

# Medial Amygdala *Kiss1* Neurons Mediate Female Pheromone Stimulation of Luteinizing Hormone in Male Mice

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## Keywords

Amygdala · *Kiss-1* · Luteinizing hormone · Male reproduction · Neuroendocrinology

## Abstract

**Background/Aims:** The medial amygdala (MeA) responds to olfactory stimuli and alters reproductive physiology. However, the neuronal circuit that relays signals from the MeA to the reproductive axis remains poorly defined. This study aimed to test whether MeA kisspeptin (MeA<sup>Kiss</sup>) neurons in male mice are sensitive to sexually relevant olfactory stimuli and transmit signals to alter reproductive physiology. We also investigated whether MeA<sup>Kiss</sup> neurons have the capacity to elaborate glutamate and GABA neurotransmitters and potentially contribute to reproductive axis regulation. **Methods:** Using female urine as a pheromone stimulus, MeA<sup>Kiss</sup> neuronal activity was analysed and serum luteinizing hormone (LH) was measured in male mice. Next, using a chemogenetic approach, MeA<sup>Kiss</sup> neurons were bi-directionally modulated to measure the effect on serum LH and evaluate the activation of the preoptic area. Lastly, using in situ hybridization, we identified the proportion of MeA<sup>Kiss</sup> neurons

that express markers for GABAergic (Vgat) and glutamatergic (Vglut2) neurotransmission. **Results:** Male mice exposed to female urine showed a two-fold increase in the number of c-Fos-positive MeA<sup>Kiss</sup> neurons concomitant with raised LH. Chemogenetic activation of MeA<sup>Kiss</sup> neurons significantly increased LH in the absence of urine exposure, whereas inhibition of MeA<sup>Kiss</sup> neurons did not alter LH. In situ hybridization revealed that MeA<sup>Kiss</sup> neurons are a mixed neuronal population in which 71% express Vgat mRNA, 29% express Vglut2 mRNA, and 6% express both. **Conclusions:** Our results uncover, for the first time, that MeA<sup>Kiss</sup> neurons process sexually relevant olfactory signals to influence reproductive hormone levels in male mice, likely through a complex interplay of neuropeptide and neurotransmitter signalling.

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## Introduction

Highly evolved sensory and behavioural strategies enable animals to identify a mate and ensure reproductive success. The medial amygdala (MeA) is associated with mediating sexual behaviour in mammals [1], including

humans [2], and is known to influence reproductive hormone levels [3]. In rodents, chemical signals called pheromones carry sex- and species-specific information that elicits pre-programmed mating and endocrine responses. Pheromone signals activate sensory neurons in the vomeronasal organ, which relay information through the accessory olfactory bulb (AOB) for processing in the MeA [4]. Projections from the MeA to the preoptic area (POA) of the hypothalamus then influence neuroendocrine status and mating behaviours. The MeA appears to have both inhibitory and stimulatory influences on reproductive physiology and associated behaviours. MeA lesions advance puberty onset in rodents [5] and monkeys [6], but also disrupt sexual behaviours including the investigation and preference towards opposite-sex pheromones [7]. Exposure to opposite-sex pheromones leads to neuronal activation throughout the MeA [3, 8] and raised serum luteinizing hormone (LH) in male rodents [9, 10].

Although the MeA is a critical node for processing sexually relevant stimuli to influence mating behaviour and reproductive endocrine function, the underlying brain circuitry that mediates these activities remains poorly defined. A population of kisspeptin neurons located in the MeA (MeA<sup>Kiss</sup>) are cogent candidates [11] for transmitting olfactory influences to the reproductive axis. In the hypothalamus, kisspeptin regulates gonadotropin-releasing hormone (GnRH) secretion, which is critical for mammalian reproduction. Humans and mice with inactive kisspeptin (*KISS1*) or its cognate receptor (*KISS1R*) genes display a delay in or absence of puberty, reproductive hormone deficits, lack of sexual behaviour, and infertility [12, 13]. Although hypothalamic kisspeptin neurons are well established as stimulators of GnRH, the specific role of MeA<sup>Kiss</sup> neurons in the reproductive endocrine axis has not been directly demonstrated.

MeA<sup>Kiss</sup> neurons are reciprocally connected to the AOB and a subset of MeA<sup>Kiss</sup> neurons contact GnRH neurons in the POA [14]. Chemogenetic activation of MeA<sup>Kiss</sup> neurons reduces anxiety and enhances the preference for oestrous females in male mice [15]. Intra-MeA infusions of kisspeptin impact LH levels and lead to increased numbers of ex-copula erections in male rats [16], suggesting that MeA<sup>Kiss</sup> signalling regulates GnRH release and participates in sexual arousal. However, *Kiss1r* expression has not been identified in MeA<sup>Kiss</sup> neurons, therefore it remains unclear whether MeA<sup>Kiss</sup> neurons directly regulate these responses or whether another neuronal population is involved.

GnRH neurons receive both GABAergic and glutamatergic inputs [17, 18] and glutamatergic and GABAergic neurons in the MeA project to the ventromedial

hypothalamus [19]. Pharmacological manipulations of GABA and glutamate receptors directly influence GnRH pulsatility and LH levels [20]. Therefore, it is important to understand whether MeA<sup>Kiss</sup> neurons also have the capacity to elaborate these neurotransmitters that contribute to modulating reproductive hormone levels.

In the current study, we tested the hypothesis that MeA<sup>Kiss</sup> neurons are sensitive to sexually relevant olfactory stimuli and directly contribute to reproductive endocrine responses. First, we assessed whether exposure to female mouse urine led to activation of MeA<sup>Kiss</sup> neurons in male mice. Next, we employed DREADDs (designer receptors exclusively activated by designer drugs) to determine whether specific modulation of MeA<sup>Kiss</sup> neurons impacts circulating LH levels. We then quantified Fos immediate-early gene expression in GnRH neurons and the POA after chemogenetic manipulations to elucidate whether MeA<sup>Kiss</sup> modulation can directly or indirectly influence neuronal activity within this reproductive brain centre. Finally, because GABA and glutamate are known to influence GnRH activity and a subset of MeA<sup>Kiss</sup> neurons project directly to GnRH neurons, we used multiplexed in situ hybridization/immunohistochemistry (ISH/IHC) for definitive markers of GABAergic and glutamatergic transmission to study the neurotransmitter composition of MeA<sup>Kiss</sup> neurons.

## Materials and Methods

### Subjects

All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 approved by the University of St. Andrews Ethics Committee under the UK Home Office project license No. 7007924. The genetic mouse line *Kiss1<sup>CreEGFP</sup>* (*Kiss1<sup>tm1.1(cre/EGFP)Steij</sup>*) was generated and characterized by the Steiner Lab (University of Washington, USA) [21] and *Rosa26<sup>lox-stop-lox-tdTomato</sup>* mice were generated and characterized by the Zeng Lab [22].

All breeding and genotyping were performed at the University of St. Andrews. Mice were maintained on a 12:12 h light/dark cycle (lights on at 7:00 a.m.) with temperature maintained at 21 ± 5 °C and humidity at 55 ± 10%. Mice had ad libitum access to standard mouse chow (Special Diets Services, UK) and water. Mice were weaned at 21 days and genotyped by PCR analysis of ear clips. Male mice used for this study were group-housed (3–4 mice/cage) in individually ventilated cages (Sealsafe, Tecniplast, UK) and were kept in a single holding room.

Although *Kiss1<sup>CreEGFP/wt</sup>* mice express EGFP in hypothalamic Kiss neurons [21], we were unable to visualize EGFP expression in the MeA using IHC even with enhanced amplification methods. In addition, the *Kiss1* mRNA signal generated from RNAscope ISH in the MeA was too low to combine with evaluating c-Fos IHC, there-

fore to robustly mark MeA<sup>Kiss</sup> neurons with tdTomato, we generated double transgenic *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* mice by breeding *Kiss1<sup>CreEGFP</sup>* mice with *Rosa26<sup>lox-stop-lox-tdTomato</sup>*. Adult (sexually naive, 10–14 weeks old,  $n = 12$ ) gonad-intact *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* males were used for pheromone exposure and neurotransmitter co-expression studies. Adult (sexually naive, 10–14 weeks old,  $n = 30$ ) gonad-intact *Kiss1<sup>CreEGFP/wt</sup>* male mice were subjected to recombinant adeno-associated virus (rAAV) injections to enable Cre-dependent expression of DREADDs in MeA<sup>Kiss</sup> neurons.

#### Pheromone Stimuli

Urine presented as chemosensory stimuli was pooled from sexually naive ovary-intact females primarily from prooestrous and oestrous stages and stored in aliquots at  $-80^{\circ}\text{C}$ . Urine exposure experiments were conducted on adult gonad-intact *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice ( $n = 12$ ) between 9:00 and 11:30 a.m. Forty-eight hours prior to each experiment, males were housed individually in clean cages. On the morning of exposure, mice were randomly assigned to either exposure or control groups ( $n = 6$  animals in each group). Depending on the group, 50  $\mu\text{L}$  of female urine or water was pipetted onto sterile cotton pads and placed into their home cage for 15 min. A blood sample was collected from each mouse 15 min following exposure to urine. At 30 min post-exposure, males were anaesthetized with sodium pentobarbital (150 mg/kg i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed and post-fixed in 4% PFA/0.1 M PBS overnight at  $4^{\circ}\text{C}$  followed by cryoprotection in 30% sucrose for 72 h. Brains were embedded in OCT compound (Fisher Scientific, Cat. No. 12678086), placed in a cryomold, and flash frozen in cooled isopentane ( $-60^{\circ}\text{C}$ ). Frozen brains were stored at  $-80^{\circ}\text{C}$  until processing.

#### Stereotaxic Surgery

Adult *Kiss1<sup>CreEGFP/wt</sup>* mice ( $n = 30$ ) were anaesthetized using 1.5–2% isoflurane delivered using a Univentor 410 anaesthesia unit (Univentor Limited, Malta). Subjects were then fixed into a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA) on top of a homeothermic pad (Harvard Apparatus Ltd., UK) and connected to a mouse anaesthetic mask. A small incision was made to expose the skull and a small craniotomy was drilled directly above each target site. Each DREADD vector plasmid was packaged into rAAVs (serotype 1/2) using the method described [23]. One construct (pAAV-EF1a-DIO-hM3D[Gq]-mCherry [Addgene plasmid #50460], a gift from Bryan Roth) encodes a modified G pro-

tein-coupled receptor hM3D(Gq) that, after binding to its non-endogenous ligand, clozapine N-oxide (CNO), induces neuronal burst firing [24, 25]. The other construct (pAAV-hSyn-dF-HA-hKORD-IRES-mCitrine [Addgene plasmid #65417], another gift from Bryan Roth) encodes a modified G protein-coupled receptor (hKORD) that, after binding to its non-endogenous ligand, salvinorin B (SALB), activates inward-rectifying potassium channels, resulting in hyperpolarization (neuronal silencing) [26]. These two rAAVs were mixed equally (each titre  $4.2 \times 10^{11}$  genome copies/mL) and delivered bilaterally with 0.4  $\mu\text{L}$  of virus per site at the rate of 0.1  $\mu\text{L}/\text{min}$  using calibrated pulled-glass capillary needles (Drummond Scientific company, 2-000-005) by pressure injection. Stereotaxic coordinates: Bregma  $-1.7$  mm,  $\pm 2.2$  mm lateral to the midline, and  $-5$  mm below the skull at Bregma [27] was used to target the centre of the MeA with the aim of facilitating viral spread to the anterior and posterior MeA. The needle remained in place for 5 min before being withdrawn over 60 s. The incision was closed using Vetbond (3M Vetbond, 084-1469SB) and the mice were allowed to recover in a heated cage before being returned to their home cage. Post-surgery, subjects were given analgesic (Buprenex, 1  $\mu\text{g}/\text{g}$  in palatable jelly). All DREADD modulation experiments began 3 weeks after viral injections.

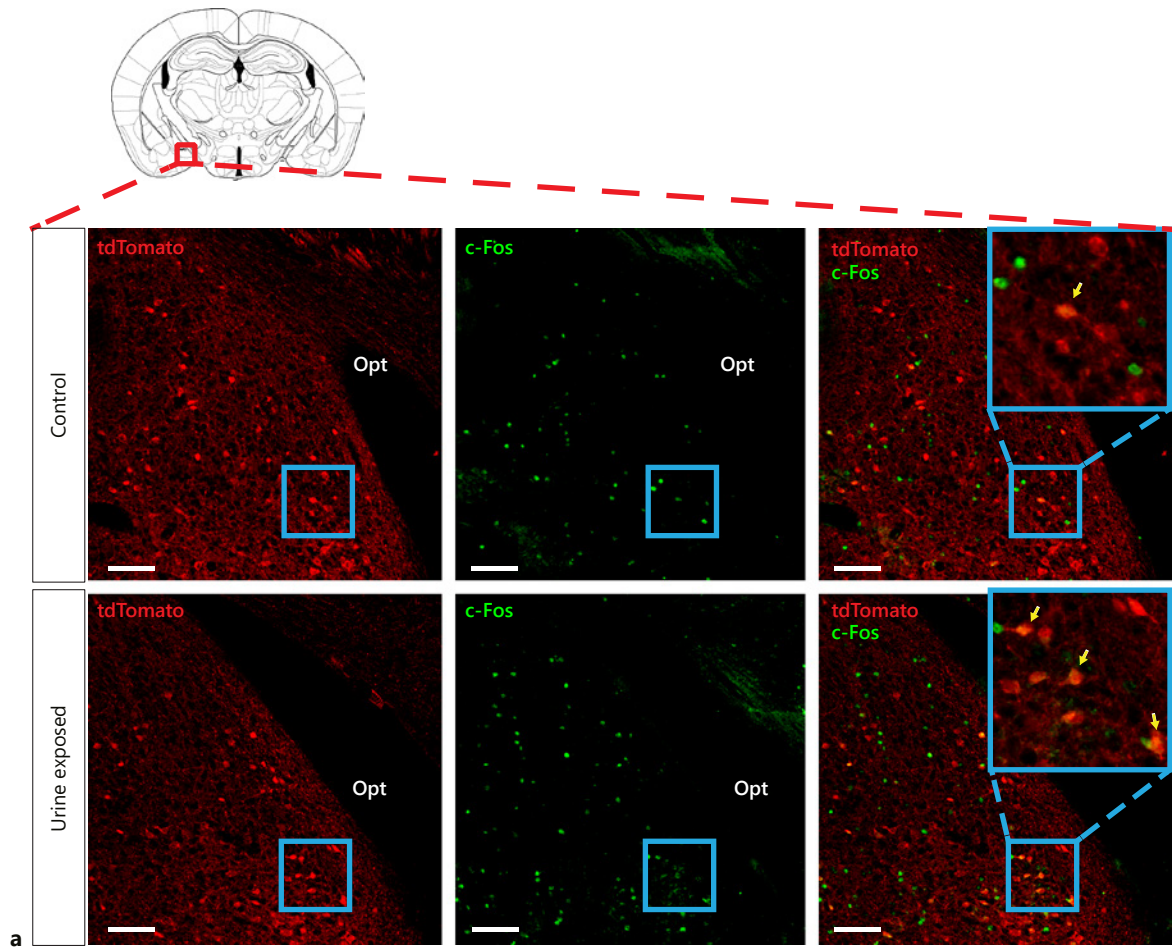
#### Chemogenetic Modulation of Reproductive Hormone Levels

CNO was obtained from Tocris Bioscience (Cat. No. 4936) and SALB was obtained from Sigma-Aldrich (Cat. No. 75250). Both were dissolved in DMSO to prepare stock solutions (CNO 10 mg/mL and SALB 5 mg/mL). A crossover study design (3-period, 3-treatment) was used for drug treatments (DMSO, SALB, CNO), where the order of the drug treatments was randomized, and each animal received all 3 treatments, with a wash-out period of 2 weeks between treatments. The treatment regimen started 3 weeks following rAAV injections, with mice housed singly for 48 h along with handling acclimation prior to the first treatment. Depending on the treatment groups, mice were injected with either CNO (3 mg/kg i.p.), SALB (10 mg/kg s.c.), or vehicle (DMSO i.p.) diluted in saline between 9:00 and 11:30 a.m. These drug doses have been used previously to activate and inhibit DREADD receptors [26] and a higher dose of SALB was used because it is administered subcutaneously, whereas CNO is administered intraperitoneally. Blood samples were collected at 30 min post-treatment. During the third and final round, at 60 min after treatments, males were euthanized with an overdose of sodium pentobarbital (150 mg/kg i.p.) and transcardially perfused with 0.1 M PBS followed by 4% PFA in 0.1 M PBS.

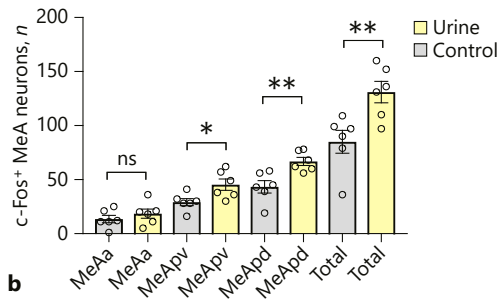
**Fig. 1.** Exposure to female urine regulates the activity of MeA<sup>Kiss</sup> neurons and impacts luteinizing hormone (LH) levels in *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice. **a** Co-localization of c-Fos (green) in MeA<sup>Kiss</sup> neurons (tdTomato<sup>+</sup>; red) of male mice exposed to control (top panel) or female urine (bottom panel). Yellow arrows indicate tdTomato<sup>+</sup> neurons that express c-Fos. Scale bars, 100  $\mu\text{m}$ . **b** Number of c-Fos<sup>+</sup> cells in the MeA subdivisions after exposure to control or urine ( $n = 6$  in each group). **c** Percentage of MeA<sup>Kiss</sup> (tdTomato<sup>+</sup>) neurons that express c-Fos in the MeA after control or urine exposure ( $n = 6$  in each group). **d** Serum LH concentrations

(ng/mL) in male mice 15 min post-exposure ( $n = 5$  in each group). **e** Percentage of c-Fos<sup>+</sup> AVPV<sup>Kiss</sup> (tdTomato<sup>+</sup>) neurons after exposure to control or urine ( $n = 4$  in each group). **f** Percentage of c-Fos<sup>+</sup> ARC<sup>Kiss</sup> (tdTomato<sup>+</sup>) neurons after exposure to control or urine ( $n = 3$  in each group). Data are represented as mean  $\pm$  SEM. Values of individual mice are plotted as circles. ns, no significant difference; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  (unpaired two-tailed Student's *t* test). opt, optic tract, MeAa, anterior medial amygdaloid nucleus, MeApv, posteroventral medial amygdaloid nucleus, MeApd, posterodorsal medial amygdaloid nucleus.

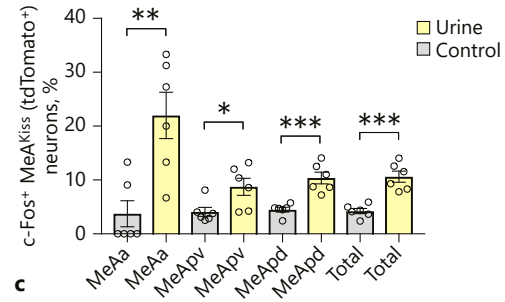
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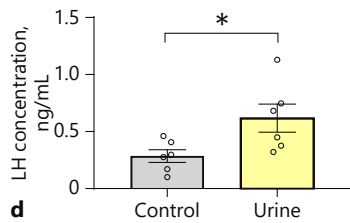
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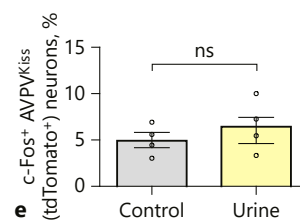
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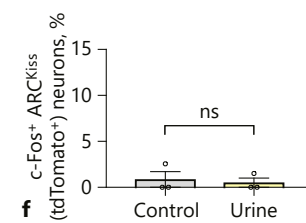
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### Hormone ELISA

Blood samples were collected in Multivette blood collection tubes (SARSTEDT; 15.1670) by submandibular cheek bleed. Serum was collected by centrifugation of whole blood at 2,000 rcf for 10 min and stored at  $-20^{\circ}\text{C}$ . LH levels were measured using a super-sensitive LH ELISA developed by the Chen Lab [28]. Serum samples were diluted 1/10 with assay buffer and 50  $\mu\text{L}$  of each diluted sample was assayed. The *o*-phenylenediamine substrate (Sigma-Aldrich; P918) was developed for 45 min at room temperature and stopped by the addition of 3 M  $\text{H}_2\text{SO}_4$ . The absorbance in each well was then measured at 490 nm. The LH ELISA had a detection limit of 0.04 ng/mL and good reproducibility with values of 6.4 and 11.5%, respectively, for the intra- and inter-assay variation coefficients.

### Histological Methods

Coronal brain sections (30  $\mu\text{m}$ ) were cut on a Leica cryostat and stored in 48-well plates in PBS containing 0.01% thimerosal at  $4^{\circ}\text{C}$ .

To verify that tdTomato-labelled MeA neurons in *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> male mice expressed *Kiss1* mRNA, we combined RNAscope ISH for *Kiss1* mRNA with immunofluorescence labelling for tdTomato on 30- $\mu\text{m}$  coronal brain sections from *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> males. The ISH was performed using the RNAscope Multiplex Fluorescent Kit v2 (Advanced Cell Diagnostics Inc.) using a mouse *Kiss1* probe set (Advanced Cell Diagnostics Inc.; Cat. No. 4762921) in accordance with the manufacturer's suggested procedure. The *Kiss1* probe set consists of a series of 20 target probes designed to hybridize to the 121- to 1,376-bp region of the mouse *Kiss1* transcript (accession No. XM\_006529679.2). Following completion of the RNAscope protocol, sections were immunolabelled using a rabbit anti-RFP polyclonal affinity purified antibody (1:1,000; Rockland, 600-401-379) [29] and a donkey anti-rabbit Alexa Fluor 555 (1:400; Invitrogen, A31572).

To investigate the responses of MeA<sup>Kiss</sup> neurons to pheromone exposure in *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> males ( $n = 6$  in each group), sections were immunolabelled using the rabbit anti-RFP antibody (1:1,000; Rockland) to label tdTomato<sup>+</sup> (MeA<sup>Kiss</sup>) neurons and a goat anti-c-Fos polyclonal affinity purified antibody (1:400; Santa Cruz Biotechnology, sc-52-G) [30] to label for c-Fos protein (a surrogate marker of neuronal activation). Negative controls for c-Fos IHC using either no primary antibody or isotype controls did not produce any significant fluorescence labelling.

**Fig. 2.** Verification and comparison of MeA neuron targeting using *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> mice and Cre-dependent rAAV infection of *Kiss1*<sup>CreEGFP/wt</sup> mice. **a** Representative photomicrograph showing tdTomato expression (red) and *Kiss1* mRNA expression (white) in the MeA of a double transgenic *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> male mouse. White arrows mark tdTomato<sup>+</sup> cells that are positive for *Kiss1* expression. **b** Representative photomicrograph showing tdTomato expression (red) and negligible RNAscope in situ signal (white) with a negative control probe (*dapB*; a bacterial gene) in the MeA of a double transgenic *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> male mouse. **c** Representative photomicrograph showing mCitrine (green) labelling in the MeA (Bregma 1.34 mm) of male *Kiss1*<sup>CreEGFP/wt</sup> mice infect-

Sections were mounted on SuperFrost Plus slides (ThermoScientific) and dried for 1 h at  $40^{\circ}\text{C}$  in a hybridization oven. The slides were washed in PBST (PBS + 0.05% Tween-20) for 10 min followed by a 5-min wash in PBS. Sections were blocked for 1 h at room temperature in blocking buffer (3% normal donkey serum in PBS with 0.4% Triton-X100) followed by overnight incubation with primary antibodies at  $4^{\circ}\text{C}$ . Sections were then washed thrice (5 min in PBS, 10 min in PBST, and 15 min in PBS) and incubated in secondary antibodies donkey anti-rabbit Alexa Fluor 594 (1:500; Jackson ImmunoResearch, 711-585-152) and donkey anti-goat Alexa Fluor 488 (1:500; Life Technologies, A11055) for 1 h at  $37^{\circ}\text{C}$ . After a 10-min wash in PBST, sections were incubated in Hoechst 33258 nuclear stain (1  $\mu\text{g}/\text{mL}$  in PBS; Sigma-Aldrich, 861405). Slides were then washed thrice before sections were mounted using Permafluor mountant (ThermoScientific; TA-030-FM) and coverslipped. Slides were allowed to dry in the dark overnight before image acquisition. This method was used for all the immunostaining experiments throughout the study. Sections from the AVPV and ARC were also processed using the same primary antibody combination to evaluate whether AVPV<sup>Kiss</sup> or ARC<sup>Kiss</sup> neurons respond to pheromone exposure. However, due to the endogenous EGFP expression in the AVPV and ARC region of *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup>, the secondary antibodies used for this experiment were donkey anti-rabbit Alexa Fluor 555 (1:500; Life Technologies, A31572) and donkey anti-goat Alexa Fluor 647 (1:600; Jackson ImmunoResearch, 705-605-147).

To visualize the localization of DREADD injections and the co-expression of hKORD and hM3D(Gq) in *Kiss1*<sup>CreEGFP/wt</sup> male mice ( $n = 3$ ), we localized mCitrine, the reporter that is co-expressed with hKORD in the viral transgene (pAAV-hSyn-dF-HA-hKORD-IRES-mCitrine) using a chicken anti-GFP antibody (1:10,000; Abcam, 13970) and we localized mCherry, which is linked to hM3D(Gq) in the viral construct (pAAV-EF1a-DIO-hM3D[Gq]-mCherry) using a rat anti-mCherry antibody (1:800, Life Technologies, M11217). The secondary antibodies used for this study were donkey anti-chicken Alexa Fluor 488 (1:500; Jackson ImmunoResearch, 703-546-155) and donkey anti-rat Alexa Fluor 594 (1:800).

To detect the level of DREADD-mediated modulation of hM3D(Gq)-expressing MeA<sup>Kiss</sup> neurons and hKORD-expressing MeA<sup>Kiss</sup> neurons in *Kiss1*<sup>CreEGFP/wt</sup> male mice ( $n = 3$  in each treatment group), we again used c-Fos as a marker of neuronal activation. We immunolabelled MeA sections using anti-mCherry and anti-c-Fos antibodies with donkey anti-rat Alexa Fluor 594 and

ed with rAAVs encoding hSyn-DIO-hKORD-IRES-mCitrine. **d** Representative photomicrograph of the MeA (Bregma 1.34 mm) labelled with tdTomato from a double transgenic *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> male mouse. **e** Comparison between the number of mCitrine<sup>+</sup> neurons in the MeA of *Kiss1*<sup>CreEGFP/wt</sup> male mice infected with AAV-hSyn-DIO-hKORD-IRES-mCitrine and tdTomato<sup>+</sup> neurons in the *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> male mice. **f** Distribution of fluorescently labelled neurons in the MeA between 1.34 and 1.94 mm posterior to Bregma in *Kiss1*<sup>CreEGFP/wt</sup> male mice infected with AAV-hSyn-DIO-hKORD-IRES-mCitrine (virally induced expression) and *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> male mice (transgenic induced expression). Scale bars, 100  $\mu\text{m}$ .

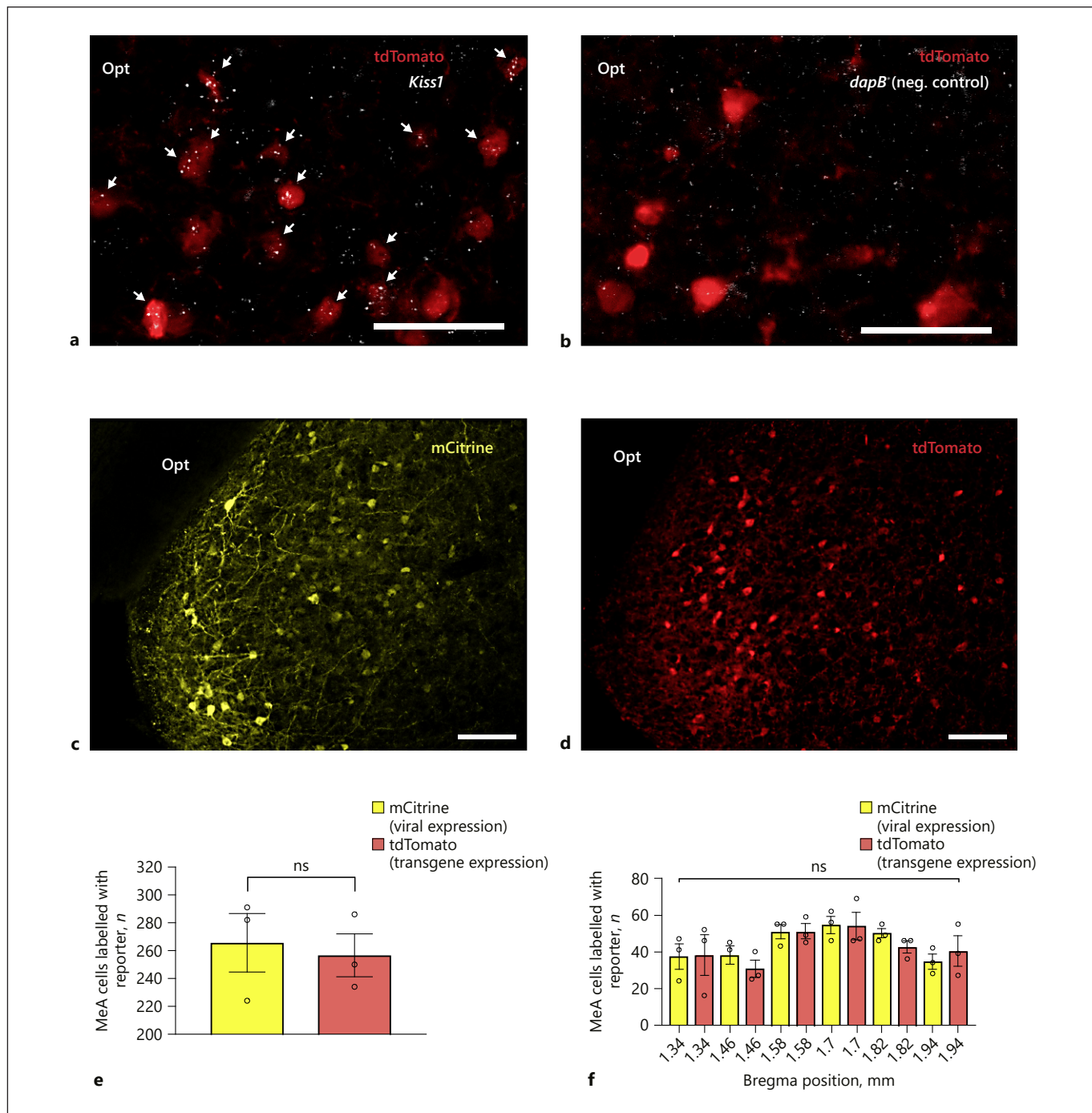
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donkey anti-goat Alexa Fluor 647 (1:600; Jackson ImmunoResearch, 705-605-147).

To interrogate how DREADD-induced modulation of MeA<sup>Kiss</sup> neurons influences neuronal activity in the POA, then to analyse c-Fos specifically in GnRH neurons, immunofluorescence analysis was carried out on every 4th section from 0.74 mm anterior to Bregma to 0.46 mm posterior to Bregma using the goat anti-c-Fos antibody and a rabbit anti-GnRH polyclonal antisera (1:1,200;

GF4, a generous gift from Prof. N. Sherwood, University of Victoria). The secondary antibodies used for this experiment were donkey anti-goat Alexa Fluor 647 and donkey anti-rabbit Alexa Fluor 488 (1:500; Invitrogen, A21206).

To identify which neurotransmitters MeA<sup>Kiss</sup> neurons express, we combined multiplexed RNAscope ISH for definitive markers of GABAergic (*Slc32a1* encoding the GABA vesicular transporter, Vgat; Advanced Cell Diagnostics Inc.; Cat. No. 319191) and gluta-



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maternal transmission (*Slc17a6* encoding the glutamate vesicular transporter, Vglut2; Advanced Cell Diagnostics Inc.; Cat. No. 319171) with immunofluorescence labelling of tdTomato<sup>+</sup> MeA<sup>Kiss</sup> neurons on coronal brain sections from *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> males ( $n = 3$ ). The dual ISH was performed using the RNAscope Multiplex Fluorescent Kit v2 (Advanced Cell Diagnostics Inc.) with probes for Vgat mRNA (*Slc32a1*; this probe set was designed to target the 894- to 2,037-bp region of the mouse *Slc32a1* transcript; accession No. NM\_009508.2) and Vglut2 mRNA (*Slc17a6*; this probe set was designed to target the 1,986- to 2,998-bp region of the mouse *Slc17a6* transcript; accession No. NM\_080853.3) in accordance with the manufacturer's suggested procedure. Following completion of the RNAscope protocol, sections were immunolabelled using the rabbit anti-RFP antibody (1:1,000; Rockland) and a donkey anti-rabbit Alexa Fluor 555 (1:400; Invitrogen, A31572) antibody.

### Imaging and Analysis

The multiplexed RNAscope ISH/immunofluorescence labelling experiments were imaged using a Leica TCS SP8 confocal laser scanning microscope equipped with a Leica 20× multi-immersion objective (NA 0.75). The z-stack images were acquired at 1.04- $\mu\text{m}$  steps through 16  $\mu\text{m}$  of tissue (1,024 × 1,024 pixels, pinhole size set at 1 Airy unit). Stacks were analysed using Leica Application Suite Advanced Fluorescence software (LAS X; Leica Microsystems, Wetzlar, Germany). To evaluate the co-expression levels of tdTomato (Kiss neurons), mRNAs for Vgat (*Slc32a1*) and Vglut2 (*Slc17a6*), z-stacks were collected using a Nyquist sampling rate and approximately 150 MeA<sup>Kiss</sup> neurons were analysed in each of 3 animals over three Bregma positions. All other fluorescence immunolabelling experiments were imaged using a Leica CTR5500 fluorescence microscope. Light intensities and image acquisition settings were kept constant across all treatments and post-imaging analysis and quantification was done using Fiji software [31]. Investigators were blinded to exposure conditions/treatments and a second investigator independently confirmed all analyses.

To quantify the number of c-Fos-positive nuclei, then to quantify the number of c-Fos-positive MeA<sup>Kiss</sup> neurons (tdTomato<sup>+</sup>) in *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> males in response to pheromone exposure, a total of 6 sections from 1.22 to 1.82 mm posterior to Bregma were analysed. One section was representative of anterior MeAa and five sections covered both

the posteroventral MeA (MeApv) and posterodorsal MeA (MeApd) from each mouse. Regions of interest were identified and selected based on the mouse brain atlas [27] by using the optic tract as a landmark. Counts were carried out manually after subtracting the background and adjusting the brightness and contrast equally for all images using Fiji software [31]. To quantify the number of c-Fos-positive AVPV<sup>Kiss</sup> neurons (tdTomato<sup>+</sup>) in *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> males in response to pheromone exposure, a total of 7 sections starting at 0.50 mm anterior to Bregma to 0.22 mm posterior to Bregma were analysed from each mouse. Regions of interest were identified based on the mouse brain atlas [27] using the third ventricle and the anterior commissure as landmarks. To quantify the number of c-Fos-positive ARC<sup>Kiss</sup> neurons (tdTomato<sup>+</sup>) in *Kiss1*<sup>CreEGFP/wt</sup>/*Rosa26*<sup>lox-stop-lox-tdTomato/wt</sup> males in response to pheromone exposure, a total of 6 sections starting at 1.34 mm posterior to Bregma to 1.94 mm posterior to Bregma were analysed from each mouse. Regions of interest were identified based on the mouse brain atlas [27] using the third ventricle and the median eminence as landmarks. Co-expression analysis was carried out manually using the ROI Manager in Fiji software [31].

Similarly, DREADD and c-Fos co-expression in MeA<sup>Kiss</sup> neurons was analysed in 6 sections from 1.22 to 1.82 mm posterior to Bregma. Quantification of c-Fos in the POA was carried out on every 4th section from 0.74 mm anterior to Bregma to 0.46 mm posterior to Bregma. Regions of interest were identified and selected based on the mouse brain atlas [27] by using the third ventricle, anterior commissure, and optic tract as landmarks. Counts were carried using the "Analyze particles" plugin in Fiji software [31] using the following selection criteria – minimum pixel area size set at 2 and maximum 30 and circularity criteria between 0 and 1. Selected nuclei were confirmed visually.

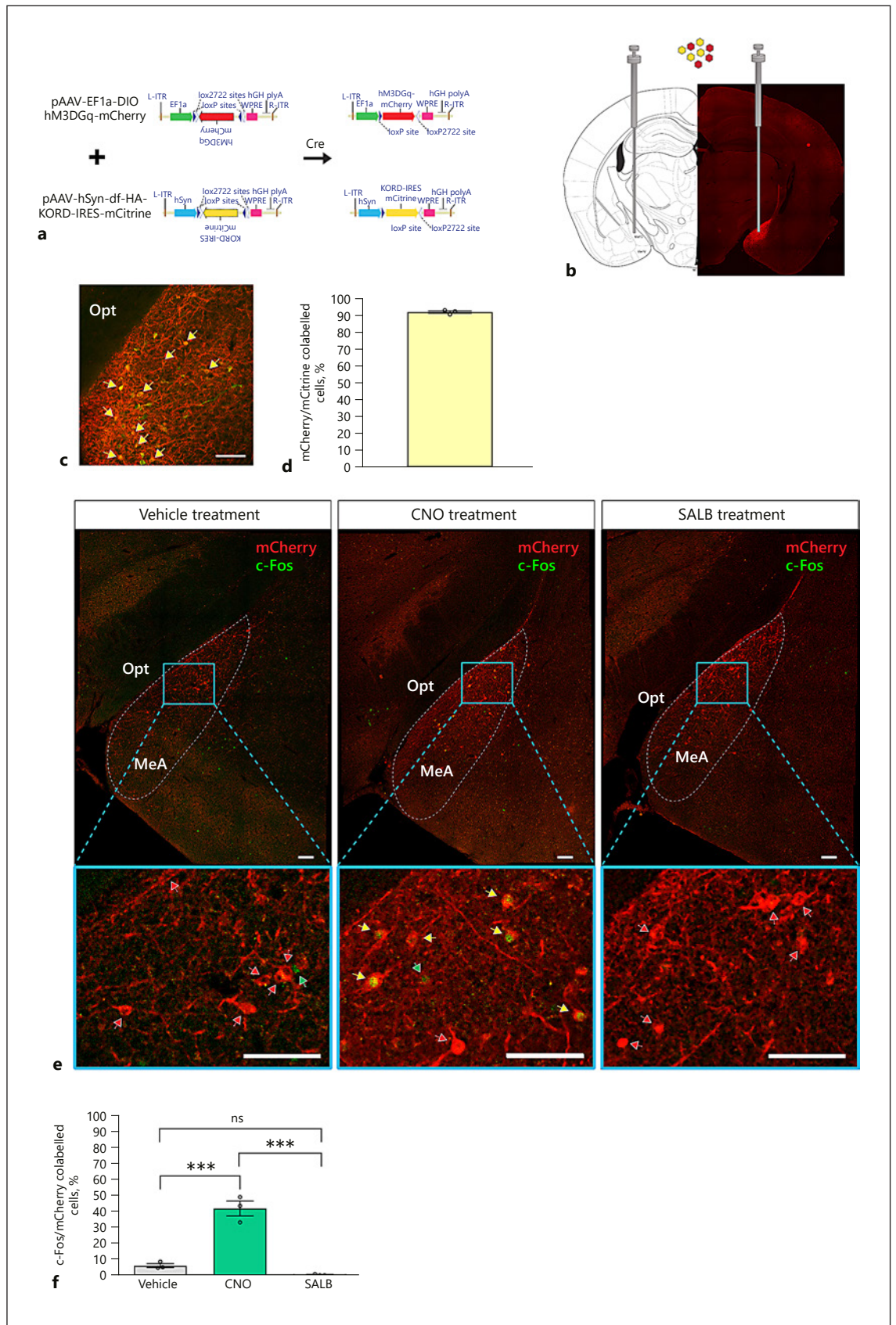
### Experimental Design and Statistical Analysis

Only male mice were used for these studies as our objective was to understand the role of MeA<sup>Kiss</sup> neurons in mediating pheromone influences on male reproductive endocrine physiology. The sample size for each individual experiment is listed in Results. Statistical analysis was conducted using GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA, USA). The number of cells per group or animals per group is indicated by  $n$ . Data were compared as dictated by distribution and experimental design; tests are spec-

**Fig. 3.** Chemogenetic control of MeA<sup>Kiss</sup> neurons in *Kiss1*<sup>CreEGFP/wt</sup> male mice. Male *Kiss1*<sup>CreEGFP/wt</sup> mice received bilateral MeA stereotaxic injections of an equal mixture of AAV1/2- EF1 $\alpha$ -DIO-hM3D(Gq)-mCherry and AAV1/2-hSyn-DIO-hKORD-IRES-mCitrine. After 3 weeks, the mice were treated with CNO (3  $\mu\text{g/g}$ ), SALB (10  $\mu\text{g/g}$ ), or vehicle (DMSO). **a** Schematic representation of the AAV vectors encoding hM3D(Gq) and hKORD, both with a FLEX switch allowing recombination (activation) under the control of Cre-recombinase. **b** Location of stereotaxic viral injection to target Cre-expressing MeA<sup>Kiss</sup> neurons and representative image montage that confirms mCherry expression restricted to the injection site. **c** Co-localization of hM3D(Gq) (mCherry) and hKORD (mCitrine) marked by yellow arrows. **d** Quantification of

hM3D(Gq) (mCherry) and hKORD (mCitrine) virus co-expression in the MeA ( $n = 3$ ). **e** Location of virally expressed hM3D(Gq) (mCherry) and induction of c-Fos (green) 60 min after treatments (vehicle, CNO, or SALB). Yellow arrows mark c-Fos<sup>+</sup> virally transduced cells (mCherry), red arrows mark mCherry cells lacking c-Fos expression, and green arrows mark c-Fos<sup>+</sup> nuclei. **f** Quantification of c-Fos<sup>+</sup> cells as a percentage of mCherry cells in MeA 60 min after vehicle, CNO, or SALB treatment ( $n = 3$  in each group). Data are represented as mean  $\pm$  SEM. Circles represent the individual values for each mouse. ns, no significant difference; \*\*\*  $p < 0.001$  (one-way ANOVA followed by Tukey's multiple comparison test). opt, optic tract, MeA, medial amygdaloid nucleus. Scale bars, 100  $\mu\text{m}$ .

(For figure see next page.)



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ified in the results. Data were analysed using a two-tailed unpaired Student's *t* test or a one-way ANOVA followed by Tukey's multiple comparison test. Data are reported as mean  $\pm$  SEM. Significance was set at  $p < 0.05$  in all analyses.

## Results

### *Female Urine Exposure Activates MeA<sup>Kiss</sup> Neurons and Raises LH in Male Mice*

To determine whether MeA<sup>Kiss</sup> neurons integrate chemosensory stimuli with reproductive endocrine status, we exposed double transgenic *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* males to female urine and measured c-Fos expression in tdTomato<sup>+</sup> MeA<sup>Kiss</sup> neurons. Counting the overall number of c-Fos<sup>+</sup> cells in each subdivision revealed that only the MeApv and MeApd subdivisions of the MeA showed significant induction of c-Fos, whereas the MeAa did not (Fig. 1a, b). Double immunofluorescence labelling (for c-Fos and tdTomato) revealed that the percentage of c-Fos immunoreactive (c-Fos-ir) MeA<sup>Kiss</sup> neurons was significantly higher in the MeApd of males exposed to female urine ( $10.4 \pm 1.1\%$ ;  $n = 80 \pm 5$  tdTomato<sup>+</sup> neurons counted in the MeApd of 6 animals) compared to water exposure ( $4.5 \pm 0.4\%$ ;  $n = 81 \pm 3$ , total number of tdTomato<sup>+</sup> neurons counted in the MeApd of 6 animals) (\*\* $p < 0.001$ ; unpaired two-tailed Student's *t* test; Fig. 1a, c). Similarly, a significantly higher percentage of c-Fos-ir MeA<sup>Kiss</sup> neurons was observed in the MeApv of males exposed to female urine ( $8.7 \pm 1.6\%$ ;  $n = 70 \pm 7$  tdTomato<sup>+</sup> neurons counted in the MeApv of 6 animals) compared to water exposure ( $4.1 \pm 0.8\%$ ;  $n = 69 \pm 4$  tdTomato<sup>+</sup> neurons counted in the MeApv of 6 animals) (\* $p < 0.05$ ; unpaired two-tailed Student's *t* test; Fig. 1c and online suppl. Fig. S1; for all online suppl. material, see [www.karger.com/doi/10.1159/000496106](http://www.karger.com/doi/10.1159/000496106)). Despite the lack of change in overall neuronal activity within the MeAa, a significantly increased number of c-Fos-ir MeA<sup>Kiss</sup> neurons was observed in the MeAa of males exposed to female urine ( $21.9 \pm 4.3\%$ ;  $n = 14 \pm 1$ , total number of tdTomato<sup>+</sup> neurons counted in the MeAa of 6 animals) compared to animals exposed to water ( $3.7 \pm 2.4\%$ ;  $n = 14 \pm 1$  tdTomato<sup>+</sup> neurons counted in the MeAa of 6 animals) (\* $p < 0.05$ ; unpaired two-tailed Student's *t* test; Fig. 1c and online suppl. Fig. S2). To test for pheromone-induced changes in serum LH levels, blood was sampled 15 min following urine exposure. Mean serum LH levels of both groups are shown in Figure 1d. Serum LH concentrations at T = 15 were significantly higher in males exposed to female urine ( $0.68 \pm 0.13$  ng/mL, monitored in 5 animals) com-

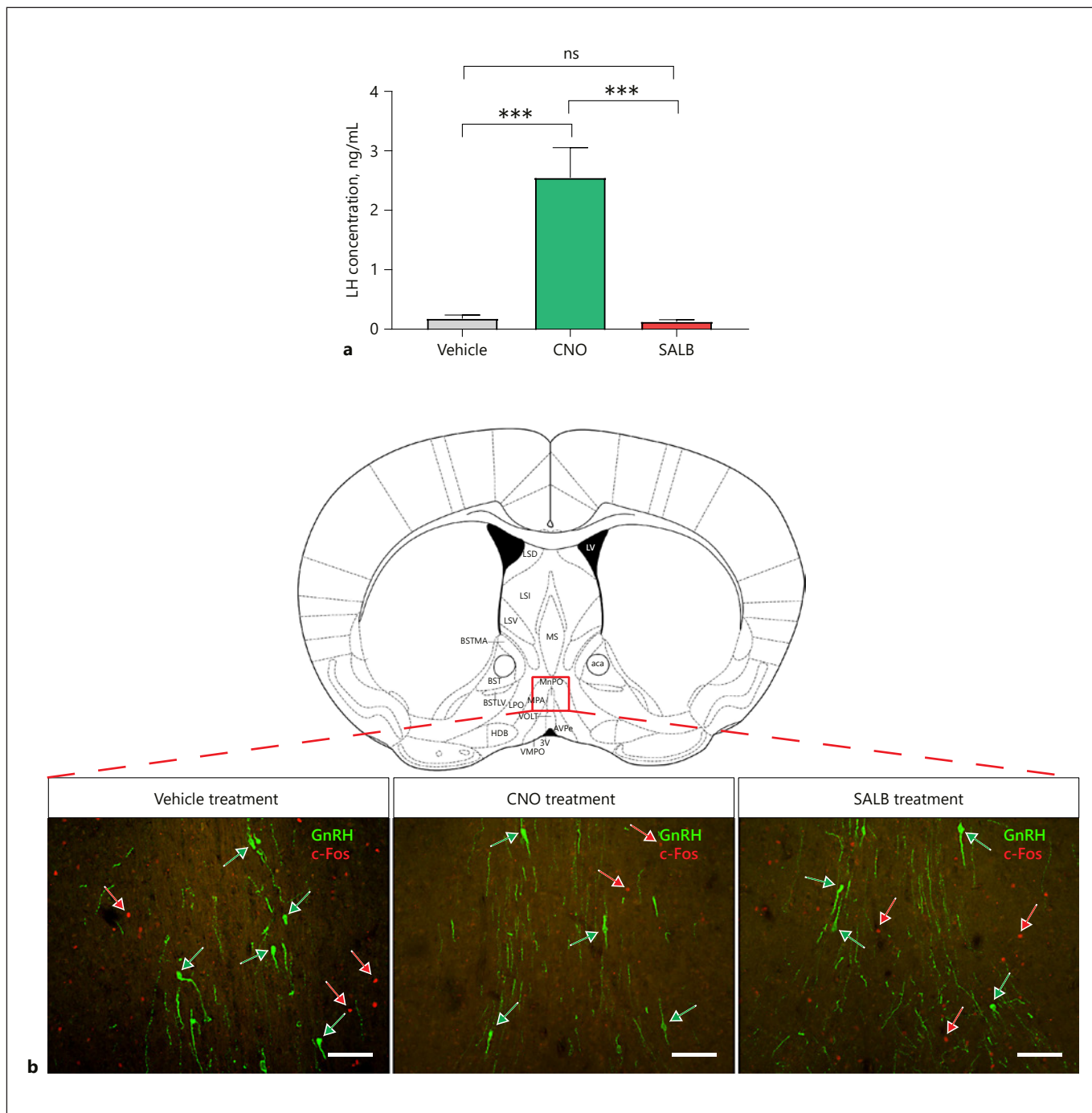
pared to water ( $0.31 \pm 0.06$  ng/mL, monitored in 5 animals;  $p < 0.05$ ; unpaired two-tailed Student's *t* test), but no significant activation of AVPV<sup>Kiss</sup> (tdTomato<sup>+</sup>) neurons was detected with urine exposure ( $6.5 \pm 1.4$  vs.  $4.9 \pm 0.8\%$  in control;  $n = 6$  in each group; no significant difference; unpaired two-tailed Student's *t* test; Fig. 1e and online suppl. Fig. S3). Similarly, no significant activation of ARC<sup>Kiss</sup> (tdTomato<sup>+</sup>) neurons was detected with urine exposure ( $0.5 \pm 0.5$  vs.  $0.85 \pm 0.85\%$  in control;  $n = 3$  in each group; no significant difference; unpaired two-tailed Student's *t* test; Fig. 1f and online suppl. Fig. S4).

### *Verification of MeA<sup>Kiss</sup> Neuronal Labelling Strategies*

To verify that fluorescently labelled neurons in the MeA of *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* mice express *Kiss1*, we analysed the number of tdTomato<sup>+</sup> neurons in the MeA that express *Kiss1* mRNA by combining RNAscope ISH and IHC (Fig. 2a). The negative control RNAscope in situ probes did not display any significant signal in tdTomato<sup>+</sup> MeA neurons in *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice (Fig. 2b). A total of 73 tdTomato<sup>+</sup> neurons were counted in 3 MeA sections (1.22, 1.46, and 1.58 mm posterior to Bregma) of which 67 tdTomato<sup>+</sup> neurons displayed a positive *Kiss1* mRNA signal (approx. 92%). We also compared the number of fluorescently labelled (tdTomato<sup>+</sup>) neurons in adult *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* mice to fluorescently labelled (mCitrine<sup>+</sup>) neurons in *Kiss1<sup>CreEGFP/wt</sup>* mice infused with Cre-dependent AAVs encoding hKORD (this DREADD is linked to mCitrine). No significant difference in the number of labelled cells was observed between the two labelling approaches (Fig. 2c, d). An average of  $265.7 \pm 21$  mCitrine<sup>+</sup> neurons were observed in 3 *Kiss1<sup>CreEGFP/wt</sup>* male mice infected with hKORD and  $256.7 \pm 15.4$  tdTomato<sup>+</sup> neurons were observed in the MeA of 3 *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice (Fig. 2e). The distribution of fluorescently labelled cells in the MeA between 1.34 and 1.94 mm posterior to Bregma revealed no significant difference (unpaired two-tailed Student's *t* test; Fig. 2f).

### *Localization of DREADD Infections and Co-expression of hM3D(Gq) and hKORD*

To investigate the influence of MeA<sup>Kiss</sup> neurons on the reproductive endocrine axis, we reciprocally modulated MeA<sup>Kiss</sup> neurons by co-infusing two Cre-dependent rAAVs encoding hM3D(Gq) (EF1 $\alpha$ -DIO-hM3D[Gq]-mCherry) and hKORD (hSyn-DIO-hKORD-IRES-mCitrine) DREADDs into the MeA of *Kiss1<sup>CreEGFP/wt</sup>* male mice (Fig. 3a, b). Approximately 92% co-expression was



**Fig. 4.** Chemogenetic activation of MeA<sup>Kiss</sup> neurons in *Kiss1<sup>CreEGFP/wt</sup>* male mice stimulates LH release but fails to activate GnRH neurons. Male *Kiss1<sup>CreEGFP/wt</sup>* mice received MeA injections of rAAVs encoding EF1 $\alpha$ -DIO-hM3D(Gq)-mCherry and hSyn-DIO-hKORD-IRES-mCitrine. After 3 weeks, the mice were treated with SALB (10  $\mu$ g/g), CNO (3  $\mu$ g/g), or vehicle (DMSO). **a** Serum LH concentration (ng/mL) in male mice 30 min following drug treatment ( $n = 23$ –24 in each group).

Data are represented as mean  $\pm$  SEM. ns, no significant difference; \*\*\*  $p < 0.001$  (one-way ANOVA followed by Tukey's multiple comparison test). **b** Representative photomicrographs showing GnRH (green) and c-Fos (red) labelling in the POA (Bregma 0.50 mm) 60 min after vehicle, CNO, or SALB treatment. Green arrows mark GnRH cell bodies and red arrows mark c-Fos<sup>+</sup> nuclei. Scale bars, 100  $\mu$ m.

observed between mCitrine- and mCherry-expressing neurons in the MeA ( $n = 145 \pm 7$ , number of mCherry<sup>+</sup> neurons expressing mCitrine monitored in 3 animals; Fig. 3c, d). This high degree of co-expression indicated that both fluorescent markers could be used interchangeably, and on-target injections were confirmed using mCherry immunofluorescence (Fig. 3b). Of the 30 *Kiss1<sup>CreEGFP/wt</sup>* male mice that received rAAV-DREADD injections, 24 mice received on-target bilateral rAAV infections. In mice that received on-target rAAV infections, CNO (the hM3D[Gq] agonist) significantly elevated c-Fos expression in MeA<sup>Kiss</sup> neurons ( $42.0 \pm 4.6\%$ ,  $n = 107 \pm 7$ , number of mCherry<sup>+</sup> neurons monitored in 3 animals) compared to the SALB (hKORD agonist)-treated group ( $0.3 \pm 0.3\%$ ,  $n = 144 \pm 15$ , mCherry<sup>+</sup> neurons monitored in 3 animals) and vehicle (DMSO) controls ( $6.1 \pm 1.2\%$ ,  $n = 115 \pm 24$  mCherry<sup>+</sup> neurons monitored in 3 control animals, \*\*\*  $p < 0.001$ ,  $F$  value = 66.56; one-way ANOVA followed by Tukey's multiple comparison test; Fig. 3e, f) with no significant difference observed between the SALB-treated group and controls.

#### *Chemogenetic Stimulation of MeA<sup>Kiss</sup> Neurons Leads to a Significant LH Rise*

To investigate whether MeA<sup>Kiss</sup> neurons can directly elicit a reproductive endocrine response, we measured serum LH concentrations after administration of chemogenetic drugs. Blood samples were collected 30 min after treating mice with CNO (3 µg/g), SALB (10 µg/g), or vehicle (DMSO). Serum LH concentrations increased significantly in mice receiving CNO compared to SALB or vehicle treatment. At 30 min post-treatment, mean serum LH was significantly elevated in the CNO group ( $2.40 \pm 0.48$  ng/mL, monitored in 24 animals) compared to either the SALB-treated group ( $0.11 \pm 0.01$  ng/mL, monitored in 24 animals) or the vehicle-treated controls ( $0.17 \pm 0.05$  ng/mL in the vehicle treated controls, monitored in 23 animals, \*\*\*  $p < 0.001$ ,  $F$  value = 23.39; one-way ANOVA followed by Tukey's multiple comparison test, Fig. 4a). To check for non-specific drug-induced effects on serum LH, blood samples from mice that were administered

chemogenetic drugs but received off-target rAAV injections were also analysed. No significant changes to LH were observed in these animals (online suppl. Fig. S5).

#### *Chemogenetic Modulation of MeA<sup>Kiss</sup> Neurons Decreases Neuronal Activity in the POA*

To determine whether the CNO-mediated rise in serum LH coincided with increased c-Fos-ir in GnRH neurons (Fig. 4b), we examined the level of c-Fos activity in GnRH neurons using double immunofluorescence labeling (for GnRH and c-Fos). Despite the elevated LH after CNO treatment, c-Fos-ir was undetectable in GnRH neurons in all groups ( $66 \pm 5$  GnRH neurons counted in 3 mice receiving DMSO,  $60 \pm 6$  GnRH neurons counted in 3 mice receiving SALB, and  $66 \pm 9$  GnRH neurons counted in 3 mice receiving CNO). Instead, CNO-mediated stimulation of hM3D(Gq)-expressing MeA<sup>Kiss</sup> neurons led to reduced neuronal activity in the POA compared to controls and SALB treatment (Fig. 5a). The number of c-Fos-ir cells in the POA after CNO treatment was  $113.2 \pm 6.9$  compared to  $173.7 \pm 17.7$  with SALB treatment and  $179.3 \pm 9.7$  in controls (\*  $p < 0.05$ ,  $F$  value = 9.055; one-way ANOVA followed by Tukey's multiple comparison test;  $n = 3$  each, Fig. 5b).

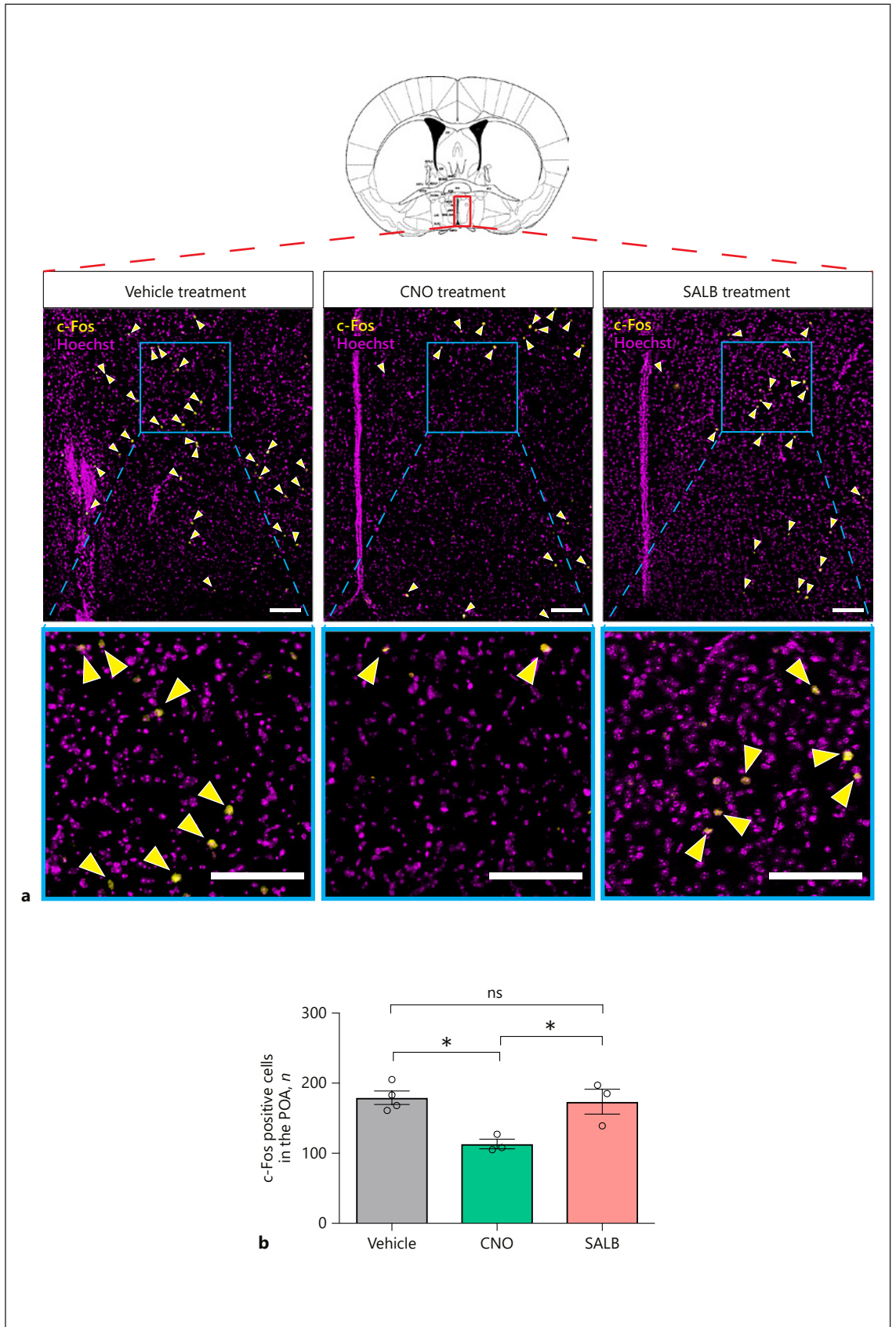
#### *A Higher Proportion of MeA<sup>Kiss</sup> Neurons Express Markers for GABAergic Transmission rather than Glutamatergic Transmission*

In order to determine which neurotransmitters MeA<sup>Kiss</sup> neurons (tdTomato<sup>+</sup>) elaborate, we carried out multiplexed RNAscope ISH for mRNA encoding the glutamate vesicular transporter type 2 (Vglut2) or the GABA vesicular transporter (Vgat) on brain sections from *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice. Using a confocal laser scanning microscope (Leica TCS SP8), z-stack images were acquired to analyse Vglut2 and Vgat mRNA expression in MeA<sup>Kiss</sup> (tdTomato<sup>+</sup>) neurons (Fig. 6a). The negative control RNAscope in situ probes designed against the bacterial *dapB* gene did not display any significant signal in the MeA in *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice

**Fig. 5.** Chemogenetic activation of MeA<sup>Kiss</sup> neurons reduces neuronal activity in the POA in male mice. Male *Kiss1<sup>CreEGFP/wt</sup>* mice received MeA injections of rAAVs encoding EF1α-DIO-hM3D(Gq)-mCherry and hSyn-DIO-hKORD-IRES-mCitrine. After 3 weeks, the mice were treated with SALB (10 µg/g), CNO (3 µg/g), or vehicle (DMSO). **a** Representative photomicrographs showing Hoechst-stained nuclei (magenta) and c-Fos<sup>+</sup> nuclei (yel-

low) in the POA (Bregma -0.10 mm) 60 min after treatments. Arrowheads mark c-Fos<sup>+</sup> nuclei. Scale bars, 100 µm. **b** Number of c-Fos<sup>+</sup> nuclei in the POA analysed 60 min after treatments ( $n = 3$  in each group). Data are represented as mean ± SEM. Circles represent the individual values for each mouse. ns, no significant difference; \*  $p < 0.05$ , (one-way ANOVA followed by Tukey's multiple comparison test).

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(Fig. 6b). In MeA<sup>Kiss</sup> neurons, the highest proportion were labelled for Vgat (71.1 ± 2.7%) compared to Vglut2 (28.7 ± 4.3%), and a small proportion expressed both (6.4% ± 1.6%, *n* = 150 tdTomato<sup>+</sup> neurons monitored in each of 3 animals; Fig. 6c, d).

## Discussion

In rodents, the MeA receives pheromonal information from the accessory olfactory system and signals to the hypothalamus to coordinate appropriate sexual behaviours and reproductive endocrine responses. Although activation of the MeA in response to opposite-sex chemosensory cues has been reported previously [8, 32], the specific neural circuit underlying the reproductive endocrine response remains poorly defined. Due to the recently revealed connectivity of MeA<sup>Kiss</sup> neurons with the AOB and GnRH neurons [14], we hypothesized that MeA<sup>Kiss</sup> neurons process sexually relevant olfactory stimuli to regulate reproductive neuroendocrine function. Male mice are preferentially attracted to ovary-intact females in proestrous and oestrous stages [33], therefore urine used as a chemosensory stimulus was collected from ovary-intact females primarily from proestrous and oestrous stages. Using double transgenic *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice, we observed a significant elevation of c-Fos in both the MeApv and MeApd, but not in the MeAa after female urine exposure. These data are in line with other studies in male mice which found elevated c-Fos throughout the MeA after female pheromone exposure, but c-Fos-ir was most prominent in the MeApd [8, 32]. We then quantified c-Fos-ir specifically within MeA tdTomato<sup>+</sup> (MeA<sup>Kiss</sup>) neurons in response to urine exposure and observed raised c-Fos levels in MeA<sup>Kiss</sup> neurons throughout the MeA, but this was most significant in the MeApd. Our data are supported by anatomical tracing studies that show

the AOB provides dense input to all three MeA subdivisions [34], but neurons sensitive to volatile molecules which induce reproductive neuroendocrine effects (oestrous induction, puberty acceleration or delay) terminate principally in the MeApd [35, 36]. Reciprocal connections between MeApd MeA<sup>Kiss</sup> neurons and the anterior AOB have been identified previously [14], suggesting that these neurons are directly targeted by pheromonal pathways but also may feedback to the AOB. When the overall number of c-Fos<sup>+</sup> neurons in the MeA was compared to the number of c-Fos<sup>+</sup> MeA<sup>Kiss</sup> (tdTomato<sup>+</sup>) neurons (online suppl. Table 1), only a small number of c-Fos<sup>+</sup> neurons were found to be MeA<sup>Kiss</sup> neurons. This result is similar to that of a recent study using another transgenic mouse line (*Kiss1-hrGFP*), which found that the majority of pheromone-induced c-Fos-ir in the MeA was not in MeA<sup>Kiss</sup> neurons [37]. This study detected increased c-Fos-ir in MeA<sup>Kiss</sup> (hrGFP<sup>+</sup>) neurons, but the increase was not found to be statistically significant, likely due to the small number of samples analysed.

Using the double transgenic *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* mouse line enabled visualization of MeA<sup>Kiss</sup> neurons in mice, which has been difficult using conventional immunological methods or other transgenic approaches (e.g., *Kiss1<sup>CreEGFP</sup>*). Acknowledging the limitation of this approach and considering that crossing the *Kiss1<sup>CreEGFP</sup>* mice with Cre-dependent reporter lines may label transient Cre-expressing cells (e.g., cells that express Cre during development) [21], we first verified *Kiss1* mRNA expression in MeA tdTomato<sup>+</sup> cells and found approximately 92% of tdTomato<sup>+</sup> cells to be positive for *Kiss1* mRNA expression. We then compared adult male mice from the *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* line with adult male *Kiss1<sup>CreEGFP/wt</sup>* mice infused with Cre-dependent rAAVs 3 weeks prior, where cell labelling occurs only if viral infection coincides with Cre expression within the period post-infusion. Counting the number of

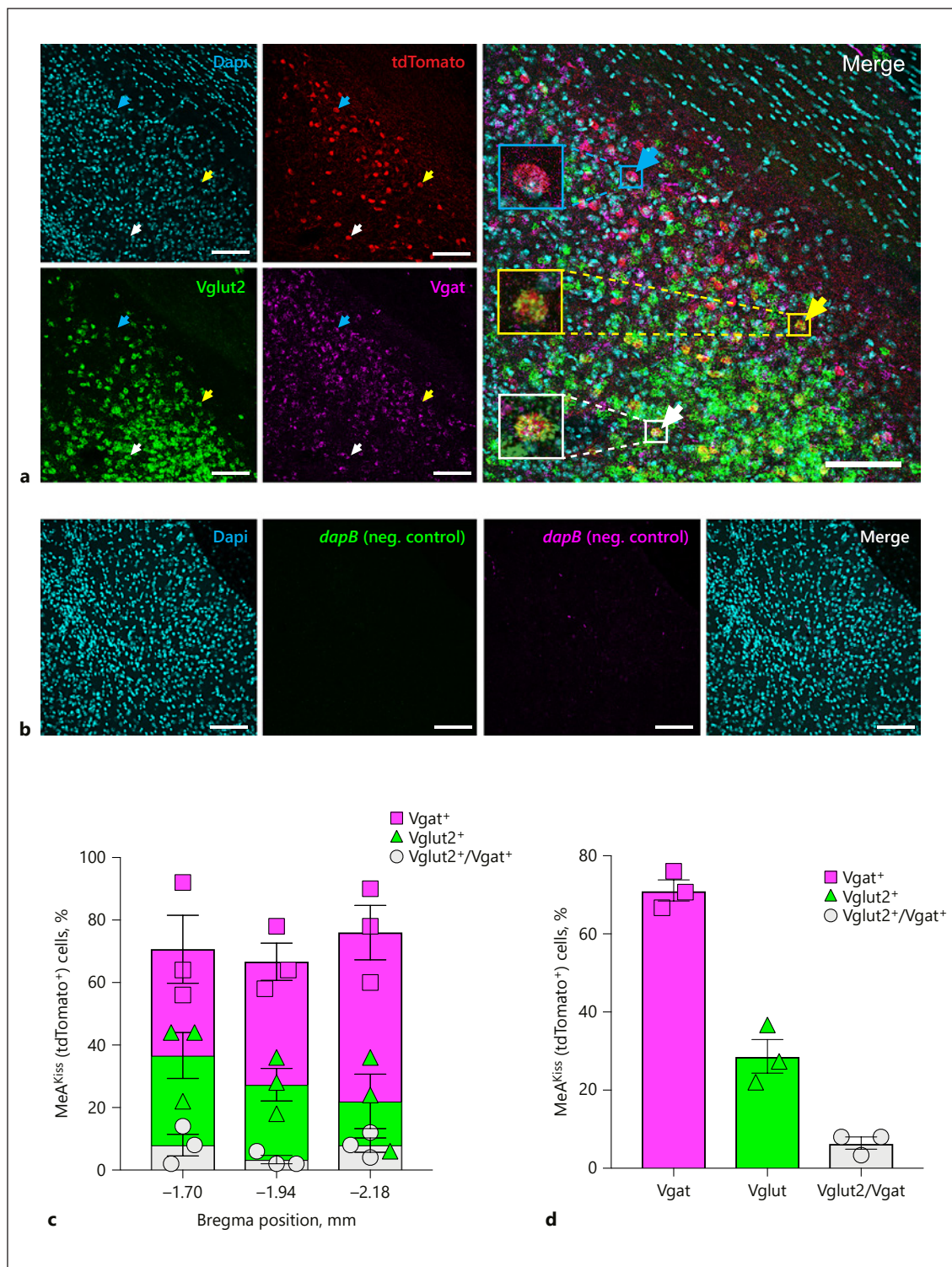
**Fig. 6.** Amygdala *Kiss1* neurons express mRNAs encoding *Vgat* and *Vglut2* in male mice. **a** A representative confocal micrograph (16 μm thickness) displays Dapi nuclear stain (cyan), tdTomato (red), *Vglut2* (green), and *Vgat* (magenta) expression in the MeA in *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice. A cyan arrow marks a tdTomato<sup>+</sup> cell that expresses *Vgat* mRNA, a yellow arrow marks a tdTomato<sup>+</sup> cell that expresses *Vglut2* mRNA, and a white arrow marks a tdTomato<sup>+</sup> cell that expresses both. Scale bars, 100 μm. **b** Representative confocal micrographs (16 μm thickness) that display negligible RNAscope in situ signals with RNAscope 3-plex negative control probes in the MeA in *Kiss1<sup>CreEGFP/wt</sup>/Rosa26<sup>lox-stop-lox-tdTomato/wt</sup>* male mice. Dapi nuclear stain (cyan); RNA-

scope 3-plex negative control probe (*dapB*; a bacterial gene) developed in the FITC channel (green); RNAscope 3-plex negative control probe (*dapB*; a bacterial gene) developed in the Cy5 channel (magenta), and Merge of all three channels. Scale bars, 100 μm. **c** Quantification of labelled MeA<sup>Kiss</sup> neurons expressing *Vgat* (squares), *Vglut2* (triangles), or both (circles) as a percentage of tdTomato<sup>+</sup> cells at three Bregma positions (*n* = 150 cells monitored in each of 3 animals). **d** The overall percentage of labelled MeA<sup>Kiss</sup> neurons expressing *Vgat* (squares), *Vglut2* (triangles), or both (circles) (*n* = 3). Data are represented as mean ± SEM with the value from each individual mouse indicated.

(For figure see next page.)

fluorescently marked neurons in the MeA revealed no difference between the two labelling approaches, supporting that these two labelling strategies mark equivalent numbers of Cre-expressing MeA (MeA<sup>Kiss</sup>) neurons,

which indicates that a negligible number of MeA neurons are labelled during development. Our imaging studies found that fluorescently labelled MeA<sup>Kiss</sup> neurons were concentrated in the MeApd but were also distributed



throughout the MeAa and MeApv, whereas previous studies have located *Kiss1* mRNA principally in the MeApd of mice [38]. In mice, kisspeptin protein is almost undetectable using conventional immunolabelling techniques, probably due to much lower expression or more rapid release compared to other rodents [38, 39]. However, recent labelling approaches using different transgenic mouse lines have confirmed that *Kiss1* gene-driven reporters localize in the MeAa and MeApv [37, 40], supporting our data.

Sex pheromones are an important means of communication between many rodent species. However, the neuronal and molecular basis of processing pheromone signals appears to be sexually dimorphic. In female rats, male odours lead to activation of AVPV<sup>Kiss</sup> neurons coincident with elevated LH and increased sexual behaviour [41]. Similarly, in mice, male urine leads to the activation of AVPV<sup>Kiss</sup> neurons and increased lordosis behaviour in females [42]. These studies in female rodents contrast with our male study, which found that female urine failed to activate AVPV<sup>Kiss</sup> or ARC<sup>Kiss</sup> neurons in male mice (online suppl. Table 2, Fig. S3, S4), but instead activated MeA<sup>Kiss</sup> neurons coincident with significantly raised LH. These data indicate that in male mice, MeA<sup>Kiss</sup> neurons have a crucial role in processing opposite-sex pheromones to influence reproductive endocrine status.

In order to precisely define whether MeA<sup>Kiss</sup> neurons regulate hypothalamic activity and orchestrate endocrine responses, we utilized a multiplexed chemogenetic approach, injecting a mixture of Cre-dependent AAVs encoding hM3D(Gq) (stimulatory) and hKORD (inhibitory) DREADDs to reciprocally modulate MeA<sup>Kiss</sup> neurons in the absence of female urine exposure. Co-expression of hM3D(Gq) and hKORD has previously been used to remotely control neural circuits in mice [26] and our study achieved over 90% co-expression of hM3D(Gq) and hKORD DREADDs in MeA<sup>Kiss</sup> neurons. Treatment with CNO depolarizes and increases the firing rate of hM3D(Gq)-expressing neurons [24], whereas SALB administration induces hyperpolarization (silencing) of hKORD-expressing neurons [26]. In our study, administration of CNO significantly elevated c-Fos-ir in hM3D(Gq)-expressing neurons and led to an impressive 15-fold higher LH level compared to controls and SALB-treated mice, whereas SALB treatments failed to reduce LH below the level in vehicle-treated controls. However, the LH levels in control animals were already very low, which may have precluded the demonstration of a decrease to near unmeasurable levels after SALB treatment.

It is also plausible that MeA<sup>Kiss</sup> neurons do not constitutively release an HPG-stimulatory factor (e.g., kisspeptin or glutamate) in the absence of an olfactory stimulus. Surprisingly, the large CNO-mediated induction of LH release was not accompanied by increased c-Fos-ir in GnRH neurons despite ~40% of the MeA<sup>Kiss</sup> neurons being activated. Previous studies in male mice have also failed to detect pheromone-induced c-Fos-ir in GnRH neurons accompanying LH rises [3] or after increased neuronal activation in the amygdala [8]. Previous research has shown that GnRH neurons in mice extend long dendritic projections to the median eminence and receive synaptic input along their length. The absence of GnRH soma activation may be due to the ability of these synapses to induce action potentials without activating the cell soma [43]. Interestingly, the median eminence is largely devoid of any MeA<sup>Kiss</sup> projections (online suppl. Fig. S6), thus it appears unlikely that MeA<sup>Kiss</sup> neurons directly influence GnRH release at GnRH nerve terminals. Therefore, the activation of the reproductive axis through MeA<sup>Kiss</sup> neurons may be multi-modal where the release of GABA, glutamate, and kisspeptin may exert effects along the GnRH dendrite or through inter-neuronal pathways.

Despite the absence of c-Fos in GnRH neurons, when analysing the POA, CNO-mediated activation of MeA<sup>Kiss</sup> neurons led to decreased neuronal activity compared to control and SALB-treated mice. Recent studies have identified MeA<sup>Kiss</sup> neurons and the POA as regulators of sexual motivation and behaviour in male rodents [15, 16, 44]. Several studies have identified various neuronal types in the POA as key players in mediating male-typical mating and paternal behaviour including neurons expressing oestrogen receptor alpha [45], progesterone receptor [46], and galanin [47]. Therefore, future studies will be required to identify the exact cellular substrate in the POA responsive to modulation by MeA<sup>Kiss</sup> neurons. A decrease in neuronal activity in the POA in association with an increase in LH secretion has been previously reported in response to peripheral administration of kisspeptin [48]. It was proposed that kisspeptin suppresses GABA neurons in the POA to reduce inhibition of GnRH neurons, which leads to increased LH secretion. Previous work had already established that kisspeptin can modulate GABA activity [49] and GABA neurons are known to inhibit GnRH activity and suppress LH secretion [50, 51]. Considering that GnRH neurons are known to be sensitive to GABA and glutamate [17, 52], we investigated whether MeA<sup>Kiss</sup> neurons may modulate GnRH neurons through elaborating GABA and/or glutamate neu-

rotransmission directly. We found that 71% of MeA<sup>Kiss</sup> neurons express the GABAergic phenotypic marker Vgat and 29% express the glutamatergic marker Vglut2, whereas 6% express both. These results reveal that MeA<sup>Kiss</sup> neurons consist of a heterogeneous population that may release inhibitory and/or excitatory transmitters to accompany peptidergic actions.

Several human studies have shown the influence of olfactory signals on sociosexual behaviour [53], but there is no direct evidence linking MeA<sup>Kiss</sup> neurons to this pathway. Instead, visual stimuli may serve a more obvious influence on sexual motivation and consummation and this has been recently linked to amygdala activation [54]. Adult males have a larger amygdalar volume than females [55] and this is thought to correlate to higher sexual drive [2]. Sexually arousing visual stimuli leads to increased activation in the male MeA [54] and kisspeptin administration in humans enhances the activity of the left amygdala in response to sexual and couple-bonding stimuli [56]. Moreover, sexual differences in amygdala responses have been linked to the differential prevalence of psychological and sexual disorders in men and women [54] and it is worth considering whether MeA<sup>Kiss</sup> neurons are involved. However, further research is required to show that MeA<sup>Kiss</sup> neurons in particular do indeed contribute to any sex differences in sexual or affective disorders.

Overall, our study is the first to demonstrate that male MeA<sup>Kiss</sup> neurons are sensitive to female urinary chemosensory stimuli, stimulate LH secretion, and suppress POA neuronal activity. These neurons could be using peptidergic as well as amino acidergic co-transmission to regulate the reproductive axis. Together, these results establish that MeA<sup>Kiss</sup> neurons functionally link olfactory cues with the reproductive neuroendocrine axis.

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## Statement of Ethics

All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 approved by the University of St. Andrews Ethics Committee under the UK Home Office project license No. 7007924 granted to J.A.T.

## Disclosure Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

S.A. designed and performed experiments, acquired, analysed, and interpreted data, and wrote the first draft of the paper. C.T. analysed and interpreted data. K.S. conducted experiments and analysed data. H.W.K. conducted experiments and analysed data. R.P.M. analysed and interpreted data and critically revised the paper. J.A.T. conceptualized the study, designed and performed experiments, acquired, analysed, and interpreted data, and wrote the paper.

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