Article

The pheromone darcin drives a circuit for innate and reinforced behaviours

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Ebru Demir[1,2], Kenneth Li[1], Natasha Bobrowski-Khoury[1,2], Joshua I. Sanders[2,3], Robert J. Beynon[4], Jane L. Hurst[5], Adam Kepecs[2,6,7]* & Richard Axel[1,8]*

Organisms have evolved diverse behavioural strategies that enhance the likelihood of encountering and assessing mates[1]. Many species use pheromones to communicate information about the location, sexual and social status of potential partners[2]. In mice, the major urinary protein darcin—which is present in the urine of males—provides a component of a scent mark that elicits approach by females and drives learning[3,4]. Here we show that darcin elicits a complex and variable behavioural repertoire that consists of attraction, ultrasonic vocalization and urinary scent marking, and also serves as a reinforcer in learning paradigms. We identify a genetically determined circuit—extending from the accessory olfactory bulb to the posterior medial amygdala—that is necessary for all behavioural responses to darcin. Moreover, optical activation of darcin-responsive neurons in the medial amygdala induces both the innate and the conditioned behaviours elicited by the pheromone. These neurons define a topographically segregated population that expresses neuronal nitric oxide synthase. We suggest that this darcin-activated neural circuit integrates pheromonal information with internal state to elicit both variable innate behaviours and reinforced behaviours that may promote mate encounters and mate selection.

Communication through scents elicits innate and learned behavioural repertoires that enhance the reproduction and survival of the species[1]. Male mice deposit scent marks that attract females and enable assessment of the quality and compatibility of potential mates[2,5]. Innate attraction in females is elicited by the non-volatile protein pheromone darcin (MUP20)[3,4], a member of the major urinary protein (MUP) family that is recognized by receptors in the vomeronasal organ[6]. Darcin not only elicits innate attraction but can also serve as an unconditioned stimulus for both place and odour conditioning, enabling a female to recognize, assess and locate males on the basis of their scent marks[3–5].

We developed a quantitative behavioural paradigm to examine the effects of darcin, and found that the pheromone elicits a complex and variable behavioural array. Female mice were placed in a chamber equipped with two ports that contained glass fibre filters embedded with different social olfactory cues, and entry to the ports was quantified. The frequency of port entry provides a measure of preference for the cues present on the individual filters. During the initial habituation each port contained a blank filter, and port entries (pokes) were infrequent (mean ± s.e.m. poke count: left port 18 ± 3, right port 14 ± 3; Fig. 1b1). The mice were then exposed in their home cage[3] to bedding that had been soiled by male mice, after which the number of pokes increased substantially without any apparent side bias (left port 247 ± 35, right port 246 ± 3; Fig. 1b2). The response to darcin was therefore examined in cycling female mice after exposure to male-soiled bedding[3]. Poke frequency was higher for the port that contained the recombinant darcin (darcin-containing port 516 ± 47, blank 326 ± 21; Fig. 1b3). Exposure to urine that contained very low levels of darcin (low-darcin urine, from male BALB/c mice)[4] also elicited more frequent port entries than did blank filters in this assay, both with and without the addition of recombinant darcin (low-darcin urine 386 ± 42, blank 154 ± 14, Fig. 1c3; recombinant darcin-added urine 391 ± 29, blank 96 ± 18, Fig. 1d3).

Innately attractive cues can often serve as a teaching signal, reinforcing both classical and instrumental learning[7]. We examined whether exposure to darcin alone or to low-darcin urine elicits a lasting preference for the darcin port after the stimulus is removed. Female mice were exposed to a social cue in one port and then placed into a clean chamber on the following day with blank filters in both ports. Poke counts were significantly greater in the port that had previously contained either darcin (285 ± 38, blank 146 ± 16; Fig. 1b4) or urine with equivalent levels of darcin (179 ± 15, blank 65 ± 9; Fig. 1d4). By contrast, exposure to urine with very low levels of darcin did not result in a port preference during recall sessions on the following day (prior exposure to low-darcin urine 147 ± 14, blank 147 ± 16; Fig. 1c4). Therefore, both low-darcin male urine and darcin elicit a port preference, but only exposure to normal levels of darcin results in a remembered preference.

We also observed that female mice that were exposed to darcin emitted ultrasonic vocalizations and engaged in urinary scent marking

[1]Department of Neuroscience, Columbia University, Mortimer B. Zuckerman Mind Brain Behavior Institute, New York, NY, USA. [2]Department of Neuroscience, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA. [3]Sanworks LLC, Stony Brook, NY, USA. [4]Centre for Proteome Research, Institute of Integrative Biology, University of Liverpool, Liverpool, UK. [5]Mammalian Behaviour and Evolution Group, Institute of Integrative Biology, University of Liverpool, Leahurst Campus, Neston, UK. [6]Department of Neuroscience, Washington University School of Medicine, St. Louis, MO, USA. [7]Department of Psychiatry, Washington University School of Medicine, St. Louis, MO, USA. [8]Howard Hughes Medical Institute, Columbia University, New York, NY, USA. *e-mail: akepecs@wustl.edu; ra27@columbia.edu

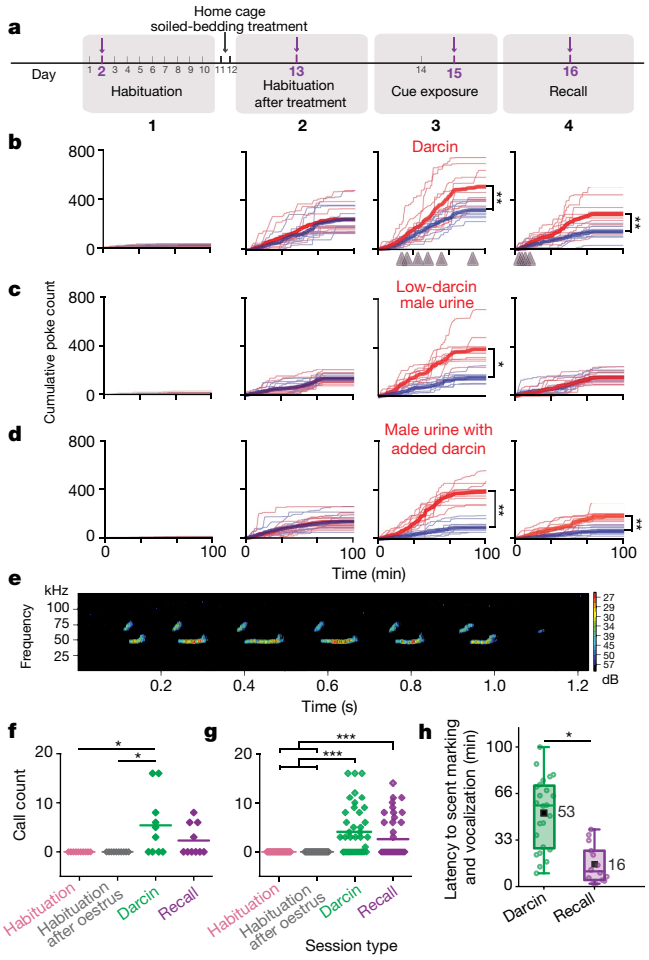

Fig. 1 | Darcin elicits an array of behaviours. a, Timeline of the two-port preference assay. **b**–**d**, Cumulative poke counts in ports containing darcin (1 µl µg^{-1}) (**b**), urine from BALB/c male mice with very low darcin levels (less than 0.1 µl µg^{-1})[4] (**c**) and BALB/c male urine with added recombinant darcin (1 µl µg^{-1}) (**d**) (red), compared with ports containing control filters (blue) during cue-exposure sessions. Counts are shown on days 2, 13, 15 and 16 (graphs 1–4, respectively), as indicated by arrows on the timeline. Mean (bold lines, $n = 30$ mice) and individual (fine lines) counts are shown. The time-stamps for ultrasonic vocalizations and scent marking are indicated as arrowheads (**b** (3, 4)). Bias in counts was assessed using the two-sided Wilcoxon signed-rank test (**b** (3, 4), $^{**}P = 0.004$, $n = 10$; **c** (3), $^{*}P = 0.006$, $n = 10$; **d** (3, 4), $^{**}P = 0.004$, $n = 10$). **e**, Spectrogram of an example song detected during darcin-exposure sessions. **f**, **g**, Mean call count (horizontal line) and total number of calls made by individual mice (diamonds); $n = 10$ mice (**f**) as tested in **b**, $n = 43$ mice (**g**) as tested across the study. Calls were compared using the two-sided Wilcoxon signed-rank test (**f**, *adjusted-$P = 0.03$; **g**, ***adjusted-$P = 0.00003$ and $P = 0.0001$). **h**, Latency to urinary marking and vocalization in response to darcin ($n = 24$ mice) and during recall ($n = 14$ mice) sessions. Mean (squares) and individual (circles) latencies are shown. The bounds in box plots are defined by the 25th and 75th percentile of the distribution. The line represents the median and the upper and lower whiskers represent 75th percentile + 1.5 × interquartile range (IQR) and 25th percentile − 1.5 × IQR, respectively. Latencies are compared using the two-sided Wilcoxon signed-rank test ($^{*}P = 0.03$).

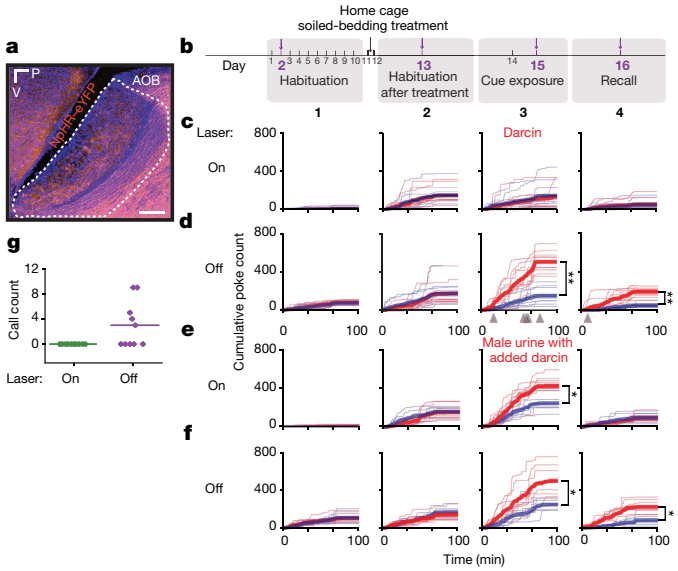

Fig. 2 | Optogenetic silencing of the AOB results in the suppression of darcin-evoked behaviours. a, eNpHR–eYFP expression in the AOB. Scale bar, 200 µm. P, posterior; V, ventral; this experiment was independently repeated with 18 mice. **b**, Timeline of the two-port preference assay. **c**–**f**, Cumulative poke counts with (**c**, **e**) and without (**d**, **f**) optical silencing of the AOB. In **c**, **d** mice were exposed to darcin (3) (1 µl µg^{-1}) ($n = 10$) and in **e**, **f** mice were exposed to C57BL/6 male urine (3) with normal levels of darcin (1 µg µl^{-1})[4] ($n = 8$) in one port (red) and a blank filter (blue) in the second port. Mean (bold lines) and individual (fine lines) counts are shown. The time-stamps for ultrasonic vocalizations and scent marking are indicated as arrowheads (**d** (3, 4)). Counts were compared using the two-sided Wilcoxon signed-rank test (**d** (3), $^{**}P < 0.001$; **e** (3), **f** (3, 4), $^{*}P < 0.008$). **g**, Mean call count (horizontal lines) and total number of calls made by individual mice (diamonds) during darcin exposure with (**c**, **e**) and without (**d**, **f**) AOB silencing; $n = 10$ mice. Calls were compared using the two-sided Wilcoxon signed-rank test.

(Fig. 1e–h). Scent marks were located closer to the darcin port (Extended Data Fig. 1a, b) and were smaller in size (Extended Data Fig. 1c) than those observed from free urination, which was consistent with the deliberate deposition of scent close to darcin; this suggests a distinction between the two behaviours. Ultrasonic vocalizations were consistently linked with urinary marking and occurred within 40 ms of one another (mean ± s.e.m. 42 ± 9 ms; Supplementary Video 1). These episodes did not occur immediately upon exposure to darcin, but appeared after a long and variable delay during a 100-min session (mean latency 53 ± 5 min, Fig. 1h). Vocalization and urinary scent marking were also observed during recall sessions (Fig. 1f–h). These episodes occurred earlier in the recall session than in the darcin-exposure session (recall sessions 16 ± 4, darcin sessions 53 ± 5 min; Fig. 1h). Male urine that contained normal levels of darcin also stimulated scent-marking behaviour during cue and recall sessions, but low-darcin urine stimulated marking only when present and not during recall sessions (Extended Data Fig. 2d). Thus, darcin induces a behavioural repertoire that comprises attraction and ultrasonic vocalization simultaneous with urine marking, behaviours that may serve as reciprocal communication. Moreover, this behavioural repertoire is also observed during recall sessions in the absence of darcin.

We next implemented genetic strategies to identify the neural circuitry that mediates these darcin-induced behaviours. Darcin binds to V2R receptors on sensory neurons in the vomeronasal organ[6]. These neurons extend axons through the skull, where they converge to form microglomeruli within the accessory olfactory bulb (AOB)[8]. Microglomeruli are innervated by mitral cells that project to multiple brain regions—including the cortical amygdala, bed nucleus of the stria terminalis, and the medial amygdala (MeA)[8,9]. We demonstrated that this pathway is responsible for the behavioural repertoire elicited by darcin by silencing the AOB. Bilateral injection of an adeno-associated virus (AAV) encoding halorhodopsin[10] fused to enhanced yellow fluorescent protein (eNpHR–eYFP) resulted in the expression of eNpHR–eYFP (Fig. 2a) in the majority of mitral cells in the AOB (73 ± 8% across mice). AOB silencing eliminated the preference for the darcin-containing port (180 ± 49, blank 149 ± 37; Fig. 2c3) and suppressed darcin-evoked

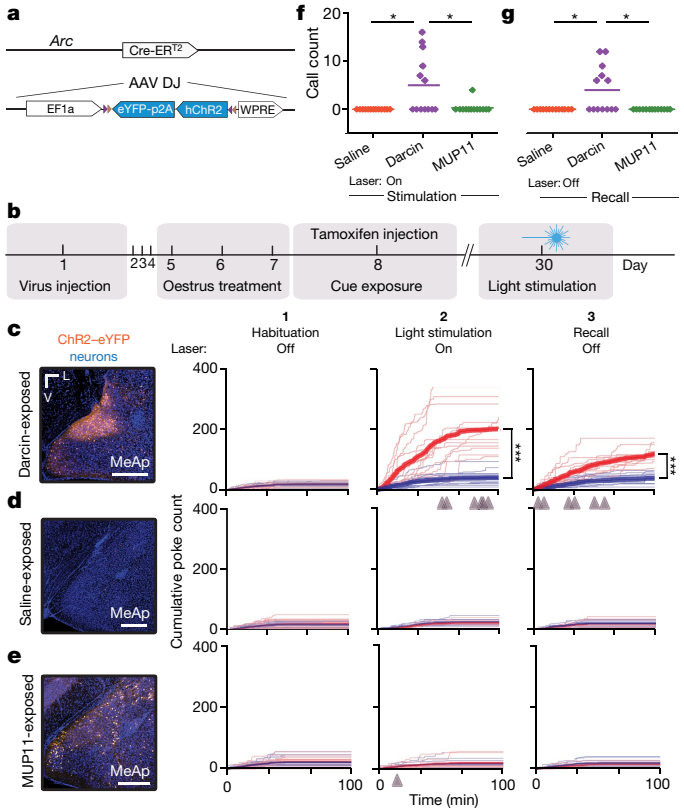

Fig. 3 | Activation of darcin-responsive neurons in the MeA recapitulates pheromone-induced behaviours. a, Genetic strategy used to express ChR2 in pheromone-responsive neurons. **b**, Timeline of experimental manipulations. **c**–**e**, Left, representative images showing eYFP expression in the posterior MeA after exposure to darcin (mean ± s.e.m.: eYFP counts, 255 ± 29 in the MeApd and 115 ± 16 in the the MeApv) (**c**), saline (16 ± 5 in the MeApd and 23 ± 7 in the MeApv) (**d**) and MUP11 (54 ± 10 in the MeApd and 42 ± 9 in the MeApv) (**e**). Scale bars, 400 μm; L, lateral; V, ventral. Right, corresponding cumulative poke counts. Mean (bold lines, $n = 13$ mice for each group, $n = 39$ mice in total) and individual (fine lines) counts are shown. The time-stamps for ultrasonic vocalizations and scent marking are indicated as arrowheads (**c** (2, 3), **e** (2)). Counts were compared using the two-sided Wilcoxon signed-rank test (**c** (2, 3), ***$P = 0.0002$). **f**, **g**, Mean (horizontal lines) and total calls made by individual mice (diamonds) during the light-stimulation sessions (**f**) and subsequent recall sessions (**g**); $n = 13$ mice per group. Calls were compared using the two-sided Mann–Whitney test adjusted for multiple comparisons (**f**, *$P < 0.05$; **g**, *$P = 0.02$).

ultrasonic vocalizations and scent marking (Fig. 2g). By contrast, the preference for male urine with normal levels of darcin was not suppressed during AOB silencing (423 ± 40, blank 243 ± 25; Fig. 2e3). AOB silencing did not affect port investigation during the initial habituation periods with the blank filters (Fig. 2c1, 2–2f1, 2).

Exposure to recombinant darcin elicited a memory for the darcin port (prior exposure to darcin 190 ± 16, blank 56 ± 6; Fig. 2d4), but a port preference was not observed if the AOB was silenced during darcin exposure (prior exposure to darcin 55 ± 15, blank 60 ± 18; Fig. 2c4). Females that were exposed to male urine containing normal levels of darcin also exhibited a persistent port preference during AOB silencing (urine 423 ± 40, blank 243 ± 25; Fig. 2e3), but failed to show a preference for this port in the recall sessions (prior exposure to urine containing normal levels of darcin 97 ± 16, blank 94 ± 15; Fig. 2e4). These observations show that the AOB is necessary for darcin-induced attraction behaviours as well as for conditioning. Other components of male urine also elicit attraction that is independent of the AOB but fail to reinforce conditioned behaviours.

The mitral cells—the projection neurons of the AOB—send axons to the MeA[8,9]. We identified the neurons of the MeA that are responsive to darcin by using the promoter of the activity-dependent gene *Arc* to express channelrhodopsin-2 (ChR2), a light-gated ion channel[11]. AAV encoding Cre-dependent channelrhodopsin fused to the fluorescent protein eYFP was injected into the MeA of transgenic mice (Arc-CreER mice; Fig. 3a) in which the *Arc* promoter drives the expression of the tamoxifen-sensitive Cre recombinase (Cre-ER)[12]. The administration of tamoxifen followed by exposure to darcin should result in the expression of ChR2–eYFP in neurons that are activated by darcin. We compared the expression of Fos with that of ChR2–eYFP and found that ChR2–eYFP is faithfully expressed in neurons that respond to darcin (78 ± 4% of the ChR2–eYFP$^+$ neurons also express endogenous Fos, and 79 ± 3% of the neurons that express endogenous Fos also express ChR2–eYFP; $n = 6$ mice).

We next determined whether the activation of neurons that express ChR2 after exposure to darcin is sufficient to recapitulate the behaviours elicited by darcin. Arc-CreER mice injected with AAV encoding Cre-dependent ChR2–eYFP in the posterior dorsal medial amygdala (MeApd) and the posterior ventral medial amygdala (MeApv) (Fig. 3a, b) were treated with tamoxifen and then exposed to darcin, saline or a control MUP (MUP11)[3,4]. Histological analysis of ChR2–eYFP expression induced by exposure to darcin revealed a dense clustering of ChR2–eYFP neurons that were restricted largely to the MeApd and the MeApv (Fig. 3c). Exposure to MUP11[3,4] revealed sparser labelling in both the MeApd and the MeApv, and even sparser labelling was observed after exposure to saline (Fig. 3d, e). Mice that expressed ChR2–eYFP induced by exposure to darcin, MUP11 or saline were introduced into the behavioural chamber after two days of habituation. We then photoactivated the MeA when the mice entered one of the two ports containing blank filters, to recapitulate exposure to darcin. Mice that expressed ChR2–eYFP induced by darcin exposure exhibited a strong preference for the stimulation port (mean poke counts: light 202 ± 21, no light 40 ± 7; Fig. 3c2). Photoactivation of the ensemble of darcin-responsive neurons also elicited ultrasonic vocalizations and scent marking (Fig. 3f, Extended Data Fig. 2a–c). Photoactivation of the MeA in mice that expressed ChR2–eYFP after exposure to saline (light 26 ± 3, no light 24 ± 2; Fig. 3d2) or MUP11 (light 19 ± 4, no light 20 ± 5; Fig. 3e2) did not elicit any preferences for the stimulation port, and did not result in ultrasonic vocalizations or urinary scent marking (Fig. 3f).

Mice that expressed ChR2–eYFP in neurons that were responsive to darcin exhibited a remembered preference for the port in which they previously received light stimulation (prior photoactivation 126 ± 8, no activation 39 ± 6; Fig. 3c3). Control mice that expressed ChR2–eYFP in neurons after exposure to MUP11 (prior photoactivation 20 ± 3, no activation 16 ± 2; Fig. 3e3) or to saline (prior photoactivation 20 ± 2, no activation 23 ± 3; Fig. 3d3) exhibited no preference for the previous light-stimulated port. In recall experiments, ultrasonic vocalizations and scent marking were detected only in mice that previously experienced photostimulation of neurons expressing ChR2–eYFP induced by exposure to darcin (Fig. 3g, Extended Data Fig. 2a–c, Supplementary Video 2), and not in mice expressing ChR2–eYFP in neurons activated by exposure to MUP11 or saline (Fig. 3g). We demonstrated that exposure to darcin could also result in conditioned place preference (Extended Data Fig. 3a, b). Therefore, photoactivation of a population of neurons that express ChR2 induced by darcin exposure can elicit innate attraction, ultrasonic vocalizations, urinary scent marking and reinforce conditioned behaviours.

Lactating females fail to exhibit attraction to darcin[13]. We therefore asked whether darcin activates MeA neurons in lactating females. Lactating Arc-CreER mice expressing Cre-dependent eYFP in the MeA were exposed to darcin three to five days postpartum. Exposure to darcin in virgin females resulted in dense labelling of posterior MeA neurons with eYFP expression (mean ± s.e.m. eYFP$^+$ cells: 255 ± 29 in the MeApd and 115 ± 16 in the MeApv). Exposure to darcin during lactation

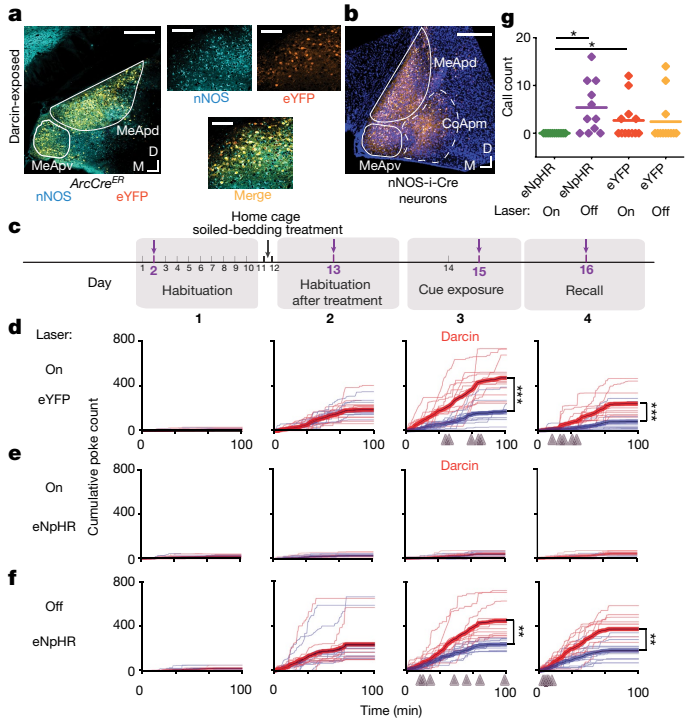

Fig. 4 | nNOS neurons in the MeA are necessary for darcin-mediated behaviours. a, Representative image showing the co-expression of eYFP expressed in darcin-responsive neurons (Fig. 3c, left) and nNOS in the posterior MeA (D, dorsal; M, medial; $n = 7$ mice). Scale bars, 400 µm (main image, left); 100 µm (smaller images, right). **b**, eYFP expression in coronal sections of the posterior MeA of an nNOS-ires-Cre mouse (scale bar, 400 µm; CoApm, posterior medial cortical amygdala; this experiment was independently repeated with 66 mice). **c**, Timeline of the two-port preference assay. **d**–**f**, Cumulative poke counts in mice expressing eNpHR (**e**, **f**) or eYFP (**d**) in nNOS neurons. Neurons expressing eYFP ($n = 12$ mice) (**d**) and eNpHR ($n = 11$ mice) (**e**) were photostimulated, with no photostimulation of neurons expressing eNpHR ($n = 11$ mice) (**f**). Mean (bold lines) and individual (fine lines) counts are shown. The time-stamps for ultrasonic vocalizations and scent marking are indicated as arrowheads (**d** (3, 4), **f** (3, 4)). Counts were compared using the two-sided Wilcoxon signed-rank test (**d** (3, 4), $^{***}P < 0.0005$; **f** (3, 4), $^{**}P < 0.005$). **g**, Vocalization counts of mice expressing eYFP ($n = 12$ mice) and eNpHR ($n = 11$ mice). Mean (horizontal lines) and total calls made by individual mice (diamonds). Calls were compared using the two-sided Wilcoxon signed-rank test, adjusted for multiple comparisons ($^{*}P < 0.05$).

resulted in a sparse labelling (23 ± 11 in the MeApd and 15 ± 12 in the MeApv) at levels similar to that observed upon saline exposure (16 ± 5 in the MeApd and 23 ± 7 in the MeApv) (Extended Data Fig. 4f–h). By contrast, darcin activates an equivalent number of mitral cells in the AOB of both virgin and lactating females (Extended Data Fig. 4a–e, Fos cells in virgin females 378 ± 35 and in lactating females 358 ± 45; $n = 6$, $P = 0.9$). Therefore, the darcin-activated circuit is likely to be gated by lactation in the MeA.

We next identified a genetic marker, neuronal nitric oxide synthase (nNOS), which defines the population of MeA neurons that mediate the darcin-induced behaviours. Immunohistochemical examination of the MeA of Arc-CreER mice revealed that a considerable fraction of neurons that express ChR2–eYFP in response to darcin also express nNOS. We found that 18% of neurons in the posterior MeA express nNOS. Double-labelling experiments demonstrated that this nNOS-expressing population (denoted nNOS neurons) consists of 55 ± 4% excitatory neurons (vGlut2$^+$ cells) and 24 ± 3% inhibitory neurons (Gad2$^+$ cells). We observed that 74 ± 2% of the ChR2–eYFP-expressing neurons that are labelled upon darcin exposure also express nNOS, whereas 66 ± 3% of the nNOS neurons also express ChR2–eYFP (Fig. 4a). Similar values are obtained in Arc-CreER mice that are exposed to male urine containing normal levels of darcin. The pheromones ESP1[14], MUP11[3,4] and cat salivary lipocalin Fel-D4[15], as well as female urine, activated less than 20% of the nNOS neurons (Extended Data Fig. 5, Extended Data Table 1). The majority of the MeA neurons activated by these stimuli do not express nNOS, which demonstrates the specificity of the response of nNOS neurons for darcin.

These observations suggest that activation of the nNOS neurons in the MeA should elicit the behavioural repertoire that is observed upon darcin exposure. We therefore injected AAV encoding Cre-dependent ChR2–eYFP into the posterior MeA of mice in which the *Nos1* promotor drives the expression of Cre (nNOS-ires-Cre) to express channelrhodopsin-2 in nNOS neurons. We then photoactivated nNOS$^+$ MeA neurons when the mouse entered one of the two ports containing blank filters, and observed a strong preference for the stimulation port (light 541 ± 45, no light 66 ± 12; Extended Data Fig. 3d2). Moreover, photostimulation of nNOS cells expressing ChR2–eYFP evoked ultrasonic vocalization and scent marking (Extended Data Figs. 2a–c, 3f). Photoactivation of these MeA neurons also reinforced conditioned behaviours (prior light 295 ± 16, no light 57 ± 11; Extended Data Fig. 3d3). Control experiments in which AAV encoding Cre-dependent eYFP was injected into the MeA of nNOS-ires-Cre mice failed to elicit any darcin-mediated behaviours upon photostimulation (light 24 ± 4, no light 25 ± 5, $P = 0.8$, Extended Data Fig. 3c2; prior light 23 ± 7, no light 25 ± 6, $P = 0.8$, Extended Data Fig. 3c3). Therefore, photoactivation of ChR2–eYFP in nNOS neurons in the MeA is sufficient to recapitulate both the innate and the reinforcing behaviours that are observed upon exposure to darcin.

These observations predict that silencing of the nNOS neurons in the MeA should impair the behavioural response to darcin. The bilateral injection of an AAV (AAVDJ-EF1a-DIO.eNpHR3.0-eYFP, Fig. 4b) encoding the Cre-dependent opsin into the nNOS-ires-Cre mice resulted in the expression of halorhodopsin[10] in nNOS neurons in the MeA. In mice in which the nNOS neurons were silenced, no preference was observed for filters that contained recombinant darcin in the poke-preference assay (darcin 35 ± 5, blank 39 ± 5; Fig. 4e3), and darcin elicited no port preference during recall sessions (prior exposure to darcin 35 ± 5, blank 42 ± 6; Fig. 4e4). Ultrasonic vocalizations and urinary scent marking were also eliminated upon light-induced silencing of nNOS neurons (Fig. 4g). As a control we showed that, when photostimulation was terminated, darcin elicited a strong port preference that was also observed during recall sessions (prior exposure to darcin 375 ± 40, blank 186 ± 28; Fig. 4f4). Light-induced silencing in the MeA of mice that expressed eYFP in nNOS neurons failed to inhibit darcin-mediated behaviours (Fig. 4d). Notably, silencing of the MeA also inhibited the port preference that was elicited by urine containing normal levels of darcin (Extended Data Fig. 6b3). These observations suggest that the components in urine other than darcin that elicit port preference also require the MeA. We found that silencing of nNOS neurons resulted in inhibition of poking to control filters after females were exposed to male scent in their home cages (blank 24 ± 5, blank 23 ± 5; Fig. 4e2). We performed additional experiments to demonstrate that the inhibition of darcin-evoked behaviours upon the silencing of nNOS neurons was not due to diminished motivation (Extended Data Fig. 7).

We then asked whether the nNOS neurons in the MeA are also required for the expression of the remembered response. Female mice were exposed to darcin, and then nNOS neurons were silenced only during recall sessions. These mice exhibited a strong preference for the port that had previously contained darcin (prior exposure to darcin 254 ± 22, blank 77 ± 17; Extended Data Fig. 6e4). Darcin-responsive neurons that express nNOS in the MeA are therefore necessary to recapitulate the innate and reinforcement behaviours elicited by darcin. Recall of darcin memory, however, no longer requires this neural population.

The array of properties elicited by darcin suggests that this pheromone does not elicit a simple behavioural response, but rather activates

a complex integrative process that may optimize mate encounters and mate selection. First, the attractive response is rapid and prolonged upon darcin exposure, whereas vocalization and scent marking are variable and often occur after long delays. Activation of the nNOS population of neurons by darcin may therefore elicit a state of 'sexual drive', which increases the probability of individual component behaviours that are suited to enhance the likelihood of mate encounters under different environmental circumstances. Darcin exposure results in exploration and assessment of the darcin source: the urine of a dominant male. In the absence of the male, after active search strategies have failed, the female may emit ultrasonic vocalizations synchronized with scent marking in an attempt to communicate her presence and her current oestrus status to the male.

Second, darcin activation of the nNOS neurons reinforces both contextual and olfactory learning—generic learning processes—which may allow the female to return to the location of the male's scent mark[4] or to track airborne scents of the territorial male[3,5]. The MeA may therefore provide a signal mediated by darcin to midbrain dopamine neurons to reinforce more traditional 'non-social' reinforcement learning[7]. The more stereotypical communication behaviours elicited by darcin—vocalization and scent marking—might also result from reinforcement of a specific set of social behaviours that coordinate a successful mate search. Whereas the nNOS neurons are required for the behavioural and reinforcing effects of darcin, recall of darcin-elicited memory no longer requires this neural population—presumably reflecting the transfer of a learned representation in other brain structures.

Third, we observe that male urine with very low levels of darcin elicits attraction but does not result in reinforcement learning or memory of port preference. This attractive response does not require the AOB but is eliminated upon silencing the nNOS neurons of the MeA. These observations suggest that the MeA is integrating pheromonal information from the vomeronasal pathway with olfactory cues from the main olfactory system to elicit both innate attraction and learning.

Finally, the response to darcin is dependent on internal state. Lactating females fail to exhibit this complex behavioural response to darcin exposure[13]. We found that darcin activates the projection neurons in the AOB in lactating females, but fails to activate the nNOS neurons in the MeA (Extended Data Fig. 4). Taken together, these observations suggest that the nNOS neurons of the MeA integrate internal state with the pheromonal cues to mediate both innate variable behaviours and reinforced behaviours that may coordinate a successful mate search.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-1967-8.

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Methods

Mice

All surgical and experimental procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals[16] from the National Institute of Health Standards, and approved by the Cold Spring Harbor Laboratory and Columbia University Medical Center Institutional Animal Care and Use Committees. Experiments were conducted with 279 female mice between 6 and 30 weeks old. Mice were purchased at 4 weeks old and were handled for at least 10 min each day for a minimum of 5 days before experimentation. Surgeries were performed on mice that were 6 weeks old in order to match their brain coordinates to the Allen Reference Atlas. The mouse lines used were as follows: Arc-CreER (a gift from C. Denny at Columbia University; also available from Jackson Laboratory, Jax stock 022357); ICR outbred (CD-1) wild-type mice (Harlan/Envigo); Ai14 (Rosa-CAG-LSL-tdTomato); nNOS-ires-Cre (Jax stock 017526); vGlut-ires-Cre (Jax stock 028863); Gad2-T2a-NLS-mCherry (Jax stock 023140). The nNOS-ires-Cre mice were crossed to ICR outbred mice (Harlan/Envigo) for 15 generations to exchange their genetic background to the ICR mice. Throughout the study, five mice were co-housed in a single cage for two to six months. This long-term co-housing has the potential to suppress oestrus cycling in females (the Lee–Boot effect)[17]. To ensure that all females had previously encountered male scent and were showing normal oestrus cycling, females were exposed to male-soiled bedding from an unfamiliar strain for at least 60 h[3]. They were then visually evaluated for their stage of oestrus before the experimental testing. One hour before testing, each mouse had its vaginal opening photographed for evaluation. After oestrus entrainment, most females (more than 90%) were evaluated to be in the pro-oestrus stage[3] of the cycle (with swollen, moist, pink and wide-open vaginal openings[18]) and advanced into behavioural testing. Mice were kept in a controlled 12-h day/night (7:00 to 19:00) cycle and tested only during the night phase (23:00 to 6:00).

Behavioural assays

Before behavioural training, mice were handled for 10 min each day for five days, and were given access to a mouse exercise cage that was enriched with spinning discs and toys for one hour every day during the experimental period. Training took place in a custom-designed sound-isolation chamber containing a behavioural arena (25 × 25 × 28 cm) integrated with two stimulus ports (circular nose port (4.6-cm diameter) with an attachable circular cup for the filter (1.3-cm diameter)), which were surrounded by distinct visual stimuli (stripe and circle stickers were used on either side (Context Kit for Conditioned Place Preference, Stoelting)) on the walls. Mice were tested under room light during the night phase of their day/night cycle (23:00 to 6:00). Mice poked their snouts into stimulus ports to sample the social stimuli. The social cue was presented on a glass microfibre filter in a portable cup attached to the nose port. Social cue ports were constructed out of metal and boiled in detergent (1–2% Alconox for at least 15 min), rinsed thoroughly with water, dipped in 3% hydrogen peroxide and ethanol, rinsed again with running distilled water and air-dried to clean off any contaminants between experiments. The frequency and duration of nose pokes were quantified by means of an infrared beam within the port. The behavioural nose poke data were acquired through a MATLAB interface and a Bpod.

Ultrasonic vocalizations in the chamber were captured using an Avisoft ultrasound microphone with a frequency range of 20–200 kHz. The microphone was connected to a portable time-code generator and reader (Horita PTG2), which generated a time code that was embeddable into both the audio and the video files. Avisoft Recorder USGH software was used to record vocalizations and integrate time codes from the PTG2. To capture urinary scent-marking behaviours with the embedded time code, a Marshall Genlock 3G-SDI HDMI camera was mounted at the base of the transparent chamber. An AJA K_i Pro Recorder, which was connected to the camera and the PTG2, was used to record video for the entire duration of the session. The time code generated by the PTG2 was visible as a display within the video window of the Marshall camera recording through the AJA recorder, and was also recorded by Adobe Captivate.

The nature of the ultrasonic vocalizations in each session was analysed with Avisoft SAS Lab Pro (Supplementary Videos 1, 2). We quantified call counts as the number of syllables in a given session of an individual mouse. Comparison of the calls emitted in response to the pheromone and the calls emitted upon the photoactivation of MeA neurons confirmed that the pheromone- and photoactivation-evoked syllables shared similar sonic qualities (Extended Data Fig. 8, Extended Data Table 2). All spectrograms were additionally parametrized using SAP 2011[19] and MUPET[20] software, and all syllables emitted by the mice during the sessions were manually extracted and classified for analysis (Extended Data Fig. 8). To analyse the urinary scent-marking behaviour of the mice, Adobe Premiere Pro was used. To determine the concurrency between urination and vocalization, Adobe Premiere Pro was used to align the video to the audio by using the time shown by the OLED display of the PTG2 (visible in the video window) in conjunction with the time code encoded in the audio file as temporal references. In addition, engagement of the poke port resulted in the simultaneous activation of a red LED, which was visible to the human eye in the video window but not to the mice, and a TTL (transistor–transistor logic) pulse, which was recorded in the ultrasonic audio track as a labelled time event by the Avisoft Recorder USGH software. Engagement of the port was thus used as an additional online reference to observe the alignment of audiovisual events, and this was recorded by Adobe Captivate. In addition, the distances from urinary drops to the base of each of the ports were quantified for the pheromone, photoactivation and free-urination sessions. Distances were extrapolated from individual frames of the video using Adobe Photoshop.

Mice were placed in the behaviour chamber for 100 min once per day for each session during the dark phase (23:00 to 6:00) of their day/night cycle (7:00 to 19:00). The behavioural chamber and the stimulus ports were thoroughly cleaned with 1–2% Alconox detergent, distilled water, 3% hydrogen peroxide and 80% ethanol, rinsed again with distilled water and air-dried in between individual sessions. The first ten sessions served as habituation sessions, during which no social cue was present in either social cue port. Therefore, there were no special cues available to the mice as they acclimatized to movement in the chamber and, for subjects involved in optogenetic experiments, movement while tethered to the patch cord. For behavioural testing, all mice—except for the optically activated mice—were exposed for 60 h in their home cage[3] to bedding soiled by male mice, followed by an extra habituation session with blank filters in both stimulus ports after this home-cage treatment. Subsequently all mice were tested with social cues or optical activation present in either port. The social cue or activation sides were randomly assigned between two ports across mice to control for any potential side bias. For the optical activation experiments, a nose poke into the stimulation port triggered an external laser pulse (473-nm light, 60 pulses, 20 Hz) using a PulsePal[21] device.

The ICR background mice that were not tested optogenetically were subjected to the following social cues in one port: recombinant darcin (1 μg μl^{-1}); male urine with low levels of darcin (<0.1 μg μl^{-1} in BALB/c J Ola-Hd urine, purchased from Harlan/Envigo)[4]; male urine with normal adult levels of darcin (1 μg μl^{-1}, C57BL/6J Ola-Hsd urine, purchased from Harlan/Envigo)[4]; or recombinant darcin added to BALB/c J Ola-Hd male urine with low levels of darcin (BALB/c J plus recombinant darcin, 1 μg μl^{-1}). In all instances, there was no odour in the other port. To confirm the presence or absence of darcin (18,893 Da MUP20), 12% SDS–PAGE gel electrophoresis of all urine samples was performed[3,4].

The C57BL/6 Arc-CreER and nNOS-ires-Cre/ICR mice tested with optical activation were subjected to optical activation in one port and no optical activation in the other port. The ICR outbred mice tested with

AOB inactivation were subjected to either recombinant darcin (11 µg in 10 µl) or male urine with normal levels of darcin (10 µl of C57BL/6 Ola-Hd urine)[4] in one port and no odour in the other port. The nNOS-ires-Cre mice tested with MeA inactivation were subjected to darcin or male urine with normal levels of darcin (C57BL/6 Ola-Hd)[4] in one port and no odour in the other port. All the optical-silencing experiments used a continuous light-on protocol during the entire test sessions. The final session for all mice was a recall session designed to quantify retained poke preferences in the absence of social cues or optical activation.

For conditioned place preference experiments, C57BL/6 Arc-CreER and nNOS-ires-Cre/ICR mice were introduced into a two-chamber conditioned place preference arena (22 × 16 × 28 cm, length × width × height) for 100 min once per day for each session. The two chambers had distinct walls decorated with visual cues (stripes and circles stickers, Context Kit for Conditioned Place Preference, Stoelting)); chambers were separated by a corridor and a divider, each containing a single nose port. Light stimulation was delivered to a port in one of the two chambers, and there was no optical activation in the other port. A nose poke into the light-stimulation port triggered an external laser pulse (473-nm light, 60 pulses, 20 Hz) using a PulsePal[21] device during the light-stimulation sessions only. During the habituation and recall sessions, a nose poke into the light-stimulation port did not trigger a laser pulse. Videos were recorded throughout the 100-min sessions. The positions of the mice were tracked using Ethovision, and the occupancy trajectories and time spent in each chamber were computed for analysis.

To demonstrate that MeA nNOS neurons are indispensable for social cue reinforcement behaviours only, we optogenetically silenced nNOS neurons in the MeA and tested the mice with water as a reinforcer rather than darcin. Mice were tested using a two-port setup without any social cues. Before behavioural training, mice were gradually water-restricted over the course of a week and kept under water scheduling until the tests were concluded. Mice were placed in the behaviour chamber for 100 min once per day for each session during the dark phase (23:00 to 6:00) of their day/night cycle (7:00 to 19:00). The first ten sessions served as habituation sessions, during which no cue was present in either port. Mice were acclimatized to the movement in the chamber while being tethered to the patch cord. They were then subjected to male-soiled bedding exposure for 60 h in their home cage and an extra habituation session with blank filters in both ports following this home-cage treatment. Subsequently, all mice were tested for cue sessions. The cue sides were evenly split in a random manner between two ports across the mice to control for any potential side bias. During cue sessions, a nose poke in one port rewarded the mice with 5 µl of water, and there was no reward for a nose poke in the other port. Behavioural training sessions lasted 100 min, during which the mice typically collected at least 4 ml of water. The final session for water-reinforcement behaviour was a recall session designed to quantify the retained poke preferences without any water reward. The behavioural hardware was controlled by custom MATLAB programs and a Bpod and PulsePal[21].

Investigators were blinded to the allocation of the mice during the experiments and data analysis.

Stereotactic surgeries

An adeno-associated virus (AAV) DJ serotype[22] (1.3×10^{13} vg ml^{-1} (genomic), 8×10^{8} IU ml^{-1} (infectious) titre, Stanford Vector Core Facility) carrying EF1a DIO hChR (E123T/T159C)-p2A–eYFP-WPRE, EF1a DIO NpHR3.0–eYFP, EF1a DIO–eYFP or EF1a NpHR3.0–eYFP construct was injected in 4- to 6-week-old mice. The mice were anaesthetized with an intraperitoneal injection of a ketamine and xylazine mixture (0.13 mg per g body weight ketamine and 0.01 mg per gram body weight xylazine). Small craniotomies were made above the posterior MeA (−2.0 mm AP and 2.3 mm ML from the bregma) or the AOB (3.2 mm AP, 1 mm ML and 0.8–1.5 mm DV). Virus was injected with a glass micropipette using a Picospritzer (General Valve). For posterior MeA injections, 20–60 pulses of 10-ms duration were delivered at 0.2 Hz starting from a depth of 4.6 mm from the brain surface up to 5.2 mm in 200-µm steps, waiting a minimum of 10 min per site to allow diffusion of the virus. After virus injection, fibre-optic cannulas were implanted. The mice received a supplementary dose of ketamine at 30- to 90-min intervals to maintain the depth of anaesthesia. The cannula was positioned with the help of a stereotaxic arm (David Kopf Instruments) and cannula holder (Doric Lenses) above the craniotomy. The optical cannula was gradually lowered close to the viral injection depth (100–300 µm above the injection site). Two miniature watch screws (Micro-Mark) were fixed into the parietal plates as anchors. The cannula was secured to the skull with light-curable dental cement (Vitrebond Plus) followed by a layer of black dental acrylic (Lang Dental Manufacturing). For post-operative analgesia, ketoprofen (5 mg per kg body weight) was administered subcutaneously. The mice were allowed to recover for one week.

Exposure of Arc-CreER mice to social cues

One week after stereotaxic viral infection and cannula surgery, 6- to 8-week-old Arc-CreER mice were transferred to a reverse day/night cycle. They were individually housed unless mentioned otherwise and oestrus was synchronized through exposure to male-soiled bedding for 60 h[3,4]. Mice were then injected with 2 mg of tamoxifen (Sigma T5648), which was prepared as a 10 mg ml^{-1} stock solution dissolved in a mixture of ethanol and sunflower seed oil (Sigma S5007). Five hours after tamoxifen injection, the mice were exposed to darcin, MUP11, saline, cat salivary lipocalin (Fel-D4)[15], ESP1[14] (exocrine-gland secreting peptide), male urine with normal levels of darcin, female urine or male urine with low levels of darcin on a glass microfibre filter (10 mm diameter) placed through the roof of their home cage; 10 µl (equivalent to 11 µg of darcin[3,4], MUP11[3,4], equivalent to 3.3 µg of Fel-D4[15] and 25 µg of ESP1[14]) was used. The lactating females were separated from their pups five hours before tamoxifen injection and exposed to recombinant darcin between postpartum days 3 and 5. Recombinant cat Fel-D4 was produced using the pMAL Protein Fusion and Purification System (New England Biolabs) and assayed by SDS–PAGE. The mouse ESP1 was synthesized by Atlantic Peptides. The mice were monitored with infrared cameras to confirm that they had interacted with the filters. Optical activation experiments were conducted three weeks after exposure to the cues.

Three weeks after the tamoxifen injection, the Arc-CreER mice that were subjected to optical stimulation were re-exposed to darcin for 2 h and then euthanized for immunohistochemistry.

Immunohistochemistry

Once the behavioural criteria for each behaviour assay were met, the mice were anaesthetized with a ketamine and xylazine mixture (0.30 mg per g body weight ketamine, 0.03 mg per g body weight xylazine) and perfused transcardially with 4% paraformaldehyde (PFA) in a phosphate buffer pH 7.4 (PBS). The brain was dissected and incubated at 4 °C in 4% PFA, washed in 1× PBS, and stored in PBS at 4 °C until sectioning. Subsequently, 50-µm coronal brain sections were made using a Leica VT1000S vibratome. The sections were incubated with a blocking solution (5% normal goat serum and 0.1% Triton in PBS (PBST)), washed in 0.1% PBST (3 washes, 15 min each) and incubated overnight at 4 °C with primary antibodies diluted in blocking solution. The following primary antibodies were used: anti-GFP (rabbit polyclonal, 1:1,000, Rockland), anti-GFP (chicken polyclonal 1:400, Aves Labs), anti-nNOS (rabbit polyclonal, 1:400, Invitrogen), anti-mCherry (rat monoclonal, 1:800, Thermo Fisher Scientific) and anti-Fos (goat and rabbit polyclonal, 1:500, Santa Cruz Biotechnology; guinea pig polyclonal, 1:5,000, with RRID: AB_2814707, generated by S. Brenner-Morton, at ZMBBI, Columbia University). The following day the sections were washed in 0.1% PBST (3 washes, 15 min each) and incubated for 2 h at room temperature with secondary antibodies at 1:500 dilutions (Alexa-594 goat anti-rabbit, Alexa-633 donkey anti-goat, Alexa-488 goat anti-rabbit, Alexa-488 goat anti-chicken, Alexa-594 goat anti-rat, Alexa-488 goat anti-guinea pig, Jackson ImmunoResearch, and NeuroTrace Alexa-640/660, Molecular

Probes). Sections were washed in 1× PBS for 15 min and mounted using Vectashield mounting medium (Vector Laboratories). Confocal images were acquired using an LSM780 Zeiss microscope at 10×, 20× and 65× magnifications. Area and cell counts were manually quantified using ImageJ (NIH) software by an individual who was blinded to the experimental conditions.

Statistical analysis

Port preferences within each session type (habituation day 2, habituation day 13, cue exposure and recall) for each subject were compared by matched Wilcoxon signed-rank tests. Port bias for the left port over the right port was computed by taking the difference in total poke count between the left and the right ports for each mouse. Comparisons were across each session (habituation day 2, habituation day 13, cue exposure, and recall) using the Wilcoxon signed-rank test. Port bias was compared across independent treatment cohorts by Mann–Whitney (for pairwise comparisons) and Kruskal–Wallis tests (for three-way comparisons). All poke count data did not approximate to normality and so non-parametric tests were used. Call counts were compared across independent cohorts of mice using a Mann–Whitney test and across different sessions of the same cohort of mice using the Wilcoxon signed-rank test. Adjusted *P* values were reported where multiple comparisons were made on the same sample set by using the Holm's sequential Bonferroni correction method. The probabilities of urinary scent-marking behaviour were compared across sessions using the McNemar test. Exact tests were performed for all comparisons, including those in which the sample sizes were small (the discordant pairs in some of our comparisons were less than 25). The mean latencies to first urinary scent marking were compared using a paired *t*-test. The latency data approximated to normality as confirmed by Shapiro–Wilk, Lilliefors, Kolmogorov–Smirnov, Anderson Darling, D'Agostino-*K* squared and Chen–Shapiro tests. All analyses were performed using R, OriginLab and MATLAB.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding authors on reasonable request.

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Author contributions E.D., R.J.B., J.L.H., A.K. and R.A. discussed the design of experiments and the results, and wrote the manuscript. J.I.S. designed the custom behaviour and stimulation systems. E.D. performed all of the experiments and analysis. K.L. and N.B.-K. helped with the experiments and analysis. The recombinant MUPs were provided by R.J.B.

Competing interests The authors declare no competing interests.

Additional information
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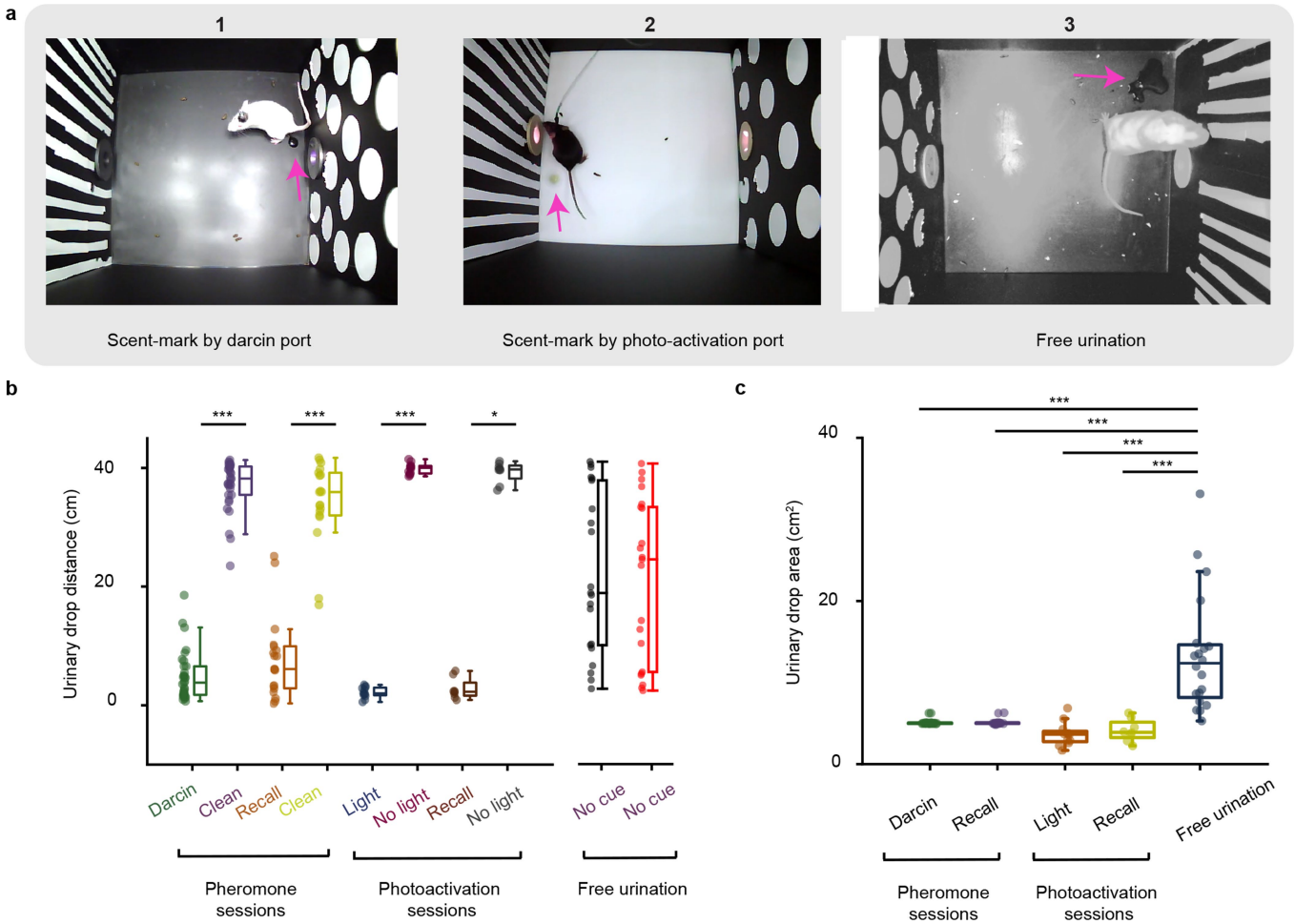
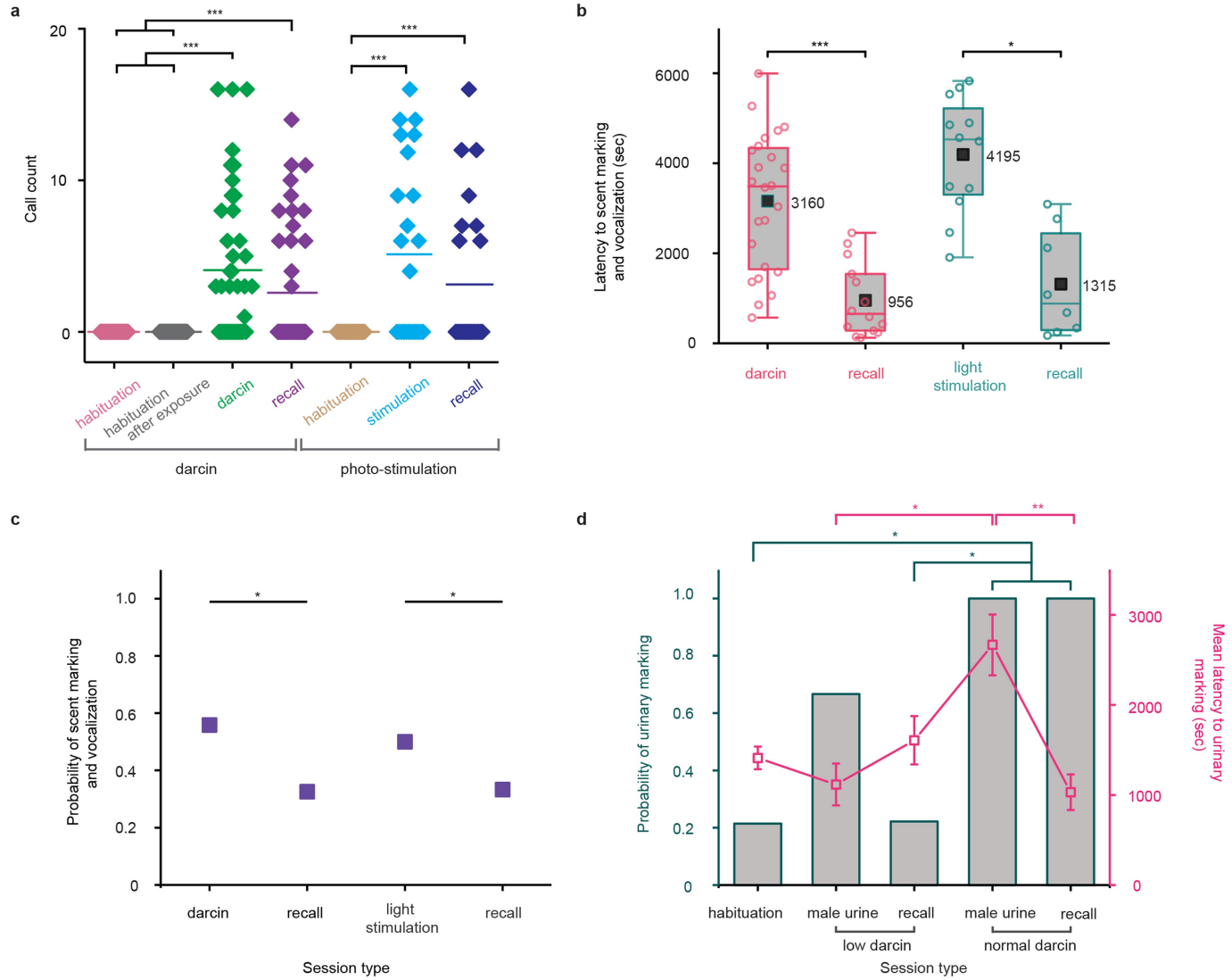
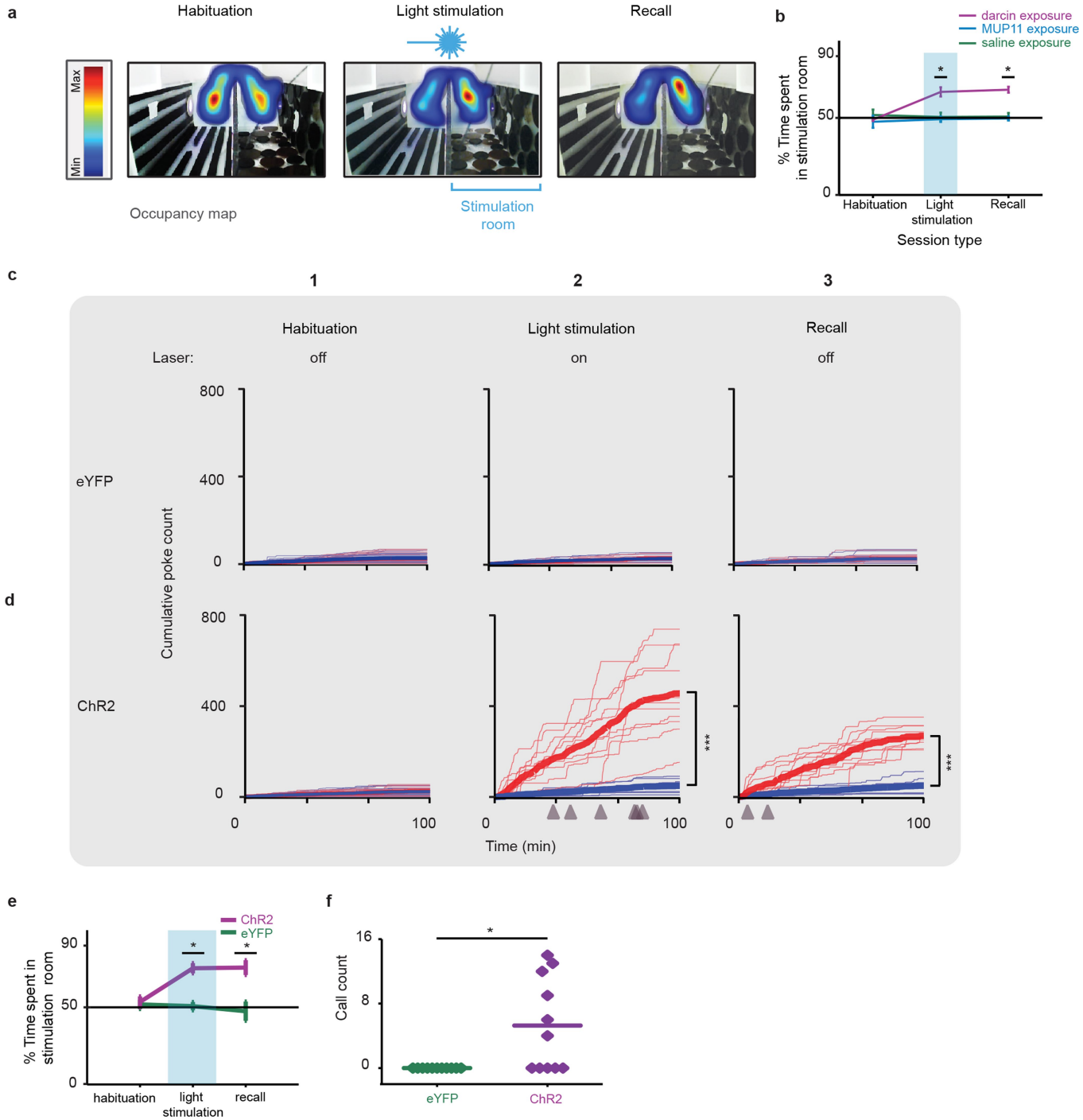


Extended Data Fig. 1 | Darcin and photoactivation of posterior MeA neurons condition scent-marking place preference. a, Representative frames from videos of the pheromone (1) and photoactivation (2) sessions, and free-range behaviours (3). **b**, Distance from urinary drop to each of the poke ports during various sessions. Individual frames were analysed using Adobe Photoshop CC to quantify the distance from the centre of a urinary drop to the base of each poke port. Units are scaled from pixels to centimetres. Distances were compared using the two-sided Wilcoxon signed-rank test (***$P < 0.0005$, *$P = 0.01$; $n = 24$ mice, (1); $n = 12$ mice, (2); $n = 20$ mice, (3)). **c**, Area of urinary drops under various conditions. Individual frames were analysed using Adobe Photoshop CC to quantify the area of the urinary marks. Units are scaled from square pixels to square centimetres. Scent-mark area (mean ± s.e.m., cm^2): darcin, 5 ± 0.05, $n = 24$ mice; recall of darcin, 5 ± 0.09, $n = 14$ mice; photoactivation, 4 ± 0.4, $n = 12$ mice; recall of photoactivation, 4 ± 0.5, $n = 8$ mice; free urination, 13 ± 2; $n = 20$ mice. Areas were compared using the two-sided Mann–Whitney test (***$P < 0.0005$), adjusted for multiple comparisons. The bounds in the box plots in **b**, **c** are defined by the 25th and 75th percentile of the distribution. The lines represent the median and the upper and lower whiskers represent the 75th percentile + 1.5 × IQR and 25th percentile − 1.5 × IQR, respectively.

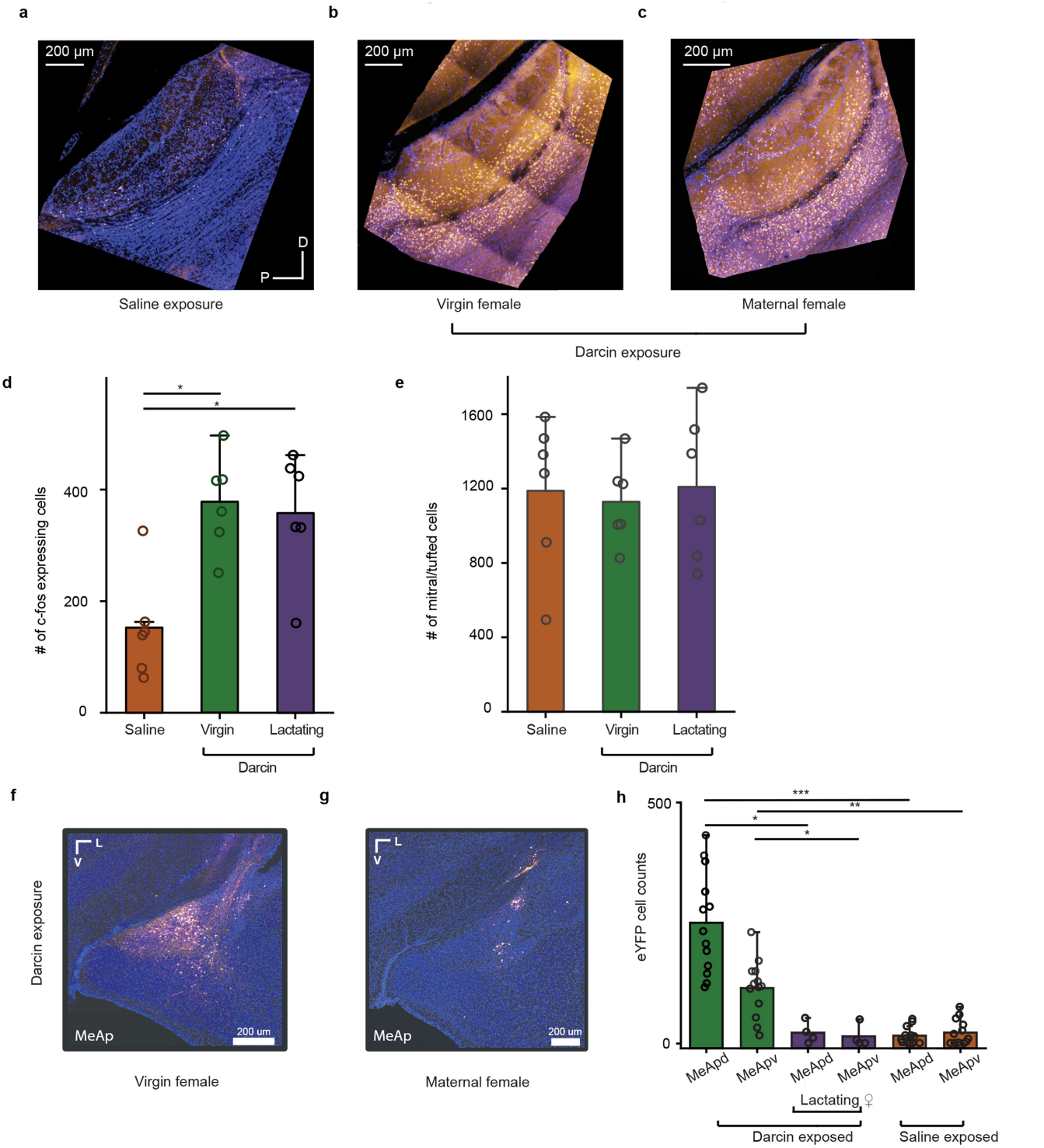


Extended Data Fig. 2 | Darcin and photoactivation of posterior MeA neurons reinforce recall of vocalization and scent-marking behaviours. a–**c**, Data for individual mice for all unique sessions across the study were pooled. **a**, Mean (horizontal line; $n = 43$ mice (darcin group), $n = 24$ mice (photostimulation group)) and total calls made by individual mice (diamonds) detected during various sessions. Call counts were compared using the two-sided Wilcoxon signed-rank test within the respective groups (***$P < 0.0005$), adjusted for multiple comparisons. **b**, Latency from the start of the session to urinary marking and vocalization behaviour (mean ± s.e.m., seconds) during exposure to darcin (3,160 ± 311, $n = 24$ mice), recall of darcin exposure (956 ± 217, $n = 14$ mice), photostimulation (4,195 ± 372, $n = 12$ mice) and subsequent recall (1,315 ± 418, $n = 8$ mice) sessions. Latencies were compared within groups using the matched-pair two-sided t-test (*$P = 0.005$, ***$P = 0.00009$). The bounds in the boxplots are defined by the 25th and 75th percentile of the distribution. The line represents the median and the upper and lower whiskers represent 75th percentile + 1.5 × IQR and 25th percentile − 1.5 × IQR, respectively. **c**, Probability of urinary scent-marking and vocalization behaviours. Mean probabilities are given for the darcin session (0.6, $n = 43$ mice), recall of darcin session (0.3, $n = 43$ mice), photostimulation-evoked urinary marking and vocalization (0.5, $n = 24$ mice) and recall of photostimulation-evoked behaviours (0.3, $n = 24$). Probabilities were compared using the two-sided McNemar test (*$P < 0.05$). **d**, Probability and mean latency to first urinary scent marking in the different sessions ($n = 9$ mice). Data from 100-min habituation sessions (mean ± s.e.m., latency for urination, seconds) and after exposure to male-soiled bedding in the home cage (1,411 ± 126), low-darcin urine from BALB/c mice (1,116 ± 232), recall session of BALB/c urine (1,607 ± 268), urine from C57BL6/6J mice containing normal levels of darcin (2,666 ± 337) and recall of C57BL6/6J urine (1,032 ± 198) are shown. Probabilities were compared using the two-sided McNemar test (*$P = 0.02$), adjusted for multiple comparisons. Latencies were compared within groups using the matched-pair two-sided t-test and across groups using the unpaired two-sided t-test (**$P = 0.0008$, *$P = 0.02$), adjusted for multiple comparisons. Scent-marking behaviours in response to low-darcin urine during the subsequent recall sessions were compared (habituation to recall, $P = 1$, cue to recall session comparison, $P = 0.1$, two-sided McNemar test).

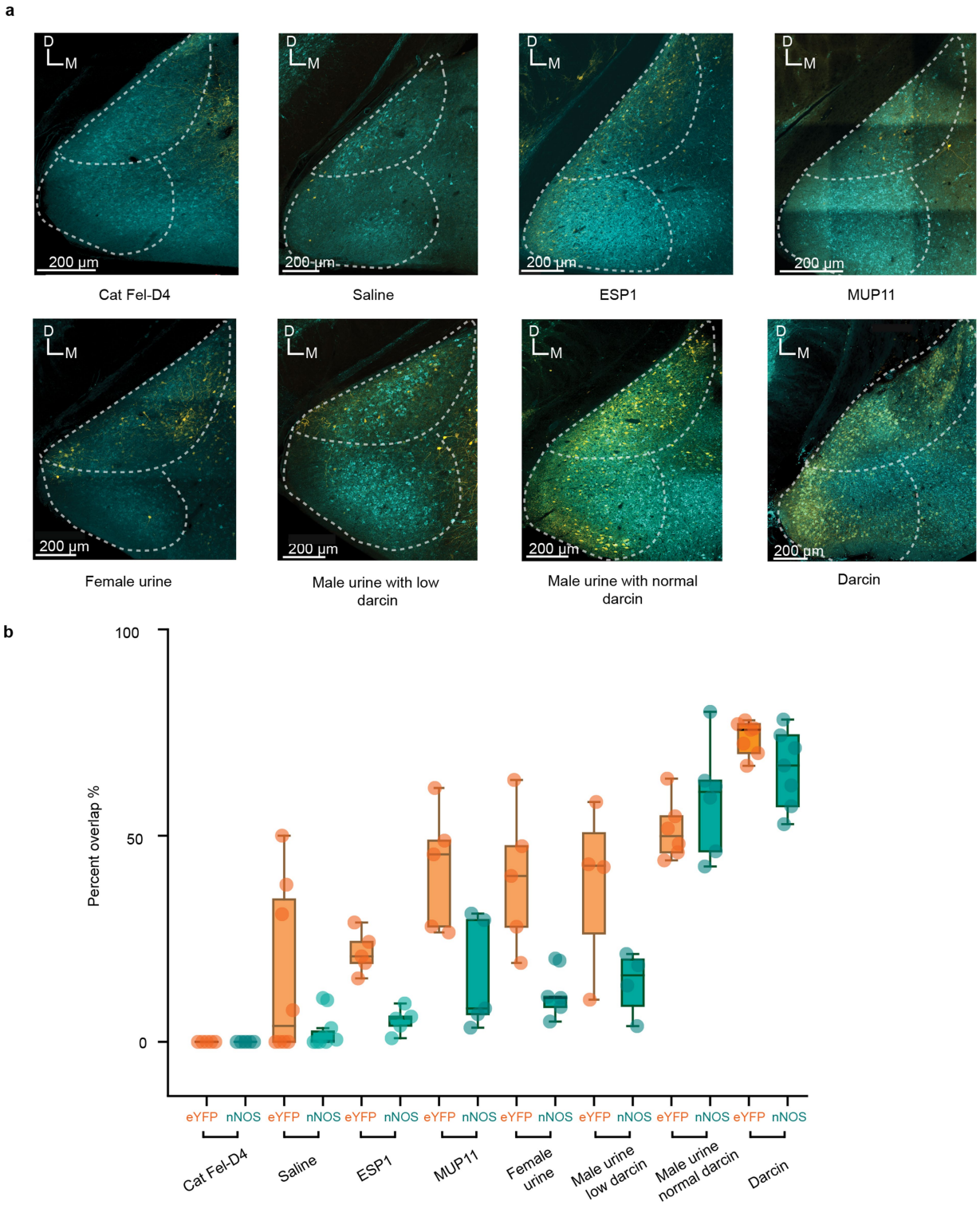


Extended Data Fig. 3 | See next page for caption.

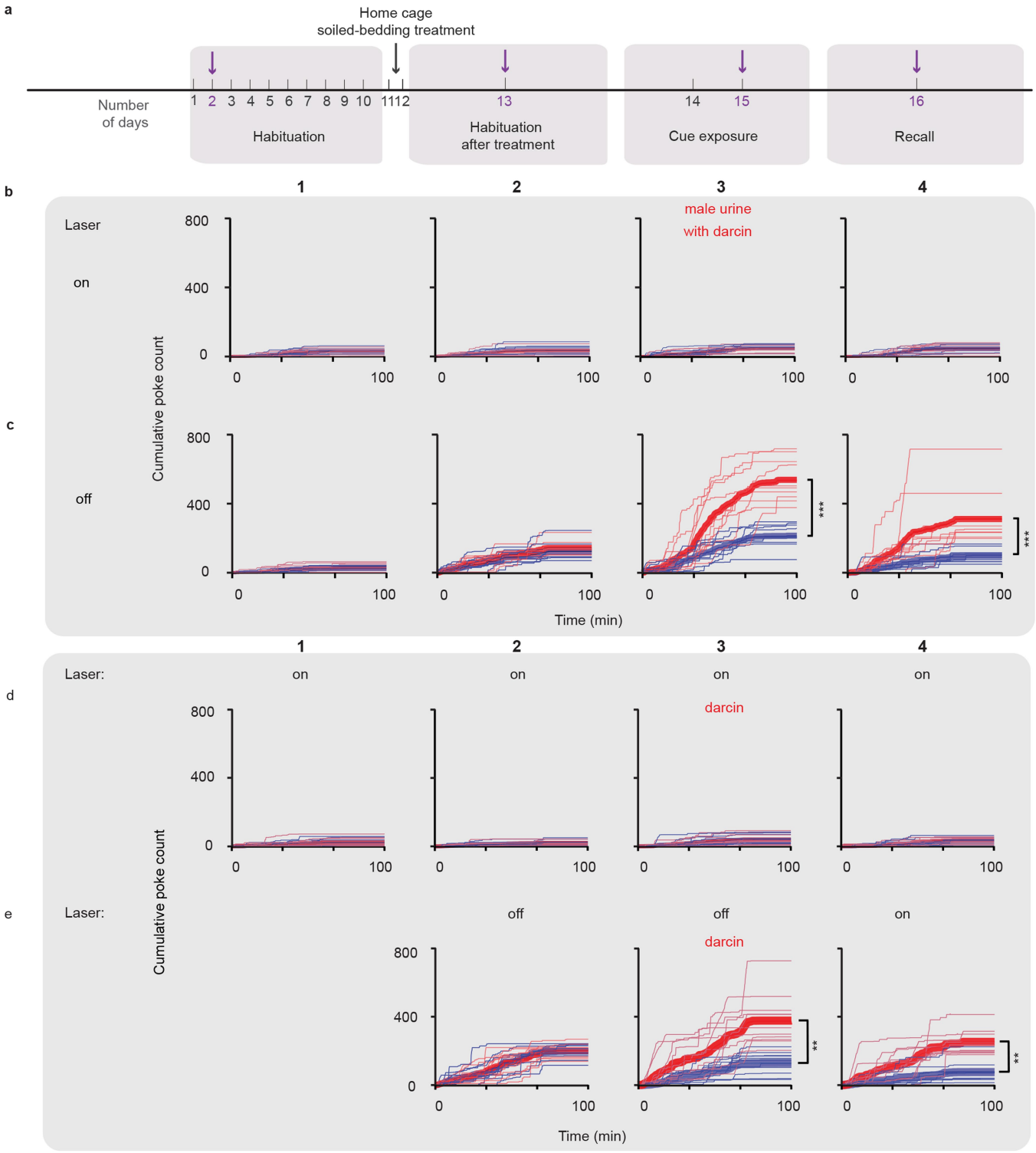
Extended Data Fig. 3 | Activation of darcin-responsive neurons in the posterior MeA recapitulates darcin-induced behaviours. a, Heat map showing occupancy of the chamber during a habituation, photostimulation and recall session. **b**, Occupancy plot showing the percentage of time spent in the photostimulation room. Arc-CreER mice were exposed to darcin (magenta), saline (green) or MUP11 (blue). The plot shows the mean ± s.e.m. ($n = 5$ mice per group, total $n = 15$ mice) percentage of time spent in stimulation room during habituation, photostimulation and recall sessions. For occupancy time, pairwise comparisons were performed using the two-sided Mann–Whitney test ($^*P < 0.05$) and three-way comparisons were performed using Kruskal–Wallis tests (habituation $P = 0.6$, light stimulation $P = 0.009$ and recall sessions $P = 0.008$). **c**–**f**, Activation of nNOS neurons in the posterior MeA recapitulates darcin-induced behaviours. **c**, **d**, Cumulative poke counts during habituation (laser off; 1), light stimulation (laser on; 2), and recall (laser off; 3) sessions in mice expressing eYFP (**c**) or ChR2 (**d**) in nNOS neurons. Light stimulation was performed in one port (red) and not in the second port (blue). During habituation (1) and recall (3) sessions, no light stimulation was given, and red and blue reflect right and left ports, respectively. Mean (bold lines, $n = 11$ mice for each group) and individual (fine lines) cumulative poke counts are shown. The time-stamps for ultrasonic vocalization and scent-marking behaviours are indicated as arrowheads (**d** (2, 3)). Poke counts were compared using the two-sided Wilcoxon signed-rank test ($^{***}P = 0.0001$). Control group (eYFP) port entries (**c**) are contrasted to the ChR2 group (**d**) during light stimulation (red entries for ChR2 (**d** (2)) compared to eYFP (**c** (2)); $P = 0.0002$) and recall sessions (red entries for ChR2 (**d** (3)) compared to eYFP (**c** (3)); $P = 0.0002$, two-sided Mann–Whitney test, adjusted for multiple comparisons). **e**, Occupancy plot showing the mean percentage of time spent in the photostimulation room by all mice during various sessions. nNOS-ires-Cre mice were injected with AAV encoding either eYFP (green) or ChR2–eYFP (purple); plots are colour-coded to their respective groups; $n = 6$ mice per group, $n = 12$ mice total. Occupancy times were compared using a two-sided Mann–Whitney test ($^*P < 0.05$). **f**, Mean (horizontal lines, $n = 11$ per group, $n = 22$ total) and total calls made by individual mice (diamonds) detected during the photostimulation (2) sessions in mice expressing eYFP (**c** (2)) or ChR2 (**d** (2)) in nNOS neurons. Call counts were compared using the two-sided Mann–Whitney test ($^*P = 0.007$).



Extended Data Fig. 4 | In lactating females, darcin activates mitral cells in the AOB but fails to activate MeA neurons. a–**c**, Representative images showing Fos expression (orange) and NeuroTrace (blue) in sagittal sections of the AOB following exposure to saline (**a**) or darcin in virgin females (**b**) and lactating females (**c**). Experiment was independently repeated on 6 mice for each group. **d**, Bar plots quantifying Fos-expressing cells in the AOB. Fos counts (mean ± s.e.m.): saline 153 ± 38, darcin in virgin females 378 ± 35, darcin in lactating females 358 ± 45; $n = 6$ mice per group. Cell counts were compared using the two-sided Mann–Whitney test (*$P = 0.02$), adjusted for multiple comparisons. **e**, Bar plots quantifying the mitral/tufted cells in the AOB. Number of cells (mean ± s.e.m.): saline 1,188 ± 167, darcin in virgin females 1,129 ± 93, darcin in lactating females 1,210 ± 163; $n = 6$ mice per group. Cell counts were compared using the two-sided Mann–Whitney test. **f**, **g**, Representative images showing eYFP expression in coronal sections of the posterior MeA of Arc-CreER mice after exposure to darcin in virgin females (**f**) and lactating females (**g**). Experiment was repeated on 13 mice and 4 mice in **f** and **g**, respectively. **h**, Bar plots quantifying eYFP-expressing cells in the MeApd and the MeApv. Cell counts were compared using the two-sided Mann–Whitney test, adjusted for multiple comparisons. *$P = 0.008$, **$P = 0.0006$, ***$P < 0.0005$. Mean ± s.e.m. eYFP-expressing cell counts: saline, 16 ± 5 in the MeApd and 23 ± 7 in the MeApv, $n = 13$ mice; darcin exposure in virgin females, 251 ± 29 in the MeApd and 115 ± 16 in the MeApv, $n = 13$ mice; darcin exposure in lactating females, 23 ± 11 in the MeApd and 15 ± 12 in the MeApv, $n = 4$ mice.

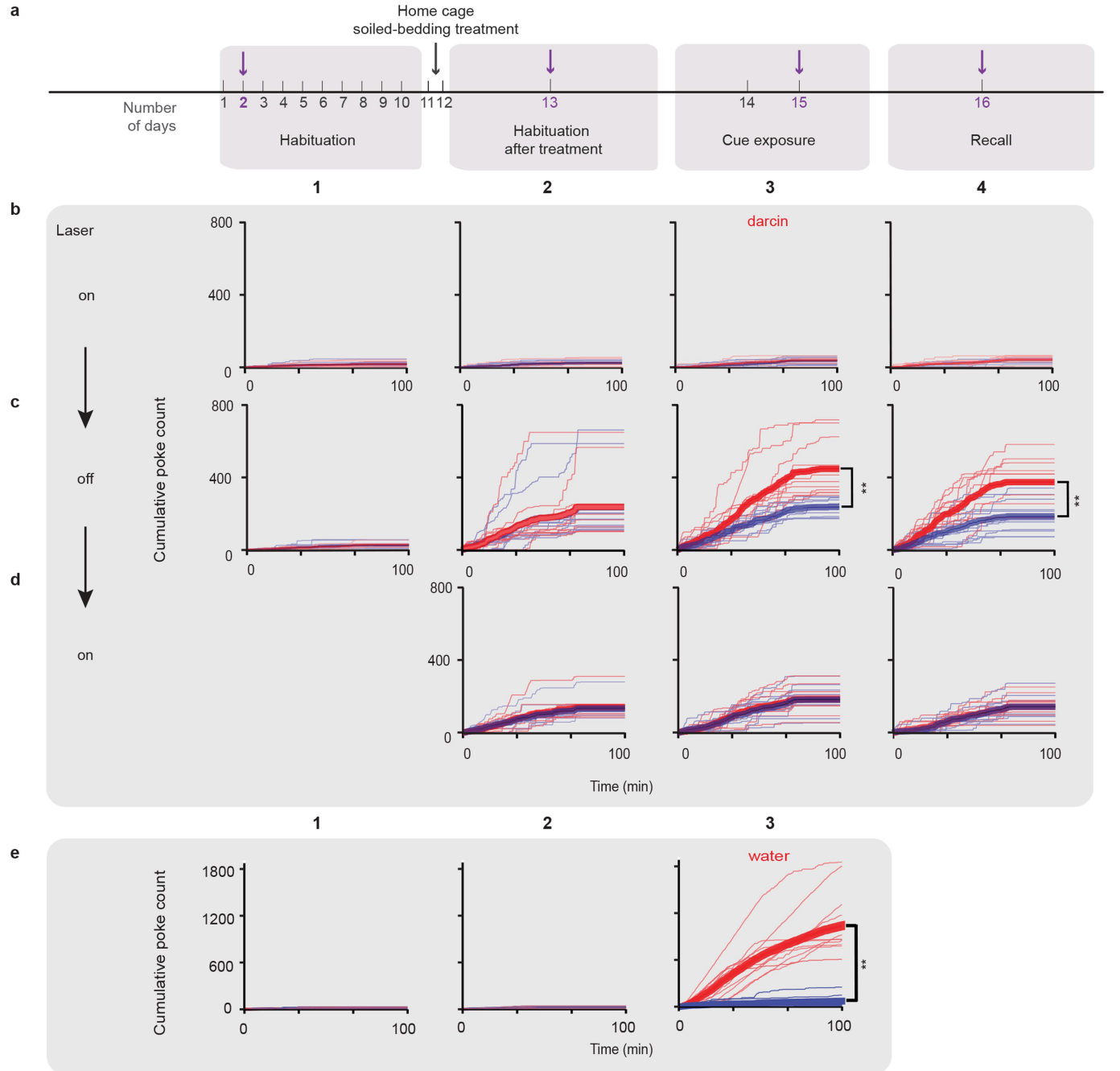


Extended Data Fig. 5 | Identification of neurons in the posterior MeA that respond to vomeronasal stimuli and their overlap with the genetic marker nNOS. a, Representative images showing the stimulus-responsive (eYFP, orange) and nNOS-expressing (cyan) neurons in the posterior MeA of Arc-CreER mice exposed to cat salivary lipocalin Fel-D4 ($n = 5$ mice), saline ($n = 8$ mice), ESP1 ($n = 5$ mice), MUP11 ($n = 5$ mice), female urine ($n = 5$ mice), male urine with low levels of darcin ($n = 4$ mice), male urine with normal levels of darcin ($n = 9$ mice) and darcin ($n = 7$ mice). **b**, Corresponding box plots quantifying the percentage overlaps between the stimulus-responsive (eYFP) and nNOS$^+$ neurons in the posterior MeA of mice exposed to the various stimuli. Orange plots represent the percentage of YFP cells that overlap with nNOS; cyan plots represent the percentage of nNOS cells that overlap with YFP. The bounds in box plots are defined by the 25th and 75th percentile of the distribution. The lines represent the median and the upper and lower whiskers represent the 75th percentile + 1.5 × IQR and 25th percentile − 1.5 × IQR, respectively.

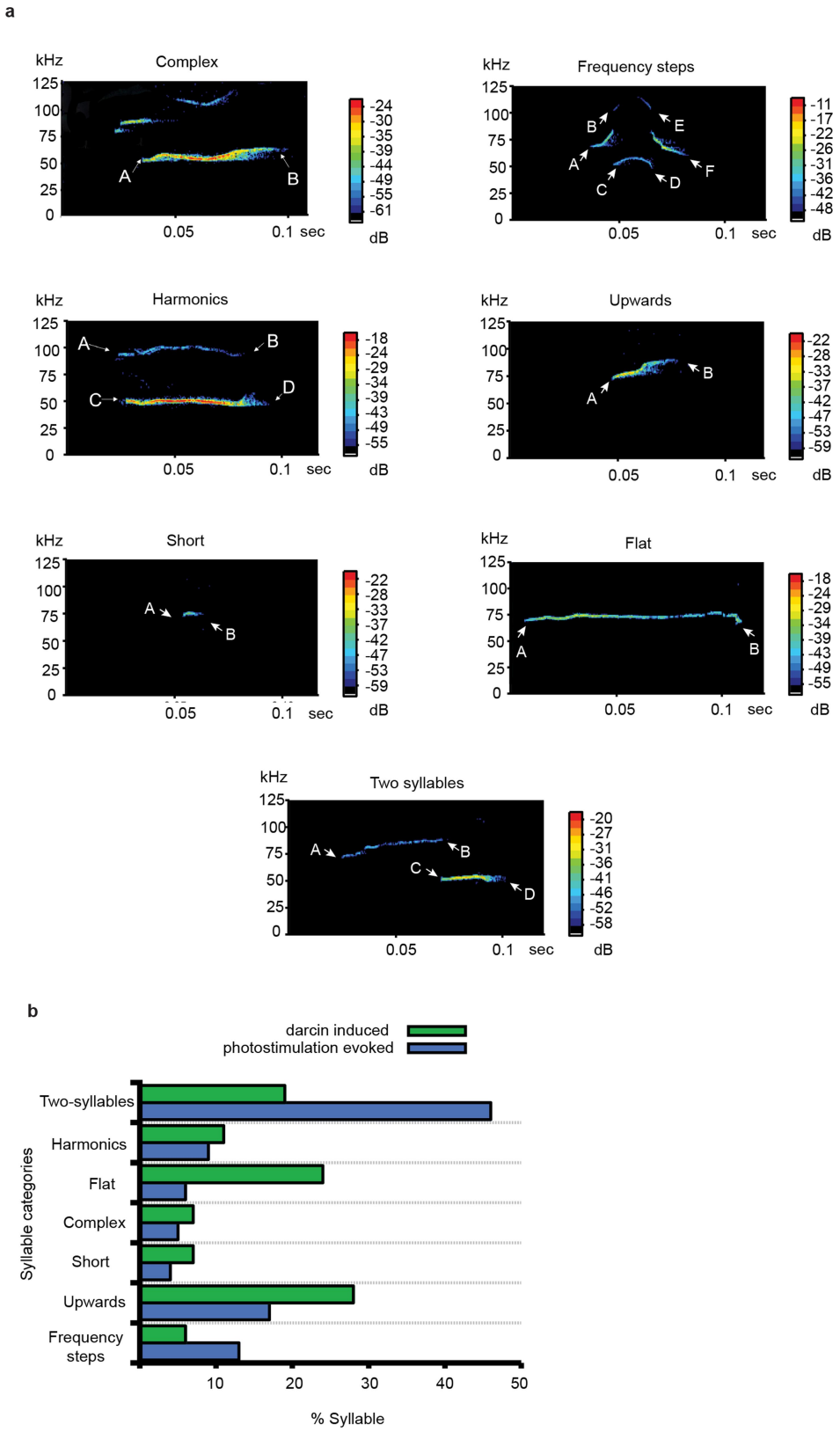


Extended Data Fig. 6 | See next page for caption.

Extended Data Fig. 6 | The additional effects of silencing nNOS neurons in the posterior MeA. a–**c**, Functional convergence of both olfactory systems mediated by the posterior MeA is pivotal for male urine reinforcement. **a**, Timeline of the preference assay. Mice were habituated in the chamber for ten days (1), then exposed to male-soiled bedding for 60 h in their home cage (2), followed by one additional day of habituation before male urine (with normal levels of darcin (1 µg µl^{-1})) was presented in one of the two ports (3). Urine was removed for the recall session one day later (4). Port preference was quantified from port entries. **b**, **c**, Cumulative poke counts during habituation (1), habituation after treatment (2), exposure to male urine (3) and recall (4) sessions for mice expressing eNpHR–eYFP (n = 10) with (**b**) and without (**c**) optical silencing of nNOS neurons. Poke counts were recorded on the days indicated by purple arrows in **a**. Mice were exposed to male urine in one port (red) and a blank filter (blue) in the second port (3). During habituation (1, 2) and recall (4) sessions both ports contained a blank filter. Mean (bold lines) and individual (fine lines) cumulative poke counts are shown. Poke counts were compared using the two-sided Wilcoxon signed-rank test (***P = 0.0002). The effect of silencing nNOS neurons is quantified with matched pair differences (male urine session comparisons, **b** (3) to **c** (3), P = 0.002) and recall of male urine with darcin (recall session comparisons, **b** (4) to **c** (4), P = 0.002) using the two-sided Wilcoxon signed-rank test, adjusted for multiple comparisons. **d**, **e**, Optical silencing of nNOS neurons does not affect recall of darcin memory. Cumulative poke counts during habituation (1), habituation after treatment (2), darcin (3) and recall (4) sessions in mice expressing eNpHR (n = 11) with optical silencing during all sessions (**d** (1–4)) and with optical silencing during recall sessions only (**e** (4)). Poke counts were recorded on the days indicated by purple arrows in **a**. Mice were exposed to darcin in one port (red) and a blank filter (blue) in the second port (3). During habituation (1, 2) and recall (4) sessions both ports contained a blank filter. Mean (bold lines) and individual (fine lines) cumulative poke counts are shown. Poke counts were compared using the two-sided Wilcoxon signed-rank test (**P = 0.001). The effect of silencing nNOS neurons during recall sessions was tested with matched pair differences (**c**, cue (**e** (3)) to recall (**e** (4)) comparisons, laser off (**e** (3)) and on (**e** (4)), P = 0.1, using the two-sided Wilcoxon signed-rank test, adjusted for multiple comparisons.



Extended Data Fig. 7 | Mice subjected to optical silencing of nNOS neurons retained a motivation to poke. To establish the primacy of the MeA in mediating darcin-evoked behaviours rather than altering general motivation, mice expressing eNpHR in nNOS neurons were also tested. **a**, Timeline of the two-port preference assay. **b**–**d**, Cumulative poke counts during habituation (1), habituation after exposure to male-soiled bedding in the home cage (2), darcin exposure (3) and recall (4) sessions with (**b**) and without (**c**) optical silencing of nNOS neurons, and with optical silencing again after 4 weeks (**d**) ($n = 11$ mice). Poke counts were recorded on the days indicated by purple arrows in **a**. Mice were exposed to darcin in one port (red) and a blank filter (blue) in the second port. During habituation (1, 2) and recall (4) sessions both ports contained a blank filter. Mean (bold lines) and individual (fine lines) cumulative poke counts are shown. Poke counts were compared using the two-sided Wilcoxon signed-rank test (**$P = 0.001$). The effect of silencing nNOS neurons after a learning experience is quantified during habituation sessions after exposure to soiled bedding in the home cage (port entries to the same port (red) with blank filters are compared during habituation after home-cage treatment sessions in **b** (2) and **c** (2), laser on and off, $P = 0.002$, in **b** (2) and **d** (2), laser on, $P = 0.001$, and **c** (2) and **d** (2), laser off and on, $P = 0.5$). The paired count differences (red–blue port) are compared across darcin sessions (**b** (3) to **d** (3), laser on, $P = 0.5$, and **c** (3) to **d** (3), laser off and on, $P = 0.0001$) and recall of darcin (recall session comparison **b** (4) to **d** (4), $P = 0.9$, and **c** (4) to **d** (4), $P = 0.0001$) using the two-sided Wilcoxon signed-rank test, adjusted for multiple comparisons. **e**, Optical silencing of nNOS neurons in the MeA does not affect non-social reinforcement behaviour. Cumulative poke counts during habituation (1), habituation after treatment (2), and water (3) sessions in mice expressing eNpHR ($n = 12$) in nNOS neurons in the MeA with silencing. Poke counts were recorded on the days indicated by purple arrows in **a**. Water-restricted mice were rewarded with a drop of water (5 µl) in one port (red) and a blank filter in the second port (blue). During habituation (1, 2) sessions both ports contained a blank filter. Mean (bold lines) and individual (fine lines) cumulative poke counts are shown. Poke counts were compared using the two-sided Wilcoxon signed-rank test (**$P = 0.0005$).



Extended Data Fig. 8 | Ultrasonic vocalizations that are emitted by mice exposed to darcin or stimulated optogenetically consist of seven unique syllable categories. a, Representative spectrograms of ultrasonic vocalizations classified into seven categories of call. The heat maps show the intensities of the vocalizations. Descriptive statistics (mean ± s.d., sample sizes) for frequencies are given in Extended Data Table 2 for the locations indicated by the corresponding letters on the spectrograms. **b**, Percentages of different call categories emitted by mice exposed to darcin ($n = 24$, in green) and optogenetically stimulated ($n = 12$, in blue).

Extended Data Table 1 | Cell counts for exposure to different cue types, nNOS expression and the overlaps in the posterior MeA

Cue Type	YFP + in MeApd	nNOS + in MeApd	YFP + nNOS + in MeApd	YFP + in MeApv	nNOS + in MeApv	YFP + nNOS + in MeApv	YFP + in MeA pd+pv	nNOS + in MeA pd+pv	YFP + nNOS + in MeA pd+pv	% nNOS / YFP % Overlap
Darcin (n=7)	200 ± 18	220 ± 22	146 ± 15	155 ± 21	179 ±28	120 ± 21	311 ± 46	348 ± 54	232 ± 37	66 ± 3
Male urine normal darcin (n=9)	262 ± 45	155 ± 23	114 ± 16	168 ± 43	114 ± 25	63 ± 13	344 ± 33	275 ± 45	159 ± 21	59 ± 5
Male urine low darcin (n=4)	**102 ± 6***	152 ± 17	**36 ± 12***	64 ± 26	301 ± 123	21 ± 8	160 ± 14	250 ± 22	**41 ± 17***	**22 ± 8***
Female Urine (n=5)	**70 ± 12***	160 ± 22	**27 ± 7***	42 ± 4	172 ± 61	17 ± 5	**104 ± 15***	297 ± 47	**45 ± 11***	**11 ± 3***
MUP11 (n=5)	**49 ± 10***	206 ± 53	**17 ± 5***	53 ± 12	183 ± 49	26 ± 8	**102 ± 17***	390 ± 93	**43 ± 8***	**16 ± 6***
ESP1 (n=5)	**85 ± 18***	323 ± 48	**14 ± 3***	111 ± 30	576 ± 75	31 ± 8	197 ± 43	899 ± 93	**45 ± 11***	**5 ± 1***
Saline (n=8)	**22 ± 9***	264 ± 41	**6 ± 3***	**14 ± 8***	290 ± 73	5 ± 4	**36 ± 15***	554 ± 92	**11 ± 6***	**2 ± 1***
Fel-D4 (n=5)	**9 ± 9***	204 ± 28	**0 ± 0***	8 ± 8	189 ± 51	**0 ± 0***	**13 ± 13***	314 ± 91	**0 ± 0***	**0 ± 0***

Counts (mean ± s.e.m. cell counts) quantifying region-specific and overlapping expression of cue-responsive (eYFP$^+$ neurons) and nNOS-expressing neurons in the MeApd and the MeApv for female mice exposed to darcin, male urine with normal levels of darcin, male urine with low levels of darcin, female urine, MUP11, ESP1, cat Fel-D4 or saline. The percentage of overlap (mean ± s.e.m.) is quantified between total eYFP and nNOS-expressing neurons in the posterior MeA. Comparisons are made pairwise between darcin and all other cue types for eYFP$^+$counts and the overlaps using the two-sided Mann–Whitney test (*P < 0.05). Comparisons are made pairwise between darcin and all other cue types for the percentage of nNOS-expressing neurons overlapping with eYFP using the two-sided Mann–Whitney test (*P < 0.05).

Extended Data Table 2 | Syllable categories for darcin and light-evoked ultrasonic vocalizations

	Darcin-Evoked	Light-Evoked	P(T≤t)
	(mean ± sd)	(mean ± sd)	
Complex	n=5	n=9	
A	62kHz ± 23kHz	67kHz ± 2kHz	
B	68kHz ± 22kHz	72kHz ± 4kHz	
Duration	42ms ± 18ms	111ms ± 54ms	*
Harmonics	n=8	n=15	
A	83kHz ± 15kHz	66kHz ± 4kHz	*
B	91kHz ± 22kHz	78kHz ± 6kHz	
C	45kHz ± 8kHz	49kHz ± 3kHz	
D	46kHz ± 11kHz	49kHz ± 2kHz	
Duration	77ms ± 26ms	81ms ± 36ms	
Short	n=3	n=9	
A	75kHz ± 28kHz	74kHz ± 11kHz	
B	78kHz ± 26kHz	73kHz ± 11kHz	
Duration	8ms ± 4ms	8ms ± 3ms	
Two-syllable	n=45	n=27	
A	75kHz ± 8kHz	81kHz ± 8kHz	*
B	86kHz ± 12kHz	100kHz ± 6kHz	***
C	57kHz ± 10kHz	67kHz ± 6kHz	***
D	63kHz ± 9kHz	72kHz ± 8kHz	***
Duration	56ms ± 25ms	74ms ± 23ms	**
Upwards	n=17	n=39	
A	64kHz ± 12kHz	72kHz ± 9kHz	*
B	79kHz ± 8kHz	81kHz ± 12kHz	
Duration	19ms ± 9ms	36ms ± 17ms	***
Frequency steps	n=13	n=8	
A	74kHz ± 10kHz	77kHz ± 10kHz	
B	85kHz ± 9kHz	94kHz ± 14kHz	
C	52kHz ± 11kHz	62kHz ± 10kHz	*
D	53kHz ± 8kHz	64kHz ± 13kHz	*
E	86kHz ± 7kHz	100kHz ± 15kHz	*
F	76kHz ± 10kHz	91kHz ± 17kHz	*
Duration	71ms ± 26ms	97ms ± 36ms	
Flat	n=7	n=34	
A	62kHz ± 12kHz	70kHz ± 6kHz	
B	60kHz ± 13kHz	71kH ± 7kHz	
Duration	47ms ± 23ms	74ms ± 47ms	*

Different call categories emitted by mice exposed to darcin ($n = 24$) and optogenetically stimulated ($n = 12$). Frequencies and durations are compared with the unpaired two-sided t-test (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0006$).

nature research

Corresponding author(s): Prof. Adam Kepecs and Prof. Richard Axel

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
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☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	The behavioral nose poke data were acquired through a MATLAB 2009b-2013a (MathWorks Inc, Natick, MA) interface and an Arduino-powered device (Bpod v0.5, Sanworks, LLC, Stony Brook, NY). Avisoft Recorder USGH software was used to record vocalizations and integrate time codes from the Horita PTG2. The position of the mice was tracked using video tracking software XT10 (Noldus, Ethovision XT 10). A video recorder (AJA Ki Pro Recorder, AJA Video Systems, Grass Valley, CA, United States), which was connected to the camera and the Horita PTG2, was used to record video for the entire duration of the session. The time code generated by the Horita PTG2 was visible as an OLED (Organic Light Emitting Diode) display within the video window of the Marshall Electronics camera recording through the AJA recorder and was also recorded by screen-capturing software (Adobe Captivate CC 2017, Adobe Systems Inc.).The behavioral hardware (valves for water delivery and port sensors) and the laser for optogenetic stimulation were controlled by custom MATLAB 2009b-2013a programs (MathWorks Inc, Natick, MA) and two Arduino-powered devices (Bpod v 0.5, Sanworks LLC and PulsePal, Sanworks LLC).
Data analysis	The behavioral nose poke data were analyzed by a custom program written in MATLAB 2009b-2013a (MathWorks Inc, Natick, MA). To analyze the nature of the ultrasonic vocalizations, Avisoft SAS Lab Pro Version 5.2.12 was used. A fast Fourier transformation (FFT) was applied to the recordings to generate spectrograms; the following parameters were used: FFT length of 256, Hamming window, time window overlap of 50%, frequency resolution of 977 Hz, and time resolution of 0.5 ms. The vocalizations were frequency modulated to a human-audible range using Avisoft SAS Lab Pro Version 5.2.12. All spectrograms were additionally parametrized using SAP 2011, MUPET and MATLAB 2009b-2013a software. To analyze the urinary scent marking behavior of the animals, Adobe Premiere Pro CC 2017 (Adobe Systems Inc.) was used to process the video recordings from each session. The position of the mice was tracked using video tracking software XT10 (Noldus, Ethovision XT 10), and the occupancy trajectories and time-spent in each chamber were computed for analysis. All statistical analyses were performed using R (R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria), OriginLab Pro 2017-2019 (OriginLab Corp., Northampton, MA), and MATLAB 2009b-2013a(MathWorks Inc, Natick, MA).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
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The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical method was used to determine the sample size prior to the study, but sample sizes were consistent with the research papers in the relevant field.
Data exclusions	In very rare cases, mice that are confirmed to have no viral expression after behavioral testing were excluded, and these exclusion criteria were predetermined.
Replication	All attempts to replicate the behavioral, opto-genetics, and immunohistochemical experiments were successful, indicating the robustness of the presented results. To guarantee reliable replication of our results, we preferred to work with high infectious titer (3e6 IU/ml) and DJ serotype AAV viruses (as purchased from Stanford virus core), especially when we delivered the viruses into medial amygdala.
Randomization	Animals were randomly assigned to the test. The social cue or light stimulation (for optogenetic activation) sides were evenly split in a random manner between two ports across the animals to control for any potential side bias.
Blinding	All behavioral experiments were scored by an individual blind to the social cue or light stimulation side and experimental design.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	All antibodies used in this study are commercially available and validated by the manufacturers, except the guiena pig c-fos antibody . The following primary antibodies were used: anti-GFP (rabbit polyclonal, 1:1000, Rockland, catalog #: 600-401-215, lot#s: 28983, 33267or chicken polyclonal 1:400, Aves Lab, catalog#:GFP-1020, lot#s: GFP879484, GFP697986), anti-nNOS (rabbit polyclonal, 1:400, Invitrogen, catalog#:61-7000, lot#s: 1207899A, 987786A, 1578834A, 797629A), and anti-c-fos (goat and rabbit polyclonal, 1:500, Santa Cruz Biotechnology, catalog #: rabbit sc 52, lot#: B1115, goat sc 52-G, lot#s: 10215, J1613, and K1109, and guinea pig polyclonal c-fos, with RRID#, AB_2814707, generated by Susan Brenner-Morton at ZMBBI, Columbia University). Secondary antibodies are all used at 1:500 dilutions (alexa-594 goat anti-rabbit: Jackson ImmunoResearch, catalog#: 111-585-003, lot#: 135 626, 140268, alexa-594 goat anti-rat: BioLegend, Clone: Poly4054, catalog#: 405422, lot#: B262774, alexa-633 donkey anti-goat: Life Technologies, catalog#: A21082, lot#1711470, alexa-488 goat anti-rabbit: Jackson ImmunoResearch, catalog#: 111-545-006, lot#131752, alexa-488 goat anti-chicken: Jackson ImmunoResearch, catalog#:

103-545-155, lot#: 139170, alexa-488 donkey anti-rabbit: InVitrogen catalog#: A21206, lot#: 1981155, alexa-488 goat anti-guinea pig: Jackson ImmunoResearch, catalog#:706-545-148, lot#s: 127887, 143798, alexa-488 donkey anti-chicken: Jackson ImmunoResearch, catalog#:703-545-155, lot#s: 126602, alexa-594 donkey anti-rabbit: InVitrogen, catalog#: A21207, lot#: 1987293, and NeuroTrace alexa 640/660, Molecular Probes, catalog#: N21483, lot# 1656094).

Validation

All antibodies are validated to react with corresponding mouse antigens.

Rabbit anti-nNOS: validated by Invitrogen and Thermofisher (https://www.thermofisher.com/us/en/home/life-science/antibodies/invitrogen-antibody-validation.html?icid=ab-search-learning-ab-validation), tested with the following applications, ELISA, Western Blotting, Immunohistochemistry (frozen), see the reference: Huang, PL. et. al. (1995) Nature 377: 239-242. CiteAb database reports 53 citations for this antibody.

Rabbit anti-GFP: validated by Rockland Immunochemicals (https://rockland-inc.com/store/Antibodies-to-GFP-and-Antibodies-to-RFP-600-401-215-O4L_18562.aspx), tested with ELISA, Western Blotting, immunohistochemistry, IF microscopy applications, and reported that no reactivity was observed against Human, Mouse or Rat serum proteins. CiteAb database reports 36 citations for this antibody.

Chicken anti-GFP: validated by Aves (https://www.aveslabs.com/products/green-fluorescent-protein-gfp-antibody), tested with Western blot, and immnunohistochemistry. See the reference: Lu J. et. al. 2017(10):1377-1383 for both GFP antibodies. CiteAb database reports 702 citations for this antibody.

c-fos antibodies are validated by Santa Cruz Biotech. using immunohistochemical, Western Blotting and immunofluorescence. See the references: Choi GB. et. al. Cell 146(6):1004-15 and Root CM. et. al. (2014) Nature 515 (7526): 269-73. These c-fos antibodies are discontinued by the manufacturer, therefore we generated a guinea pig polyclonal c-fos antibody with the help of Susan Brenner-Morton at ZMBBI, Columbia University. We validated guinea pig c-fos by comparisons to commercially available c-fos antibodies from Santa Cruz. We performed immuno-histochemistry on free floating mouse brain sections with various c-fos antibodies and counted the number of cells stained by each antibody for comparisons in various mouse brain regions.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Experiments were conducted with 279 female mice between 6 and 30 weeks old. Mice were purchased at 4 weeks old. The mouse lines used were Arc-CreER (a gift from Christine Denny at Columbia University; also available from Jackson Laboratory, Jax stock #022357), ICR outbred (CD-1) wild-type mice (Harlan/Envigo), Ai14 (Rosa-CAG-LSL-tdTomato), nNOS-ires-CRE (Jax stock #017526), vGlut-ires-CRE (Jax stock #028863), Gad2-T2a-NLS-mCherry (Jax stock #023140). The nNOS-ires-CRE mice were crossed to ICR outbred mice (Harlan/Envigo) for 15 generations to exchange their genetic background to the ICR mice.

Wild animals

This study did not involve any field captured animals.

Field-collected samples

This study did not involve any field captured samples.

Ethics oversight

All surgical and experimental procedures were done in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and approved by the Cold Spring Harbor Laboratory and Columbia University Medical Center Institutional Animal Care and Use Committees.

Note that full information on the approval of the study protocol must also be provided in the manuscript.