, respectively. M, DNA marker; WT, wild-type; NC, negative control.



**Figure S2.** Colocalization of *Oprk1* and *Oprk1-Cre*-activated *tdTomato* mRNA expression in the hypothalamic nuclei, such as the arcuate nucleus (ARC), paraventricular nucleus (PVN), and supraoptic nucleus (SON) and pubertal changes in *Oprk1* expression in KNDy neurons in *Oprk1-Cre/tdTomato* reporter rats. **(a)** Schematic illustration of crossing *Oprk1-Cre* rats with *tdTomato* reporter rats to obtain *Oprk1-Cre/tdTomato* reporter rats. Schematic illustrations of the brain section showing the ARC **(b)**, PVN **(c)**, and SON **(d)** highlighted in magenta (left). *Oprk1*-expressing (green) and *Oprk1-Cre*-activated *tdTomato*-expressing (magenta) cells in the aforementioned nuclei of representative a diestrous level of estradiol-17 $\beta$  (E2)-treated ovariectomized (OVX + low E2) *Oprk1-Cre/tdTomato* reporter rats (middle). The insets indicate *Oprk1*- and *tdTomato*-coexpressing cells pointed by the solid white arrowheads at higher magnification. Note that pseudocolors were exchanged between *tdTomato* (detected by TSA Plus Fluorescein System) and *Oprk1* (detected by TSA Plus Biotin Kit and DyLight

594-conjugated streptavidin). The numbers of *Oprk1*-expressing (green), *Oprk1-Cre*-activated *tdTomato*-expressing (magenta), or *Oprk1*- and *tdTomato*-coexpressing (striped) cells in OVX + low E2 *Oprk1-Cre/tdTomato* reporter rats (right). Circles indicate the individual data of the number of *Oprk1*-expressing (green), *tdTomato*-expressing (magenta), or *Oprk1*- and *tdTomato*-coexpressing (white) cells. (e) *Tac3*-expressing (green) and *Oprk1-Cre*-activated *tdTomato*-expressing (magenta) cells in the ARC of representative intact prepubertal (left) and OVX + low E2 adult *Oprk1-Cre/tdTomato* reporter rats (middle). The insets indicate *Tac3*- and *tdTomato*-coexpressing cells pointed by the solid white arrowheads at higher magnification. Scale bars, 100  $\mu$ m. The numbers of *Tac3*-expressing (green) or *Tac3*- and *tdTomato*-coexpressing (striped) cells in the ARC (right). Values are the means  $\pm$  SEM. Circles indicate the individual data of the number of *Tac3*-expressing (white) or *Tac3*- and *tdTomato*-coexpressing (magenta) cells. Numbers in each column indicate the number of animals used. An asterisk indicates statistically significant differences ( $p < 0.05$ ) between the groups based on Student's *t*-test.