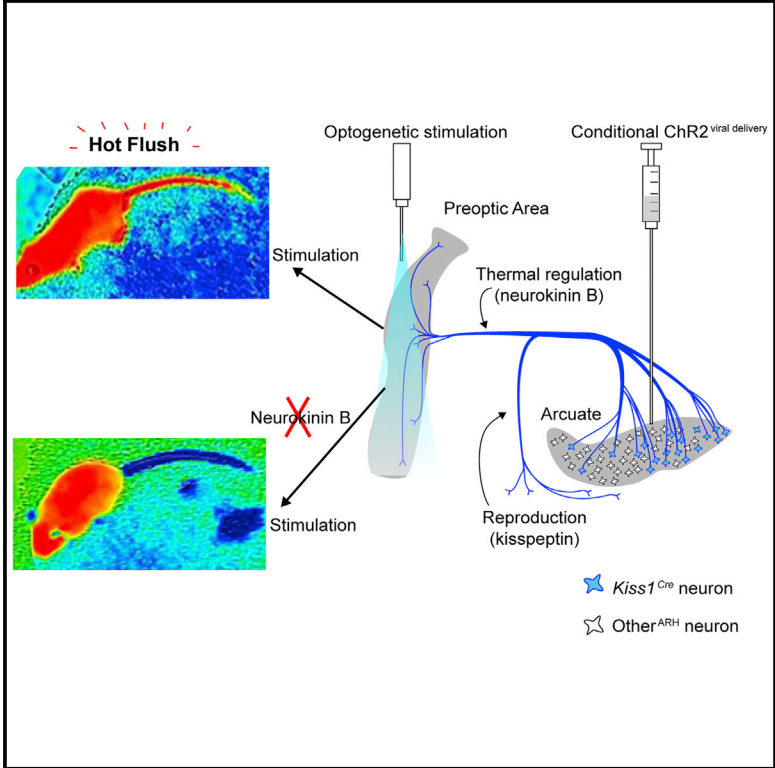


Cell Reports

A Neural Circuit Underlying the Generation of Hot Flashes

Graphical Abstract



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In Brief

The underlying cause of hot flushes is poorly understood. Padilla et al. provide evidence that a subpopulation of hypothalamic neurons can generate hot flush symptoms in mice. Establishing the mechanism of hot flush generation may allow for the development of therapies.

Highlights

- Activation of Kiss1^{ARH} neurons evokes a hot flush-like response in mice
- Ovariectomy sensitizes the hot flush response of Kiss1^{ARH} neurons
- Kiss1^{ARH} transmission to the preoptic area (POA) is sufficient to evoke a hot flush
- Neurokinin B signaling in the POA is required for a hot flush



A Neural Circuit Underlying the Generation of Hot Flashes

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SUMMARY

Hot flushes are a sudden feeling of warmth commonly associated with the decline of gonadal hormones at menopause. Neurons in the arcuate nucleus of the hypothalamus that express kisspeptin and neurokinin B (Kiss1^{ARH} neurons) are candidates for mediating hot flushes because they are negatively regulated by sex hormones. We used a combination of genetic and viral technologies in mice to demonstrate that artificial activation of Kiss1^{ARH} neurons evokes a heat-dissipation response resulting in vasodilation (flushing) and a corresponding reduction of core-body temperature in both females and males. This response is sensitized by ovariectomy. Brief activation of Kiss1^{ARH} axon terminals in the preoptic area of the hypothalamus recapitulates this response, while pharmacological blockade of neurokinin B (NkB) receptors in the same brain region abolishes it. We conclude that transient activation of Kiss1^{ARH} neurons following sex-hormone withdrawal contributes to the occurrence of hot flushes via NkB release in the rostral preoptic area.

INTRODUCTION

Hot flushes, also described in the literature as hot flashes or menopausal vasomotor symptoms, are a periodic and often overwhelming sensation of heat, sweating, and flushing that affect millions of individuals (Freeman and Sherif, 2007). Hot-flush symptoms negatively impact quality of life and are the predominant reason women seek medical intervention during menopause (Hess et al., 2012). Hot flushes are generally thought to be an inappropriate recruitment of thermoregulatory heat-effector responses to a perceived warmth; namely, an acute episode of vasodilation and a resultant drop in core body temperature as heat is lost to the environment through convection. Decades of research into mammalian thermoregulation has provided a map of brain regions involved in the maintenance of core body temperature, with the preoptic area of the hypothalamus (POA) emerging as the central hub of integration and control

(Morrison and Nakamura, 2011). The median POA receives environmental temperature information from peripheral sensory neurons via the parabrachial nucleus, and it is sufficient to modulate vasomotor responses such as those underlying hot flushes (Morrison and Nakamura, 2011; Nakamura and Morrison, 2010). However, the mechanism by which sex-hormone status is relayed to POA has not been elucidated.

Hot flushes are often coincident with a burst of luteinizing hormone (LH) release, which peaks immediately following an acute rise in skin temperature (Casper et al., 1979). When circulating estrogen is low, the pulsatile activity of GnRH neurons (expressing gonadotrophin regulatory hormone) and corresponding release of LH is thought to be determined by kisspeptin (Kiss1)-expressing neurons in the arcuate nucleus of the hypothalamus (Kiss1^{ARH}) (Clarkson et al., 2017; Navarro et al., 2009; Qiu et al., 2016). A growing body of evidence from Rance and colleagues (Rance et al., 2013) suggests that Kiss1^{ARH} neurons may be a link between sex hormone fluctuations and hot flushes. Importantly, Kiss1^{ARH} neurons project some of their axons to the median POA, and they co-express neurokinin B (NkB) (Goodman et al., 2007; Krajewski et al., 2010; Navarro et al., 2009; Yeo and Herbison, 2011). Systemic injection of an NkB receptor agonist (senktide), or microinfusion of senktide directly into the POA, is sufficient to evoke hypothermia in rodents (Dacks et al., 2011; Krull et al., 2017), and pharmacological blockade of NkB receptors markedly reduces hot-flush frequency in menopausal women (Prague et al., 2017).

Kiss1^{ARH} neurons are negatively regulated by estrogen as part of a normal feedback mechanism that is necessary to sustain fertility (Goubillon et al., 2000; Han et al., 2015; Mittelman-Smith et al., 2016; Smith et al., 2005). The drop in estrogen levels during menopause leads to the hypertrophy of Kiss1^{ARH} neurons and significant increases in Kiss1 and NkB expression levels in humans (Rance and Young, 1991; Rometo et al., 2007; Sheehan and Kovács, 1966) or following ovariectomy in mice (Smith et al., 2005), rats (Roa et al., 2006), sheep (Goodman et al., 2007), and primates (Rometo et al., 2007). Altered activity of Kiss1^{ARH} neurons during states of estrogen withdrawal has, therefore, been proposed as a mechanism that predisposes women to hot-flush generation, specifically via disrupted communication to the median POA (Rance et al., 2013). While this is an attractive hypothesis, hot flushes are an episodic



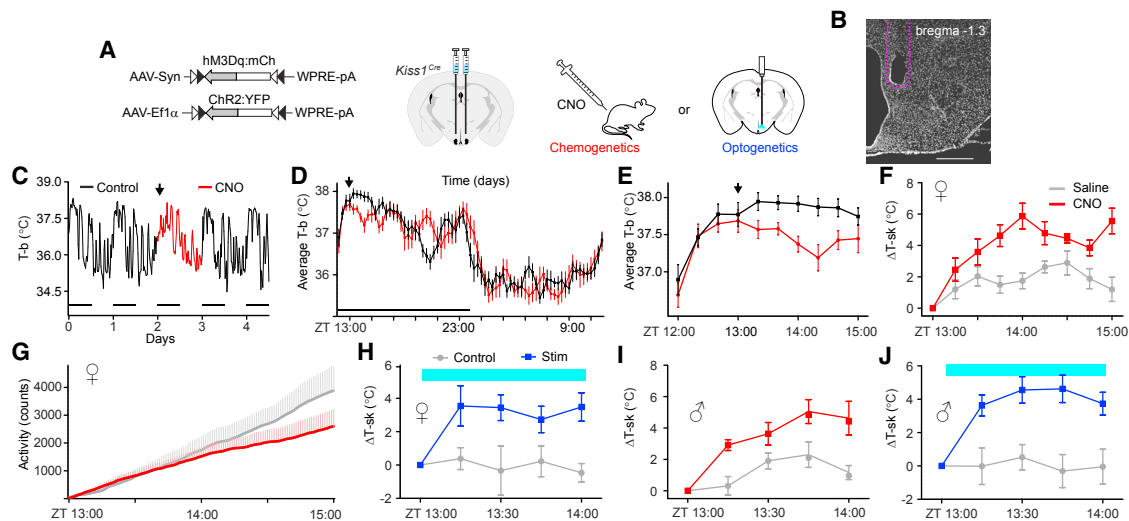


Figure 1. Artificial Activation of *Kiss1*^{ARH} Neurons Is Sufficient to Drive Heat Dissipation in Both Female and Male Mice

(A) Schematic representation of the viral vectors and injection targets. A unilateral fiber-optic ferrule was implanted above the ARH.
 (B) DAPI staining of a coronal brain section indicates the fiber-optic terminal in the ARH. Scale bar, 500 μ m.
 (C–E) Telemetry recordings of core body temperature (T-b) show that *Kiss1*^{ARH} neuron stimulation (CNO, 1 mg/kg) was sufficient to transiently decrease T-b.
 (C) 5-day T-b recording of a single mouse, with CNO injected on day 2 at ZT = 13:00 (arrow). The animals were housed on a 12-hr:12-hr light:dark cycle (horizontal bars); zeitgeber time (ZT) = 12:00 refers to the onset of the dark.
 (D) Average T-b comparing baseline versus CNO treatment on a scale of 24 hr.
 (E) Average T-b comparing baseline vs. CNO-treatment on a scale of 2 hr; $n = 9$; $F(1, 144) = 7.50$, $p = 0.0146$.
 (F) Thermal imaging of tail-skin temperature (T-sk), saline versus CNO-treated females; $n = 9$; $F(1, 128) = 17.07$, $p < 0.00001$.
 (G) Telemetry recordings of locomotor activity; $n = 9$; $F(1, 2178) = 46.48$, $p < 0.00001$.
 (H) Optogenetic stimulation (2 Hz) of *Kiss1*^{ARH} neurons was sufficient to increase T-sk in females; $n = 6$; $F(1, 40) = 11.23$, $p < 0.00001$.
 (I) T-sk in saline versus CNO-treated males; $n = 7$; $F(1, 48) = 26.35$, $p < 0.00001$.
 (J) Optogenetic stimulation (2 Hz) of *Kiss1*^{ARH} neurons was sufficient to increase T-sk in males; $n = 9$; $F(1, 64)$ (male) = 12.97, $p = 0.0024$.
 F statistics represent the main effect of treatment by two-way repeated measures (RMs)-ANOVA. Error bars represent \pm SEM. Crossover design with randomized starts.

phenomenon, and there has been no direct evidence that transient activation of *Kiss1* neurons can elicit hot flush-like activity.

RESULTS AND DISCUSSION

Contemporary technologies provide a means to functionally manipulate genetically defined neurons and circuits in mice. These tools allowed us to directly test the impact of *Kiss1*^{ARH} neuron activity and circuitry on vasomotor thermoregulation. We generated a new mouse line that expresses Cre recombinase from the *Kiss1* gene locus (*Kiss1*^{Cre} mice) to allow for selective manipulation of *Kiss1*-expressing neurons (Figure S1). We verified that Cre expression in the *Kiss1*^{Cre} mouse line is consistent with published literature on the central expression of *Kiss1*, particularly in the arcuate hypothalamus (ARH) and in the rostral periventricular area of the hypothalamus, two of the most studied populations of *Kiss1*-expressing neurons (Figures S1 and S2). *Kiss1*^{Cre} homozygous mice are infertile (Figure S2), similar to previous *Kiss1* knockout mice (d'Anglemont de Tassigny et al., 2007).

We co-injected adeno-associated virus, serotype 1 (AAV1) viruses containing fluorescently tagged Cre-dependent versions of the excitatory channelrhodopsin (DIO-ChR2:YFP) and the excitatory designer receptor exclusively activated by designer

drugs (DREADD), hM3Dq (DIO-hM3Dq:mCherry), into the ARH of female *Kiss1*^{Cre} mice and implanted a fiber-optic cannula above the ARH to allow artificial activation of *Kiss1*^{ARH} neurons (Figures 1A and 1B). We hypothesized that activating *Kiss1*^{ARH} neurons would initiate a hot-flush-like vasodilation, resulting in increased skin temperature and consequent drop in core body temperature, consistent with the clinical presentation of hot flushes (Freedman, 1998). Indeed, we found that chemogenetic activation of *Kiss1*^{ARH} neurons with clozapine N-oxide (CNO, the ligand for hM3Dq) produced hot-flush-like symptoms in experimental female mice compared to controls. Figure 1C shows the fluctuation in core body temperature of a typical mouse over 5 days, while Figure 1D shows the average of all the mice for 24 hr with CNO injection indicated by the arrow. Figure 1E shows the decrease in core body temperature compared to controls during the first 2 hr after CNO administration, while Figure 1F reveals the rise in tail-skin temperature of the same female mice. Artificial stimulation of *Kiss1*^{ARH} neurons also suppressed home-cage locomotor activity, a response similar to that observed when activating warm-sensitive POA neurons (Yu et al., 2016) (Figure 1G). Optogenetic activation of channelrhodopsin (ChR2)-expressing *Kiss1*^{ARH} cell bodies for 1 hr similarly evoked a pronounced increase in tail-skin temperature (Figure 1H).

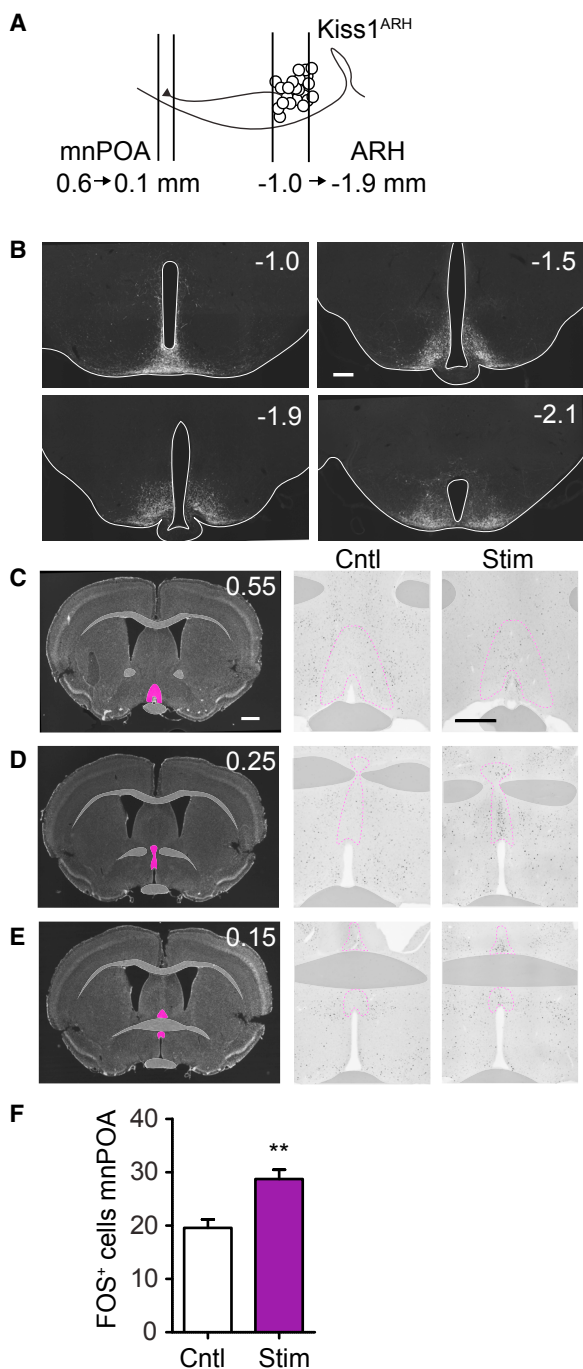


Figure 2. Activation of $Kiss1^{ARH}$ Neurons Induces Fos Expression in the Rostral POA

(A) Schematic diagram of $Kiss1^{ARH}$ projections to the POA (numbers indicate distance from Bregma in mm).

(B) Serial, coronal sections of hM3Dq:mCherry-transduced $Kiss1^{Cre}$ neurons with immunohistochemistry for mCherry.

(C–E) Representative Fos staining in POA from rostral to caudal (top to bottom). 0.55 (C), 0.25 (D), and 0.15 (E) from Bregma. Left panels: DAPI-stained coronal sections, with pink highlights indicating the POA. Middle and right panels: Fos immunoreactivity in the POA comparing control versus stimulated mice, respectively.

While the vast majority of patients who suffer from hot flushes are menopausal women, hot flushes can affect both sexes in response to rapid decreases in sex hormone levels, such as that which occurs in men undergoing androgen deprivation therapy for prostate cancer or after castration (Karling et al., 1994; Kumar et al., 2005). To examine whether $Kiss1^{ARH}$ neurons may underlie hot-flush-like symptoms in general, we co-injected AAV1 viruses containing Cre-dependent hM3Dq and ChR2 into the ARH of male $Kiss1^{Cre}$ mice. We found that activation of $Kiss1^{ARH}$ neurons using either CNO administration or light delivery in male mice increased tail-skin temperature to the same extent as that in female mice (Figures 1I and 1J).

To confirm that our viral strategy was efficiently transducing $Kiss1^{ARH}$ neurons, we verified the presence of hM3Dq:mCherry-expressing neurons in the ARH (Figures 2A and 2B). Because $Kiss1^{ARH}$ neuronal signaling can be excitatory via co-release of Kiss1, NkB, and glutamate, we then asked whether artificial activation of $Kiss1^{ARH}$ neurons is sufficient to stimulate downstream neurons in and around the predicted temperature-effector region in the POA. We examined Fos, an immediate-early gene used as a marker of activity, throughout the POA following stimulation of $Kiss1^{ARH}$ neurons compared to controls (Figures 2C–2E). Stimulation of $Kiss1^{ARH}$ neurons significantly increased Fos expression in the POA (Figure 2F).

Based on the hypothesis that $Kiss1^{ARH}$ neurons relay information to warm-sensing neurons in the POA (Rance et al., 2013), we predicted that selective activation of $Kiss1^{ARH}$ neuron terminals in the POA would recapitulate the hot-flush-like response seen after cell-body stimulation. To test this idea, we injected an AAV1 virus containing Cre-dependent ChR2 into the ARH of $Kiss1^{Cre}$ mice as before, but we implanted a fiber-optic cannula over the $Kiss1^{ARH}$ fibers in the rostral POA (Figure 3A). Figures 3B and 3C show the typical 6-day and average 24-hr fluctuations of core body temperature, with the photoactivation of $Kiss1^{ARH}$ neurons shown as a vertical blue bar. There was a significant decrease in core body temperature during the 2 hr after photoactivation of the $Kiss1$ axon terminals in the POA (Figure 3D). Activation of ChR2 in $Kiss1^{ARH}$ -neuron fibers in the POA also increased tail-skin temperature (Figure 3E) and decreased home-cage locomotor activity (Figure 3F), as was observed following cell-body stimulation. The POA contains neurons that are intrinsically temperature sensitive (Nakayama et al., 1961). To test whether our laser stimulation paradigm alone (2 Hz, 10 mW) was sufficient to induce hot-flush-like symptoms, a conditional mCherry virus (AAV1-DIO-mCherry) was injected into the ARH of $Kiss1^{Cre}$ female mice and the fiber-optic cannula implanted above the rostral POA. Laser exposure in the POA of these mice failed to evoke physiological responses, confirming that the responses obtained with ChR2 photoactivation were not induced by heating temperature-sensitive neurons within the POA (Figure S3).

(F) There were more Fos-positive cells in the rostral POA of stimulated mice; for Cntl, $M = 19.57$, $SD = 7.56$; for Stim, $M = 28.73$, $SD = 13.56$; $t(8) = 3.06$; $**p = 0.003$.

All scale bars, 500 μ m.

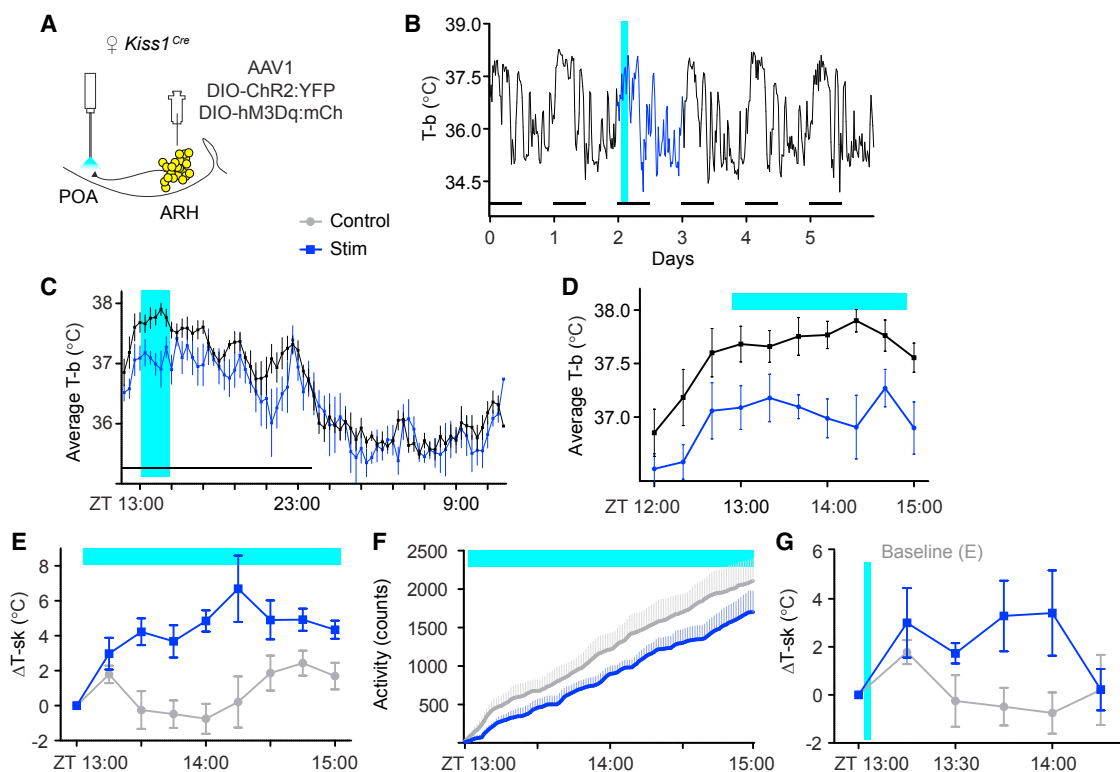


Figure 3. Optogenetic Stimulation of Kiss1^{ARH} Axons in the Rostral POA Promotes a Heat-Dissipation Response in Female Mice

(A) Schematic representation of the targeted viral injection into the ARH and fiber-optic placement 0.5 mm above the POA.

(B–D) Optogenetic stimulation (2 Hz) of Kiss1^{ARH} neuron fibers in the POA was sufficient to transiently decrease T-b. Within-subject comparison of unstimulated baseline (black trace) vs. stimulated (blue trace).

(B) 6-day T-b recording of a single mouse, subjected to blue-light stimulation from ZT = 13:00–15:00 on day 2 (blue).

(C) Average T-b comparing baseline versus stimulation on a scale of 24 hr.

(D) Average T-b comparing baseline vs. stimulation on a scale of 2 hr; n = 5; F(1, 72) = 9.589, p = 0.0147.

(E) T-sk in control versus terminal-stimulated females; n = 5; F(1, 64) = 17.83, p = 0.0029.

(F) Telemetry recording of locomotor activity, n = 5, F(1, 960) (activity) = 185.8, p < 0.00001.

(G) T-sk following a brief (2-min, 2 Hz) stimulation; n = 5; F(1, 48) = 9.41, p = 0.0035.

F statistics represent the main effect of treatment by two-way RM-ANOVA. Error bars represent ± SEM. Crossover design with randomized starts.

Hot flushes in humans are typically brief, lasting anywhere from seconds to 10 min (Freedman, 1998). To examine a more physiologically relevant time course, we optogenetically stimulated ChR2 in Kiss1^{ARH}-neuron fibers in the POA for only 2 min. Tail-skin temperature rose significantly above baseline for over 45 min before dropping back down to baseline (Figure 3G). These data confirm that even brief activation of this circuit is sufficient to engage a prolonged, thermoregulatory heat-effector response.

Because clinical hot flushes typically present after acute sex hormone withdrawal, we next examined the influence of sex-hormone status on Kiss1^{ARH}-mediated flushing. It has been shown that Kiss1^{ARH} neurons are more sensitive to artificial activation following gonadectomy (Han et al., 2015), so we predicted that the vasomotor response to Kiss1^{ARH} stimulation would be sensitized. To test this hypothesis, we first established a graded tail-skin temperature response to activation of hM3Dq-expressing Kiss1^{ARH} neurons by administering a range of CNO concentrations from 0.05 to 1 mg/kg. Intact females demonstrated a dose-dependent increase of tail-skin temperature in response

to increasing concentrations of CNO, with a partial effect at 0.3 mg/kg and a full effect at 1 mg/kg (Figure 4A). Based on these data, we then ovariectomized the mice and re-tested them with a dose of 0.3 mg/kg. After ovariectomy, this lower dose of CNO was now sufficient to induce a significant increase in tail-skin temperature (Figure 4A), a result consistent with the hypothesis that altered properties of Kiss1^{ARH} neurons after sex-hormone withdrawal could contribute to hot-flush susceptibility.

It has been proposed that NkB signaling from Kiss1^{ARH} neurons may mediate hot-flush responses (Rance et al., 2013), because NkB-receptor-expressing neurons are in the median POA (Shughrue et al., 1996), and engaging those receptors through injection of a NkB receptor agonist into the median POA induces hypothermia in rats (Dacks et al., 2011). To test this hypothesis directly, we infused neurokinin receptor antagonists into the POA before chemogenetically activating Kiss1^{ARH} neurons (Figure 4B). Because there are three neurokinin receptors (NK1R, NK2R, and NK3R), and NkB has some affinity for all of them, we infused a cocktail of antagonists for all three (de Croft et al., 2013). Infusion of this antagonist cocktail 30 min prior

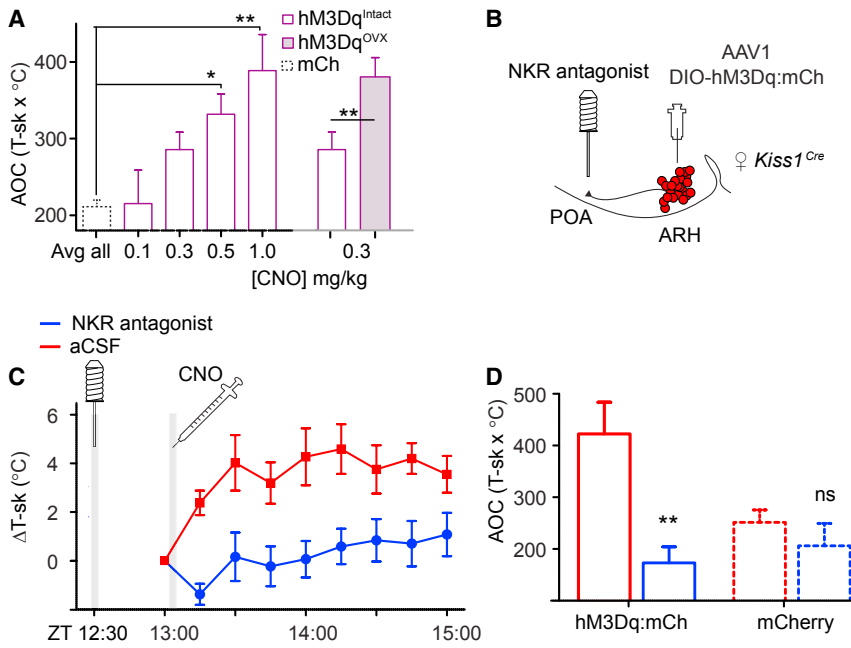


Figure 4. Evoked Hot Flushes Are Sensitive to Ovarian Estrogens and Are Blocked by Neurokinin Receptor Antagonists Delivered to the POA

(A) Dose response of T-sk to CNO. CNO was injected at ZT = 13:00, and T-sk was monitored for 2 hr; data are presented as the average area under the curve (AOC; hours × °C). mCherry-expressing controls were treated with CNO (0.05 to 1 mg/kg) and averaged. One-way ANOVA: for mCh, n = 8; for hM3Dq, n = 6; **p = 0.0006, asterisks indicate Bonferroni post-test results. Following ovariectomy, females were significantly more sensitive to 0.3 mg/kg CNO. Paired t test, n = 6. Intact: M = 285.6, SD = 22.74; ovariectomized, M = 393.1, SD = 29.31. t(5) = 2.605; *p = 0.0480.

(B) Schematic representation of targeted viral injection into the ARH and placement of infusion cannula in the POA.

(C) Infusion of the cocktail of antagonists was sufficient to block CNO-induced flushing. 30 min prior to recording, either saline or antagonists were infused into the POA of hM3Dq-expressing females. T-sk was recorded every 15 min starting immediately prior to CNO delivery at ZT = 13:00. Two-way RM-ANOVA, n = 6; F(1, 45) = 84.05, p < 0.00001.

(D) T-sk from ZT = 13:00–15:00 represented as AOC. hM3Dq data were extracted from (B) and compared to mCherry-expressing controls that were treated in the same manner. Two-way RM-ANOVA main effect of antagonists, n = 6 or 8 per group (hM3Dq or mCh); F(1, 12) = 16.49, **p = 0.0016; Bonferroni post-tests compare artificial cerebral spinal fluid (aCSF) with antagonists for paired animals in each group. ns, not significant. Experiments were conducted using a crossover design with randomized start times. All error bars represent SEM.

to chemogenetic activation of Kiss1^{ARH} neurons prevented the increase in tail-skin temperature (Figures 4C and 4D).

These data are consistent with the idea that Kiss1^{ARH} neurons contribute directly to the initiation of hot-flush-like symptoms, specifically via NkB signaling to the POA. Complete abolishment of this effect by infusion of neurokinin receptor antagonists into the POA suggests that NkB release from this projection of Kiss1 neurons is the most relevant for generating hot-flush-like activity (Yeo and Herbison, 2011). The functional relevance of Kiss1^{ARH} innervation of the POA for normal thermoregulation has not been elucidated, although cyclic changes in core body temperature that correlate with fertility and stages of menstrual cycles in women are implicated (Sanchez-Alavez et al., 2011). The process of implantation and carrying offspring to term may require an ability to modulate core body temperature that is related to, but separate from, circadian body temperature fluctuations (Smarr et al., 2016). Whether this circuit represents an independent pathway linking core body temperature to the reproductive axis or is one facet of a larger network of thermoregulatory circuits remains to be established. Alternatively, the POA is involved in a number of homeostatic mechanisms beyond thermoregulation (McKinley et al., 2015), and it is possible that the hypertrophic state of Kiss1^{ARH} neurons when sex-hormone levels fall activates NkB receptors in the POA without having functional significance under normal conditions.

Hot flushes are sporadic with an irregular frequency, and some women do not experience vasomotor symptoms during menopause. Both Kiss1 expression and NkB expression are elevated in Kiss1^{ARH} neurons after menopause or gonadectomy

(Goodman et al., 2007; Rance and Young, 1991; Roa et al., 2006; Rometo et al., 2007; Sheehan and Kovács, 1966; Smith et al., 2005), priming these neurons to evoke GnRH-mediated LH release as well as NkB-mediated hot flushes. Kiss1^{ARH} neuron activity is synchronized by local autoregulation mechanisms (Qiu et al., 2016), and following gonadectomy in mice, Kiss1^{ARH} neurons demonstrate a pulsatile activity pattern with a fixed period that is correlated with peaks of LH release (Clarkson et al., 2017). This pulsatile activity pattern, however, is not sufficient to explain the sporadic, episodic nature of hot flushes. Epidemiological data indicate that numerous sensory and interoceptive cues can evoke flushing in menopausal women, including: higher ambient temperature, consumption of spicy foods, anxiety, altitude, and acute physiological stressors (Federici et al., 2016; Hunter et al., 2013; Swartzman et al., 1990). Thus, we hypothesize that flushes occur when excitatory external cues converge on Kiss1^{ARH} neurons during an episode of endogenous activity. Our experiments demonstrate that transient activation of Kiss1^{ARH} neurons by either chemogenetic or optogenetic means is sufficient to elicit hot-flush-like vasomotor responses in mice and that this effect is enhanced by ovariectomy.

The most prevalent current therapy for hot flushes in the United States is estrogen replacement, the long-term safety of which has been questioned (Rossouw et al., 2013). The identity and function of POA neurons innervated by Kiss1^{ARH} neurons, specifically those containing the NkB receptor, may provide additional targets, e.g., receptors, for the development of drugs that can treat hot-flush symptoms. Additionally, the identification of genetically defined neuronal subpopulations in the POA that

mediate heat-effector responses is an area of growing interest (Song et al., 2016; Tan et al., 2016; Yu et al., 2016), and how neurons expressing NkB receptors fit into this larger picture remains to be seen.

EXPERIMENTAL PROCEDURES

Additional information and resources are included in the [Supplemental Experimental Procedures](#).

Study Approval

All experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with approval from the University of Washington's Animal Care and Use Committee. Adult mice (ages 2–8 months) had *ad libitum* access to standard rodent chow and water and were housed under a 12-hr:12-hr light:dark cycle at $21.51 \pm 0.09^\circ\text{C}$. Zeitgeber time (ZT) of 0:00 indicates the onset of the light cycle each day.

Data Analysis

Statistical comparisons were performed with GraphPad's Prism software and are reported in detail in the figure legends. A p value of 0.05 was the threshold for significance (*p < 0.05, **p < 0.01, and ***p < 0.001), and all error bars represent SEM. The ns represent either individual mice or histological sections, as indicated in the text.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.06.037>.

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AUTHOR CONTRIBUTIONS

S.L.P. and C.W.J. designed the study under the guidance of R.D.P. C.W.J. and S.L.P. executed the experiments and analyzed the data. R.D.P. generated the *Kiss1^{Cre}* knockin mouse with help from S. Tsang and K. Kafer. F.D.B. performed experiments to validate the *Kiss1^{Cre}* (v2) mouse line. M.A.P. wrote the Python script that was used to process data acquired from the infrared camera. C.W.J. and S.L.P. wrote the manuscript with input from R.D.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

**A Neural Circuit Underlying
the Generation of Hot Flushes**

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Supplemental Experimental Procedures

Generation and maintenance of *Kiss1^{Cre}* (v2) mice

The previous line of *Kiss1^{Cre:Gfp}* knock-in mice (v1) were prone to ectopic expression when genetically crossed to conditional reporter mice, and also retained a low level of *Kiss1* expression (Popa et al., 2013). Barring genetic crosses, this v1 line remains a useful tool for visualizing Kiss1 neurons for electrophysiological recordings with the Gfp-fused-Cre-reporter transgene. Also, viral injections into adult v1 mice, as opposed genetic crosses, can be used to gain control of those Kiss1 neurons and thereby avoid developmental ectopic recombination.

In this study we generated a second *Kiss1^{Cre}* line (v2) modified by: (a) removing the nuclear localization signal from Cre, (b) using a non-optimal initiation codon, (c) removing part of the N-terminal sequence of Cre, (d) adding a 3' untranslated region from the *Myc* gene that promotes a short mRNA half-life, (e) deleting part of exon 1 that includes the signal peptide, and (f) performing the gene targeting in C57Bl/6 ES cells. Fig. S1 legend contains a detailed description of the genetic construct that was used to engineer this transgenic mouse line. When crossed to a conditional reporter line, Ai14, v2 faithfully reports Kiss1 neurons in the rostral periventricular area, bed nucleus of the stria terminalis, arcuate nucleus and medial amygdala, with little to no ectopic expression (Fig. S1C). Genotyping was performed by 3-primer PCR. (a) *Kiss1* forward: 5'GACCTAGGCTCTGGTGAAGTACG; (b) *Kiss1* reverse: 5'AGCCTCCAGTGCTCACAGCAG; (c) Cre reverse: 5'CTTGCGAACCTCATCACTCGTTGC.

Viruses and stereotactic injections

Mice under 3 months of age received bilateral injections of either a control adeno-associated virus (AAV, serotype 1) vector encoding Cre-dependent mCherry fluorescent reporter (AAV1-EF1 α -DIO-mCherry) or co-injections of AAV1 vectors encoding Cre-dependent ChR2:mCherry (AAV1-Ef1 α -DIO-ChR2:mCherry) and Cre-dependent hM3Dq:YFP (AAV1-Ef1 α -DIO-hM3Dq:YFP); 500 nl of virus (~10⁹ particles per ml) was delivered to each targeted injection site. Viruses were prepared at the University of Washington as described (Gore et al., 2013). Surgeries were performed aseptically under constant infusion of isoflurane (2% isoflurane/O₂) while head-fixed to a stereotaxic frame. Targeted injections were either determined by manual placement on a stereotaxic frame (David Kopf Instruments) with glass pipette

delivery or using a Neurostar Drill and Injection Robot system (Tubingen, Germany) with a 33-Ga needle (NanoFil NF33BL). Viruses were injected bilaterally into the ARH (with respect to Bregma, anteroposterior (AP): -1.25 mm; mediolateral (ML): \pm 0.30 mm; dorsoventral (DV): 5.80 mm) over 5 min, and injectors were left in place for 10 min before slow withdrawal. Fiber-optic cannulas were made in-house using 2.5-mm Ceramic Stick Ferrules (Precision Fiber Products) and 0.22 NA multimode fiber (Thor Labs) as described (Sparta et al., 2011), and were positioned such that the fiber tip was \sim 0.5 mm above the targeted cell population. ARH fibers were placed unilaterally (AP: -1.25; ML: +0.3; DV: 5.3), while the single rostral POA fiber was placed centrally (AP: +0.45; ML: 0.0; DV 4.25). Fiber optic cannulas were fixed in place using Metabond (Parkell) and dental cement. Animals included in the results of this manuscript were qualified by post-hoc immunohistochemistry to be either unilateral or bilateral hits for viral expression in the ARH.

Stimulation of Kiss1^{ARH} neurons

CNO (1.0 mg/kg) or saline (10 μ l/g BW) was administered intraperitoneally immediately following baseline temperature recordings at ZT 13:00. For optogenetic experiments, mice were patched to tethers for \geq 6 h before recording. Laser emission with 473-nm light (calibrated to 10 mW at the fiber tip) was delivered at 2 Hz with 10-ms pulse width as described (Qiu et al., 2016).

Pharmacological blockade of neurokinin receptors in the POA

Guide cannulas were stereotaxically positioned at, AP: +0.5; ML: 0.0. The ventral tip of the guide or injection cannula (4.75 mm or 4.25 mm, respectively) was implanted 4 mm from the dura and cemented in place. Mice were handled daily for two weeks prior to testing. During this habituation period, mice were regularly restrained, tethered to injection tubing, and acclimated to intraperitoneal saline injections.

On test day, mice were tethered to injection tubing at ZT = 12:30 and treated with 300 μ l of either ACSF or a cocktail of NkB receptor antagonists: SDZ-NKT 343, 167 pmol (NK1R; Tocris cat. #2394); GR 94800, 167 pmol (NK2R; Tocris cat. #1667); and SB222200, 500 pmol (NK3R; Tocris cat. #1393). At ZT = 13:00 the first tail-skin recording was taken immediately prior to CNO injection (1 mg/kg). This study was performed as a crossover design with randomized starts.

Determining effect of ovariectomy

Mice were randomly assigned concentrations of 0.1, 0.3, 0.5, and 1.0 mg/kg CNO and their tail-skin temperatures were recorded after administration. Allowing at least two days for recovery in between each trial, mice were then re-assigned a different concentration until, over the course of 5 experimental days, each mouse had received each concentration once. Mice were then ovariectomised, allowed to recover for 2 weeks, and then re-tested with the 0.3 mg/kg dose that gave approximately half-maximal response in the prior dose-response experiment.

Ovariectomy: Animals were anesthetized with 2% isoflurane/O₂, fur was removed from a ~5 mm area of the flank on both sides. The skin was sterilized and a small incision was made to expose the peritoneum. The ovary was clamped with a hemostat and removed. Dissolvable sutures were used to close the fascia. For the validation of the new *Kiss1^{Cre}* line (Supplemental Figure 2), during the same surgery a small perforated tube was also implanted in the subscapular region of the back, which contained either sesame oil (20 µl) or 17β-estradiol, E₂ dissolved in oil (20 µl, 1 mg/ml); animals were euthanized and prepared for histology one week after surgery.

Physiological recordings

Tail-skin temperature (T-sk) was recorded using an infrared camera (FLIR E4; FLIR Instruments). Images were uploaded using the software provided (FLIR Tools) and a custom Python script was used to extract temperature values taken from the base of the tail. Core-body temperature (T-b) and locomotor activity were recorded using an implantable telemetry system (G2 E-Mitters, Starr Life Sciences) and analyzed using the software provided (VitalView).

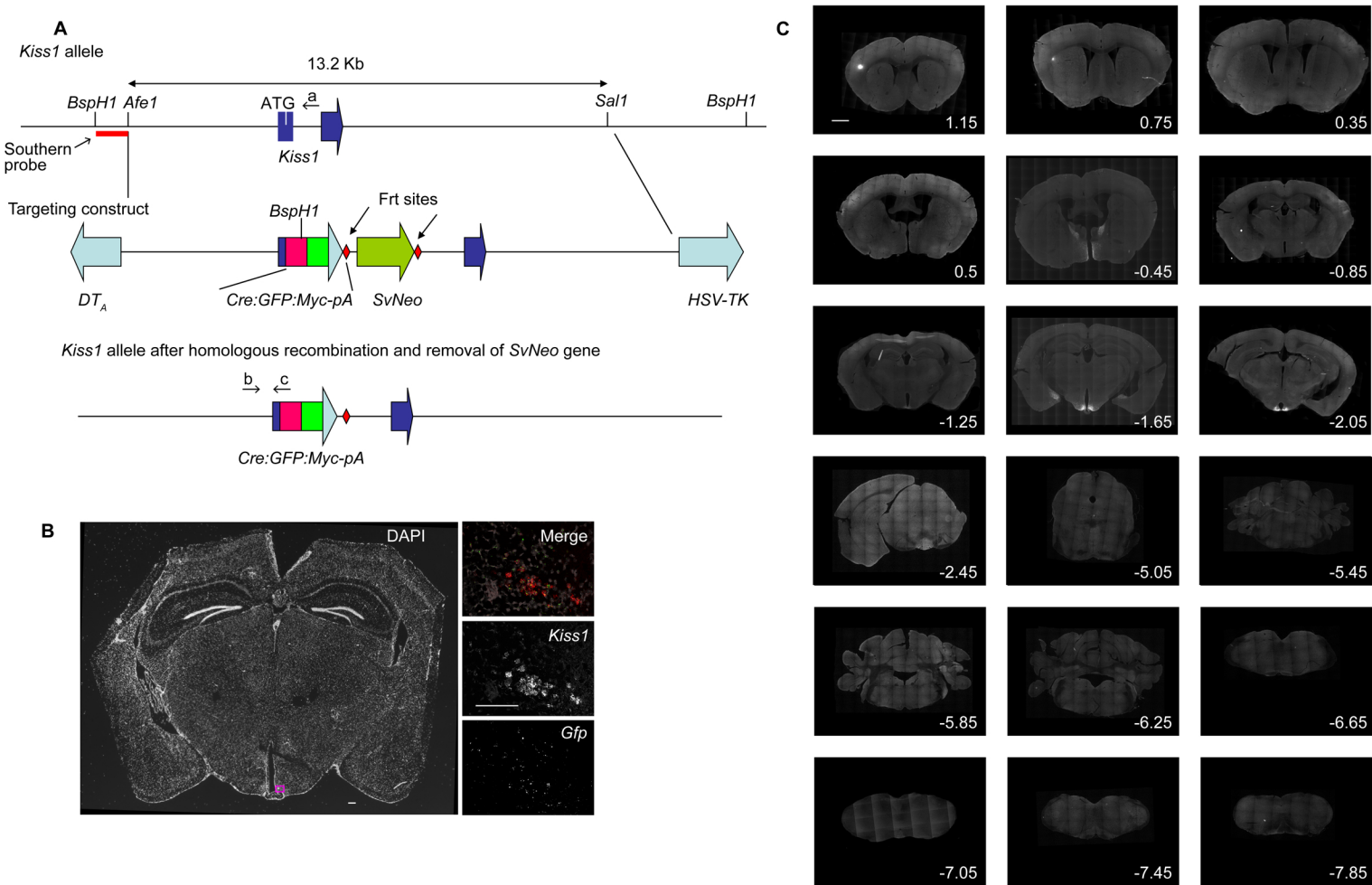
Immunohistochemistry

Mice were given Beuthanasia-D Special (effective dose 320 mg/kg pentobarbital) and transcardially perfused by flushing the circulation with cold saline, followed by administration of 40 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PFA). Brains were rapidly dissected and post-fixed in PFA at 4°C overnight, followed by subsequent cryoprotection in 30% sucrose. Brains were then rapidly frozen at -

30°C in a 2-methylbutane/Dry Ice slurry and stored at -80°C until processed. Floating 30-µm coronal sections were incubated in PBS and processed within 1 week of sectioning. For immunohistochemistry, sections were first blocked for 1 hour at room temperature (RT) in PBS with 0.2% Triton X-100, and 3% normal donkey serum. Sections were incubated overnight with the primary antibody at 4°C and the next day they were exposed to a corresponding secondary antibody for 2 hours at RT. Primary antibodies: rabbit anti-DsRed (Clontech 632496; diluted 1:1000) or goat anti-Fos (Santa Cruz SC-52-G; diluted 1:300). Processed sections were mounted onto glass slides and coverslipped with DAPI Fluoromount-G (Southern Biotech, 0100-290). Images were acquired with an Olympus FV1200 confocal microscope.

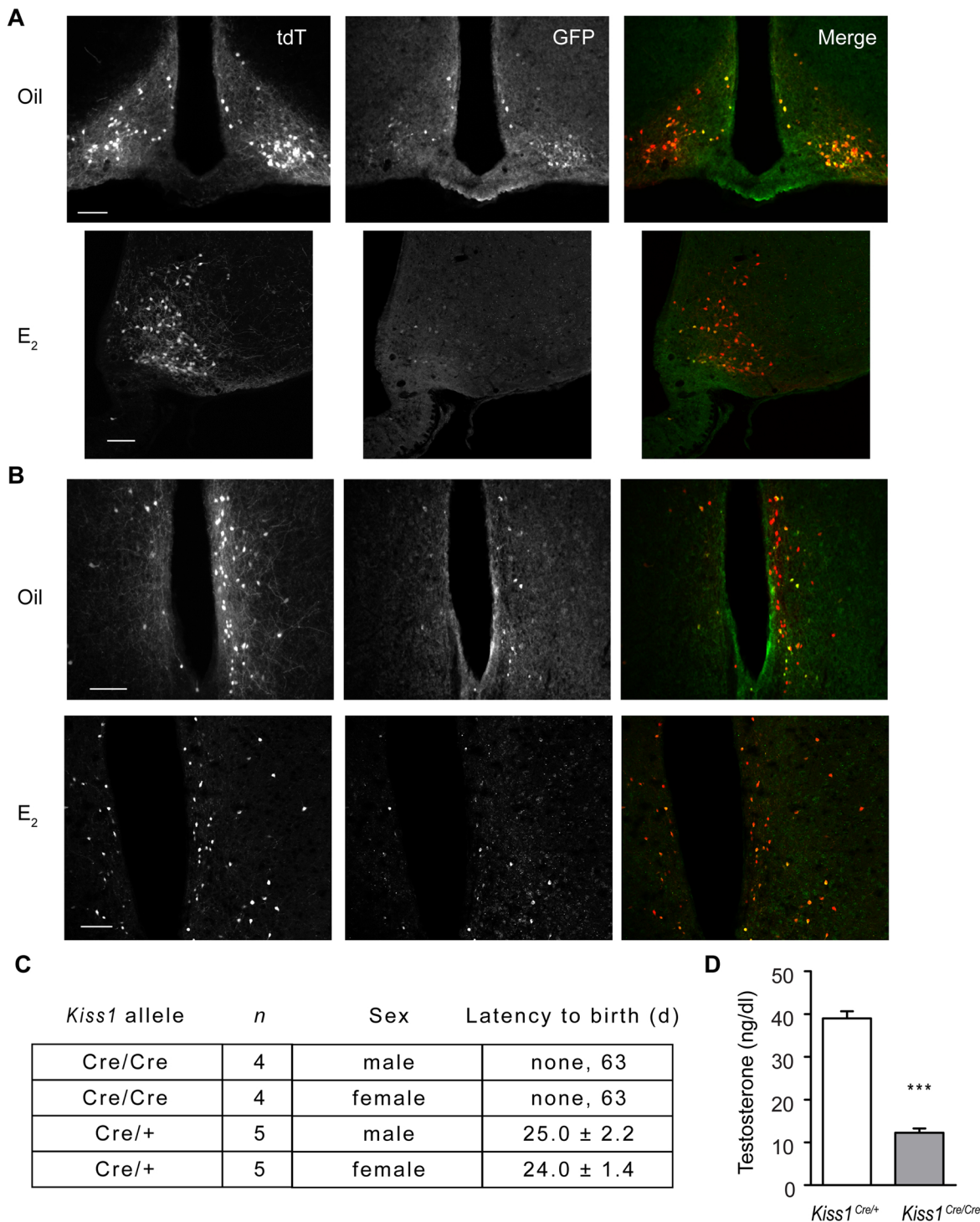
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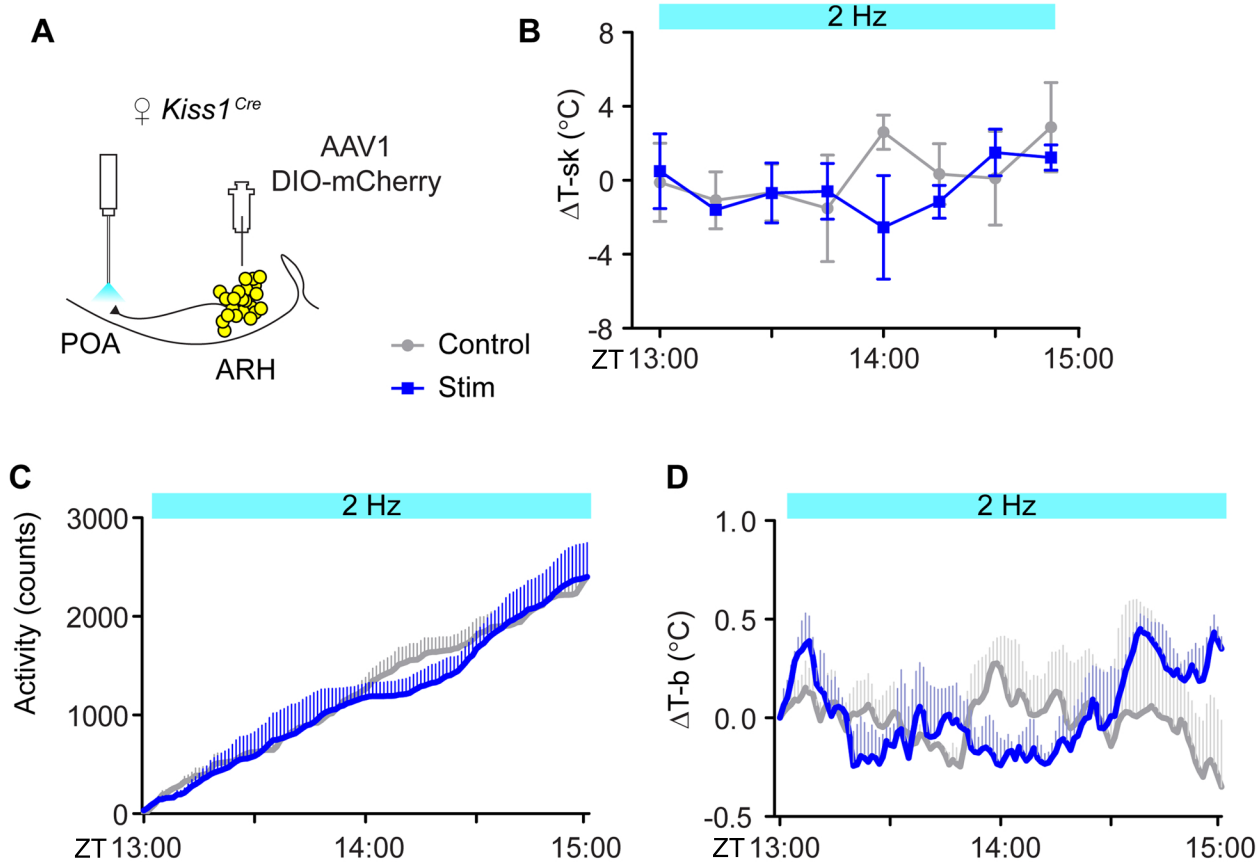


Supplemental Figure S1. Generation of *Kiss1*^{Cre:GFP(v2)} Mice and Expression Analysis. Related to Figure 1.

(A) The targeting construct for *Kiss1* included ~5.0 kb DNA 5' of the translation start site and ~8 kb on the 3' side. The targeting construct contained a Cre:GFP fusion protein (the nucleotide sequence at the encoding the N-terminus of Cre was modified to be: CCCC ATG GCT GCA TTA CCG GTC) followed by the 3' untranslated region and poly-A addition sequence from the *Myc* gene. This cassette was inserted just 5' to the *Kiss1* initiation codon. A frt-flanked *SvNeo* gene (for positive selection) was inserted at the end of the first exon, such that the sequence between the start codon and the end of that exon was deleted. The construct also contained *Pgk*-DTA and HSV-TK genes for negative selection. C57Bl/6 ES cells were electroporated and 7 positive clones were identified by digesting DNA with *BspH1* and performing a Southern blot using a 350 bp probe located just outside the 5' boundary of the targeting construct. Positive clones were injected into C57Bl/6 blastocysts and high-percentage chimeras were bred to achieve germline transfer. The frt-Neo gene was removed by breeding the heterozygous mice with Gt(*ROSA*)26Sor-FLP recombinase; mice were subsequently bred with c57Bl/6. (B) *In situ* hybridization demonstrating the relationship between endogenous *Kiss1* and GFP in the arcuate hypothalamus of an ovariectomized *Kiss1*^{Cre:GFP} female. Scale bar, 100 μ m (C) *Kiss1*^{Cre:GFP}::*Ai14*, Gt(*ROSA*)26Sor^{tm14(CAG-td-Tomato)} bisgenic mice were used to reveal the lineage of *Kiss1*^{Cre:GFP}-expressing cells. tdT expression is shown in serial coronal sections from rostral to caudal (Bregma coordinates indicated). Scale bar, 1 mm.



Supplemental Figure S2. Analysis of *Kiss1^{Cre:GFP}* (v2) Mice. Related to Figure 1. (A, B) Immunohistochemistry of ovariectomized *Kiss1^{Cre:GFP}::Ai14* mice treated with either oil or 17 β -estradiol, E₂. GFP reveals active *Kiss1/Cre* expression, while tdTomato reports all cells that have ever expressed *Kiss1^{Cre:GFP}*. (A) Sections from the ARH or (B) rostral periventricular area (R3PV). Scale bars, 100 μ m. (C) *Kiss1^{Cre/Cre}* homozygous mice, males and females, did not yield litters following 63 days of exposure to fertile mates, whereas *Kiss1^{Cre/+}* heterozygous mice became pregnant/sired litters within the first 5 d of exposure to a fertile mate and had an average litter size of 8.20 \pm 0.42 with a male to female ratio of 1.35 \pm 0.26. (D) Homozygous *Kiss1^{Cre/Cre}* male mice had diminished testosterone levels compared to heterozygous controls; Cre/+ (*n* = 8) *M* = 39.00 ng/dl, *SD* = 4.76; Cre/Cre (*n* = 8) *M* = 12.28 ng/dl *SD* = 2.81; *t*(14) = 13.68; *p* < 0.0001 (*).**



Supplemental Figure S3. Control Experiments with only AAV-DIO-mCherry Expression in POA. Related to Figure 3. (A) Schematic representation of the targeted viral injection into the ARH and fiber placement 0.5 mm above the POA. (B to D) Mice were tethered to a patch cord, a baseline measurement was taken at ZT 13:00, then they were either laser stimulated (blue line, 2 Hz) or not (grey line, controls) for 2 h; paired 2-way ANOVA comparisons, $n = 3$. Continuous light-stimulation in the POA did not change (B) tail-skin temperature (T-sk); $F(1,16) = 1.17$; $p = 0.30$, (C) locomotor activity counts or (D) core-body temperature (T-b) with respect to controls; $F(1,242) = 1.48$; $p = 0.23$.