Darcin activates a neural circuit that elicits a complex behavioral array

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Organisms have evolved diverse behavioral 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, darcin, a major urinary protein (MUP) present in male urine, provides a component of a scent mark that elicits approach by females and drives learning[3,4]. We observed that darcin elicits a more complex and variable behavioral repertoire consisting of attraction, ultrasonic vocalization, and urinary scent-marking, and also serves as a reinforcer in

learning paradigms. We then identified a genetically determined circuit extending from the accessory olfactory bulb (AOB) to the posterior medial amygdala (MeA) that is necessary for all behavioral responses to darcin. Moreover, optical activation of darcin-responsive neurons in the MeA induces both the innate and conditioned behaviors elicited by the pheromone. These neurons define a topographically segregated population that express neuronal nitric oxide synthase (nNOS) specifically responsive to darcin. These observations suggest that darcin activates a neural circuit that integrates pheromonal information with internal state and the external world to elicit a complex and variable array of innate and learned behaviors that may promote mate encounters and mate selection.

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

We developed a quantitative behavioral paradigm to examine the effects of darcin and observed that the pheromone elicits a more complex and variable behavioral array. Female mice were placed in a chamber equipped with two ports containing glass fiber filters embedded with different social olfactory cues and port entry 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±sem poke counts, left port, 18±3 vs. right port, 14±3, **Fig. 1b$_1$**). Pokes increased dramatically after exposure to male-soiled bedding in the home cage[3] without any apparent side bias (left, 247±35 vs. right, 246±3, **Fig. 1b$_2$)**. The response to darcin was therefore examined in cycling females after exposure to male-soiled bedding[3]. Poke frequency was higher for the port with the recombinant darcin (darcin-containing port 516±47 vs. blank 326±21, **Fig. 1b$_3$)**. Male urine with very low levels of darcin (low darcin BALB/c urine)[4] also elicited more frequent port entries than blank filters in this assay with or without the addition of recombinant darcin (low

58 darcin urine 386±42 vs. blank 154±14, **Fig. 1c$_3$** or recombinant darcin-added urine 391±29 vs.
59 blank 96±18, **Fig. 1d$_3$**).
60 Innately attractive cues can often serve as a teaching signal, reinforcing both classical and
61 instrumental learning[7]. We examined whether exposure to darcin alone or to low darcin urine
62 elicits a lasting preference for the darcin port once the stimulus is removed. Females were
63 exposed to a social cue in one port and then placed into a clean chamber on the following day
64 with blank filters in both ports. Poke counts were significantly greater in the port that had
65 previously contained either darcin (285±38 vs. blank 146±16, **Fig. 1b$_4$**) or urine with equivalent
66 levels of darcin (179±15 vs. blank 65±9, **Fig. 1d$_4$**). In contrast, exposure to urine with very low
67 levels of darcin did not result in a port preference during recall sessions on the following day
68 (prior exposure to low levels of darcin urine 147±14 vs. blank 147±16, **Fig. 1c$_4$**). Thus, both low
69 darcin male urine and darcin elicit port preference but only exposure to normal levels of darcin
70 results in a remembered preference.
71
72 Interestingly, we also observed that female mice exposed to darcin emitted ultrasonic
73 vocalizations and urinary scent marking (**Fig. 1e-h**). Scent marks were located closer to the
74 darcin port (**Extended Fig. 1a-b**) and were smaller in size (**Extended Fig. 1c**) than we observe
75 with free urination, consistent with the deliberate deposition of scent near darcin. This suggests a
76 distinction between the two behaviors. Ultrasonic vocalizations were consistently linked with
77 urinary marking and occurred within 40 milliseconds (mean±sem: 42±9 ms) of one another (See
78 **Supp. Video 1**). These episodes did not occur immediately upon darcin exposure but rather
79 appeared with a long and variable delay during a 100-minute session (mean latency:53±5
80 minutes, **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 (mean latencies: recall=16±4, darcin sessions=53±5 minutes, **Fig. 1h)**. Male urine containing normal levels of darcin also stimulated scent marking behavior during cue and recall sessions, but male urine with very low levels of darcin stimulated marking only when present and not during recall (**Extended Fig. 2d**). Thus, darcin induces a behavioral repertoire comprised of attraction and ultrasonic vocalization synchronous with urine marking, behaviors that may serve as reciprocal communication. Moreover, this behavioral repertoire is also observed during recall sessions in the absence of darcin.

We have implemented genetic strategies to identify the neural circuitry that mediates these darcin-induced behaviors. 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 medial amygdala (MeA)[8,9]. We demonstrated that this pathway is responsible for the behavioral 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 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 vs. blank 149±37, **Fig. 2c$_3$**) and suppressed darcin-evoked ultrasonic vocalizations and scent marking (**Fig. 2g**). In contrast, the preference for male urine with normal levels of darcin was not suppressed in the presence of AOB silencing

(423±40 vs. blank 243±25, **Fig. 2e$_3$**). AOB silencing did not affect port investigation during the initial habituation periods with the blank filters (**Fig. 2c$_{1,2}$-f$_{1,2}$**).

Exposure to recombinant darcin elicited a memory for the darcin port (prior exposure to darcin 190±16 vs. blank 56±6, **Fig. 2d$_4$**) but a port preference was not observed if the AOB was silenced during darcin exposure (prior exposure to darcin 55±15 vs. blank 60±18, **Fig. 2c$_4$**). Females that experienced male urine with normal levels of darcin also exhibited a persistent port preference during AOB silencing (urine 423±40 vs. blank 243±25, **Fig. 2e$_3$**), but failed to show preference for this port in the recall sessions (prior exposure to urine with normal levels of darcin 97±16 vs. blank 94±15, **Fig. 2e$_4$**). These observations show that the AOB is necessary for darcin-induced attraction behaviors, as well as for conditioning. Other components of male urine also elicit attraction that is independent of the AOB but fail to reinforce conditioned behaviors.

The projection neurons of the AOB, the mitral cells, send axons to the medial amygdala (MeA)[8,9]. We identified the neurons of the MeA responsive to darcin, using the promoter of the activity-dependent gene, Arc, to express the light-gated ion channel, channelrhodopsin[11]. AAV encoding Cre-dependent channelrhodopsin fused to the fluorescent protein eYFP was injected into the MeA of transgenic 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 the neurons activated by darcin. We compared the expression of c-fos with ChR2-eYFP to demonstrate that ChR2–eYFP is faithfully expressed in neurons that respond to darcin (78±4% of the ChR2-eYFP+ neurons

also express endogenous c-fos and 79±3% of the neurons expressing endogenous c-fos also expressed ChR2–eYFP (n=6)).

We next determined whether the activation of neurons expressing ChR2 induced by darcin exposure is sufficient to recapitulate the behaviors elicited by darcin. Arc-CreER mice injected with AAV encoding Cre-dependent ChR2-eYFP in the posterior dorsal (MeApd) and ventral (MeApv) medial amygdala of Arc-CreER mice (**Fig. 3a, b**) were treated with tamoxifen and then exposed to either darcin, saline, or control MUP (MUP11)[3,4]. Histologic analysis of ChR2-eYFP expression induced by darcin exposure revealed a dense clustering of ChR2-eYFP neurons restricted largely to the MeApd and MeApv (**Fig. 3c, left panel**). Exposure to MUP11[3,4] revealed sparser labeling in both MeApd and MeApv, and even sparser labeling was observed after exposure to saline (**Fig. 3d-e, left panels**). Mice expressing ChR2-eYFP induced by exposure to darcin, MUP11, or saline were introduced into the behavioral chamber after two days of habituation. We then photo-activated the MeA upon entry into one of the two ports with blank filters, to recapitulate exposure to darcin. Mice expressing ChR2-eYFP induced by darcin exposure exhibited a strong preference for the stimulation port (mean poke counts light 202±21 vs. no light 40±7, **Fig. 3c$_2$**). Photo-activation of the ensemble of darcin responsive neurons also elicited ultrasonic vocalizations and scent marking (**Fig. 3f** and **Extended Fig. 2a-c**). Photo-activation of MeA in mice expressing ChR2-eYFP after exposure to saline (light 26±3 vs. no light 24±2, **Fig. 3d$_2$**) or MUP11 (light 19±4 vs. no light 20±5, **Fig. 3e$_2$**) did not elicit any preferences for the stimulation port and did not result in USVs or urinary scent marking upon photo-activation (**Fig. 3f**).

Mice expressing ChR2-eYFP in neurons responsive to darcin exhibited a remembered preference for the port in which they previously received light stimulation (prior photo activation 126±8 vs. no activation 39±6, **Fig. 3c$_3$**). Control animals expressing ChR2-eYFP in neurons after MUP11 (prior photo-activation 20±3 vs. no activation 16±2, **Fig. 3e$_3$**) or saline exposure (prior photo-activation 20±2 vs. no activation 23±3, **Fig. 3d$_3$**) exhibited no preference for the previous light-stimulated port. Ultrasonic vocalizations and scent marking were detected in recall experiments only in mice that previously experienced photo-stimulation of neurons expressing ChR2-eYFP induced by darcin exposure (**Fig. 3g**, **Extended Fig. 2a-c and Supp. Video 2**), but 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 Fig. 3a-b)**. Thus, photo-activation of a population of neurons expressing ChR2 induced by darcin exposure can elicit innate attraction, ultrasonic vocalizations, urinary scent marking and reinforce conditioned behaviors.

Lactating females fail to exhibit attraction to darcin[13]. We therefore asked whether darcin activates medial amygdala neurons in lactating females. Lactating Arc-CreER mice expressing Cre dependent eYFP in the MeA were exposed to darcin 3-5 days postpartum. Exposure to darcin in virgin females resulted in dense labeling of posterior medial amygdala neurons (eYFP cells, mean±sem 255±29 in MeApd, and 115±16 in MeApv). Exposure to darcin during lactation resulted in a sparse labeling (eYFP cells, mean±sem 23±11 in MeApd, and 15±12 in MeApv) at levels similar to that observed upon saline exposure (16±5 in MeApd and 23±7 in MeApv) (**Extended Fig. 4f-h**). In contrast, darcin activates an equivalent number of mitral cells in the AOB of virgin and lactating females (**Extended Fig. 4a-e,** c-fos cells in virgin 378±35, and

358±45 in lactating, n=6, p=0.9). Thus, 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) that defines the population of MeA neurons that mediate the darcin-induced behaviors. Immuno-histochemical examination of the medial amygdala of Arc-CreER mice revealed that a significant fraction of neurons expressing ChR2-eYFP in response to darcin also express neuronal nitric oxide synthase (nNOS). We found that 18% of neurons in posterior MeA express nNOS. Double labelling experiments demonstrate that this nNOS population consists of 55±4 % excitatory neurons (vGlut2 positive cells) and 24±3 % inhibitory neurons (Gad2 positive neurons). We observed that 74±2% of the ChR2-eYFP neurons labelled upon darcin exposure express nNOS, whereas 66±3% of the nNOS neurons express ChR2-eYFP (**Fig. 4a**). Similar values are obtained in Arc-Cre ER mice exposed to male urine with normal levels of darcin. The pheromones ESP1[14], MUP11[3-4], cat salivary lipocalin Fel-D4[15], and female urine activated less than 20% of the nNOS neurons (**Extended Fig. 5 and Table I**). The majority of the MeA neurons activated by these stimuli do not express nNOS demonstrating 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 behavioral repertoire observed upon darcin exposure. We therefore injected AAV encoding Cre-dependent ChR2-eYFP into the posterior medial amygdala of mice in which the *nNOS* promotor drives the expression of Cre (*nNOS-ires-Cre*) to express channelrhodopsin in nNOS neurons. We then photo-activated nNOS+ MeA neurons upon entry into one of the two ports with blank

filters and observed a strong preference for the stimulation port (light 541±45 vs. no light 66±12**,** **Extended Fig. 3d$_2$**). Moreover, photo-stimulation of nNOS cells expressing ChR2-eYFP evoked ultrasonic vocalization and scent marking (**Extended Fig. 3f** and **Extended Fig. 2a-c**). Photo-activation of these MeA neurons also reinforced conditioned behaviors (prior light 295±16 vs. no light 57±11, **Extended Fig. 3d$_3$**). Control experiments in which AAV encoding Cre-dependent eYFP were injected into the MeA of nNOS-ires-Cre mice failed to elicit any of the darcin mediated behaviors upon photo-stimulation (light 24±4 vs. no light 25±5, p=0.8, **Extended Fig. 3c$_2$** and prior light 23±7 vs. no light 25±6, p=0.8, **Extended Fig. 3c$_3$**). Thus, photo-activation of ChR2-eYFP in nNOS neurons in the medial amygdala is sufficient to recapitulate both the innate and reinforcing behaviors observed upon exposure to darcin.

These observations predict that silencing of the nNOS neurons in the MeA should impair the behavioral response to darcin. We therefore expressed halorhodopsin[10] in nNOS neurons in the medial amygdala after bilateral injection of an AAV (AAVDJ-*EF1a-DIO.eNpHR3.0-eYFP*, **Fig. 4b**) encoding the Cre-dependent opsin. In mice in which the nNOS neurons are silenced, no preference was observed for filters containing recombinant darcin in the poke preference assay (darcin 35±5 vs. blank 39±5, **Fig. 4e$_3$**) and darcin elicited no port preference during recall (prior exposure to darcin 35±5 vs. blank 42±6, **Fig. 4e$_4$)**. Ultrasonic vocalizations and urinary scent marking were also eliminated upon light induced silencing (**Fig. 4g**). As a control, we demonstrate that when photo-stimulation was terminated, darcin elicited a strong port preference that was also observed during recall (prior exposure to darcin 375±40 vs. blank 186±28, **Fig. 4f$_4$**). Light-induced silencing in MeA of mice expressing eYFP in nNOS neurons failed to inhibit darcin-mediated behaviors (**Fig. 4d and g)**. Interestingly, silencing of the MeA also inhibited the

port preference elicited by urine containing normal levels of darcin (**Extended Fig. 6b$_3$**). These observations suggest that the components in urine other than darcin that elicit port preference also require the MeA. We noted 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 vs. blank 23±5, **Fig. 4e$_2$**). We performed additional experiments to demonstrate that the inhibition of darcin-evoked behaviors upon silencing of nNOS neurons is not due to diminished motivation (See **Extended Fig. 7**).

We also 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 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 vs. blank 77±17**, Extended Fig. 6e$_4$**). Thus, darcin-responsive neurons expressing nNOS in the MeA are necessary to recapitulate the innate and reinforcement behaviors 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 behavioral 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 with long delays. Darcin activation of the nNOS population of neurons may therefore elicit a state of "sexual drive" which increases the probability of individual component behaviors suited to enhance the likelihood of mate encounters under different environmental circumstances. Darcin exposure

240 results in exploration and assessment of the darcin source, the urine of a dominant male. In the
241 absence of the male, after active search strategies have failed, the female may emit ultrasonic
242 vocalizations synchronized with scent marking in an attempt to communicate her presence and
243 her current estrus status to the male.
244 Second, darcin activation of the nNOS neurons reinforces both contextual and olfactory learning,
245 generic learning processes, that may allow the female to return to the location of the male's scent
246 mark[4] or to track airborne scents of the territorial male[3,5]. The MeA may therefore provide a
247 signal mediated by darcin to midbrain dopamine neurons to reinforce more traditional "non-
248 social" reinforcement learning[7]. The more stereotyped communication behaviors elicited by
249 darcin, vocalization, and scent marking, might also result from reinforcement of a specific set of
250 social behaviors that coordinate a successful mate search. Whereas the nNOS neurons are
251 required for the behavioral and reinforcing effects of darcin, recall of darcin-elicited memory no
252 longer requires this neural population, presumably reflecting the transfer of a learned
253 representation in other brain structures.
254
255 Third, we observe that male urine with very low darcin levels elicits attraction but does not result
256 in reinforcement learning or memory of port preference. This attractive response does not require
257 the AOB but is eliminated upon silencing the nNOS neurons of the MeA. These observations
258 suggest that the MeA is integrating pheromonal information from the vomeronasal pathway with
259 olfactory cues from the main olfactory system to elicit both innate attraction and learning.
260
261 Finally, the response to darcin is dependent on internal state. Lactating females fail to exhibit the
262 complex behavioral response to darcin exposure[13]. We observe that darcin activates the

projection neurons in AOB in lactating females, but fails to activate the nNOS neurons in MeA (**Extended Fig. 4**). Taken together, these observations suggest that the nNOS neurons of MeA integrate an innate but variable behavioral repertoire with reinforcement learning to enhance mate selection and mate encounters.

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Figure Legends

Figure 1 | Darcin elicits an array of behaviors. a, Timeline of two-port preference assay. **b-d**, Cumulative poke counts towards (b) darcin (1 µl/µg) (c) BALB/c male urine with very low darcin (<0.1 µl/µg)[4] and (d) BALB/c male urine with added recombinant darcin (1 µl/µg) (red)

vs control filters (blue) during cue exposure sessions. Counts are shown from days indicated by arrows on the timeline. Mean (bold, n=30) and individual (fine) counts are shown. The time-stamps for USVs and scent marking are indicated as arrowheads ($b_{3,4}$). Bias in counts are assessed with two-sided Wilcoxon Signed Rank test (in $\mathbf{b_{3,4}}$ ** p = 0.004, n=10, $\mathbf{c_3}$ *p = 0.006, n=10 and $\mathbf{d_{3,4}}$ ** p = 0.004, n=10). **e,** Spectrogram of an example song detected during darcin sessions. **f-g,** Mean (horizontal line, n=10 (f), n=43 (g)) and total calls by individual mice (diamonds). Calls are compared with two-sided Wilcoxon Signed Rank test (in f, * adjusted-p = 0.03, in g, ** adjusted-p = 0.00003 and p=0.0001). **h,** Latency to urinary marking and vocalization in response to darcin (n=24) and during recall (n=14) sessions. Mean (square) and individual (circle) latencies are shown. The bounds in boxplots are defined by the 25th and 75th percentile of the distribution. The line represents the median and the upper and lower whiskers represent 75^{th} percentile + 1.5*IQR (interquartile range) and 25^{th} percentile - 1.5*IQR, respectively. Latencies are compared with two-sided Wilcoxon Signed Rank test (*p = 0.03).

Figure 2 | Optogenetic silencing of the AOB results in suppression of darcin-evoked behaviors. a, eYFP expression in AOB (Scale bar: 200 μm, V=Ventral, P=Posterior, n=18 animals). **b,** Timeline **c-f,** Cumulative poke counts with (c,e) and without optical silencing (d,f). Mice in (c,d) were exposed to darcin (3) (1 μl/μg) (n=10) and mice in (e,f) exposed to C57BL6 male urine (3) (with normal levels of darcin (1 μg/μl)[4]) (n=8) in one port (red) and a blank filter (blue) in the second port. Mean (bold) and individual (fine) counts are shown. The time-stamps for USVs and scent marking are indicated as arrowheads (d_3, d_4). Counts are compared with two-sided Wilcoxon Signed Rank test (in d_3** $p < 0.001$, e_3, $f_{3\text{-}4}$ * $p < 0.008$). **g,** Mean (horizontal

line, n=10) and total calls by individual mice (diamonds) during the darcin exposure with (c,e) and without AOB silencing (d,f). Calls are compared with two-sided Wilcoxon Signed Rank test.

Figure 3 | Activation of darcin-responsive neurons in the MeA recapitulates pheromone induced behaviors. a, Genetic strategy used to express ChR2 in pheromone responsive neurons. **b,** Timeline for experimental manipulations. **(c-e, left panels),** Representative images (scale bar 400 μm, V=Ventral, L=Lateral) showing eYFP expression in MeAp following exposure to (c) darcin (mean±sem: eYFP counts, 255 ± 29 in MeApd and 115±16 in MeApv) (d) saline (16 ± 5 in MeApd and 23 ± 7 in MeApv), and (e) MUP11 (54 ± 10 in MeApd and 42 ± 9 in MeApv); (**c-e, right panels**) show corresponding cumulative poke counts. Mean (bold, n=13 for each group, n=39 total) and individual (fine) counts are shown. The time-stamps for USVs and scent marking are indicated as arrowheads ($c_{2,3}$ and e_2). Counts are compared with two-sided Wilcoxon Signed Rank test (in $c_{2\text{-}3}$, *** p = 0.0002). **f-g,** Mean (horizontal line, n=13 per group) and total calls by individual mice (diamonds) during the light stimulation (f) and subsequent recall (g) sessions. Calls are compared with two-sided Mann-Whitney test adjusted for multiple comparisons (in f * p <0.05, g * p= 0.02).

Figure 4 | nNOS neurons in the MeA are necessary for darcin mediated behaviors. a, Representative image showing co-expression of eYFP expressed in darcin responsive neurons (See Fig 3c, left panel) and nNOS in the MeAp (D=Dorsal, M=Medial, n=7 animals). **b,** eYFP expression in coronal sections of MeAp of a *nNOS-ires-Cre* mouse (scale bar:400μm, D=dorsal, M=medial, n=66 animals). **c,** Timeline. **d-f,** Cumulative poke counts in mice expressing eNpHR (e, f) or eYFP (d) in nNOS neurons. (d) photostimulation of neurons expressing eYFP (n=12) or

(e) eNpHR (n=11), and (f) no photostimulation of neurons expressing eNpHR (n=11). Mean (bold) and individual (fine) counts are shown. The time-stamps for USVs and scent marking are indicated as arrowheads ($d_{3\text{-}4}$, $e_{3\text{-}4}$). Counts are compared with two-sided Wilcoxon Signed Rank test (in $d_{3\text{-}4}$ *** $p < 0.0005$, $f_{3\text{-}4}$ ** $p < 0.005$). **g,** Vocalization counts of mice expressing eYFP (n=12) and eNpHR (n=11) groups. Mean (horizontal line) and total calls by individual animals (diamonds). Calls are compared with two-sided Wilcoxon Signed Rank test, adjusted for multiple comparisons (* $p < 0.05$).

Methods

Animals

All surgical and experimental procedures were done pursuant to the National Institute of Health standards's *Guide for the Care and Use of Laboratory Animals*[1] 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 handled for at least 10 minutes each day for minimum of 5 days before experimentation. Surgeries were performed on mice that were 6 weeks old to match their brain coordinates to the Allen Reference Atlas. 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

377 background to the ICR mice. Throughout the study, five mice were co-housed in a single cage
378 for 2 to 6 months. This long-term co-housing has the potential to suppress estrus cycling in
379 females (Lee Boot effect)[2]. To ensure that all females had previously encountered male scent and
380 were showing normal estrus cycling, females were exposed to male-soiled bedding from an
381 unfamiliar strain for at least 60 hours[3]. They were then visually evaluated for their stage of estrus
382 before the experimental testing. One hour before testing, each mouse had its vaginal opening
383 photographed for evaluation. Following estrus entrainment, most females (>90%) were evaluated
384 to be in proestrus stage[3] (with swollen, moist, pink and wide-open vaginal openings)[4] of the
385 cycle and advanced into behavioral testing. Mice were kept in a controlled 12-hour day/night (7
386 am to 7 pm) cycle and tested only during the night phase (11 pm to 6 am).
387
388 **Behavioral assays**
389 Before behavioral training, mice were handled for 10 minutes each day for five days, and were
390 given access to a mouse exercise cage that was enriched with spinning discs and toys for one
391 hour every day during the experimental period. Animal training took place in a custom-designed
392 sound isolation chamber containing a behavioral arena (25 × 25 × 28 cm) integrated with two
393 stimulus ports (circular nose port (4.6 cm diameter) with an attachable circular cup for the filter
394 (1.3 cm diameter)) which were surrounded by distinct visual stimuli (stripe and circle stickers
395 were used on either side (Context Kit for Conditioned Place Preference, Stoelting Inc, US)) on
396 the walls. Mice were tested under room light during the night phase of their day/night cycle (11
397 pm to 6 am). Mice poked their snouts into stimulus ports to sample the social stimuli. The social
398 cue was presented on a glass microfiber filter in a portable cup attached to the nose port. Social
399 cue ports were constructed out of metal and boiled in detergent (1-2% Alconox for at least 15

minutes), rinsed thoroughly with water, dipped in 3% hydrogen peroxide and ethanol and rinsed again with running distilled water and air-dried to clean off any contaminants between experiments. The frequency and duration of the animals' nose pokes were quantified by means of an infrared beam within the port. The behavioral nose poke data were acquired through a MATLAB interface and a Bpod[7].

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 behaviors with the embedded time code, a Marshall Genlock 3G-SDI HDMI Camera was mounted at the base of the transparent chamber. An AJA Ki 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 analyzed 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 animal. Comparison of the calls emitted in response to the pheromone and the calls that are emitted by the photo-activation of MeA neurons confirmed that the pheromone and photo-activation evoked syllables shared similar sonic qualities as presented in **Extended Fig. 8 and Table 2.** All spectrograms were additionally parametrized using SAP

2011[5] and MUPET[6] software, and all syllables emitted by the animals during the sessions were manually extracted and classified for analysis (**Extended Fig. 8**). To analyze the urinary scent marking behavior of the animals, 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 utilizing 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 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. Thus, engagement of the port was 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, photo-activation and free urination sessions. Distances were extrapolated from individual frames of the video using Adobe Photoshop.

Mice were placed in the behavior chamber for 100 minutes once per day for each session during the dark phase (11 pm to 6 am) of their day/night cycle (7 am to 7 pm). The behavioral chamber and the stimulus ports were thoroughly cleaned with 1-2% Alconox detergent, distilled water, 3% hydrogen peroxide, 80% ethanol, and rinsed again with distilled water and air-dried in between individual sessions. The first 10 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 animals as they were acclimated to movement in the chamber and, for subjects involved in optogenetic experiments, movement while tethered to the patch cord. For behavioral

testing, all animals, except for the optically activated mice, were subjected to male mice soiled bedding exposure for 60 hours in their home cage[3] and an extra habituation session with blank filters in both stimulus ports following this home cage treatment. Subsequently all animals 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 animals 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[7] device.

Those ICR background mice that were not tested optogenetically were subjected to the social cues: recombinant darcin (1 µg/µl), male urine with low level of darcin (<0.1µg/µl in Balbc/J Ola-Hd urine purchased from Harlan/Envigo in the Netherlands)[8], male urine with normal adult level of darcin (1 µg/µl, C57BL/6J Ola-Hsd urine, purchased from Harlan/Envigo in the Netherlands)[8], or recombinant darcin added to Balbc/J Ola-Hd male urine with low levels of darcin (BALBc/J plus recombinant darcin, 1 µg/µl) in one port. In all instances, there was no odor in the other port. To confirm the presence or absence of darcin (18893 Da MUP20), 12% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide) gel electrophoresis of all urine samples were performed[3,8].

The C57/Bl6 Arc-CreER and nNOS-i-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 (11µg in 10µl) or male urine with normal levels of darcin (10 µl of C57/Bl6 Ola-Hd urine)[8] in one port and no odor in the other port. The nNOS-i-cre mice tested with MeA inactivation were subjected to darcin or

male urine with normal levels of darcin (C57/Bl6 Ola-Hd)[8] in one port and no odor 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, C57/Bl6 Arc-CreER and nNOS-i-Cre/ICR mice were introduced into a two-chamber conditioned place preference arena (22 × 16 × 28 cm, length ×width × height) for 100 minutes once per day for each session. Two chambers had distinct walls decorated with visual cues (stripes and circles stickers, Context Kit for Conditioned Place Preference, Stoelting Inc, US)), 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[7] device only during the light stimulation sessions. 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 only social cue reinforcement behaviors, we optogenetically silenced nNOS neurons in 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 behavioral training, animals 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 behavior chamber for 100 minutes once per day for each session during the dark phase (11 pm to 6 am) of their day/night cycle (7 am to 7 pm). The first 10 sessions served as habituation sessions, during which no cue was present in either port. Mice were acclimated to the movement in the chamber while being tethered to the patch cord. They were then subjected to male soiled bedding exposure for 60 hours in their home cage and an extra habituation session with blank filters in both ports following this home cage treatment. Subsequently all animals were tested for cue sessions. The cue sides were evenly split in a random manner between two ports across the animals 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. Behavioral training sessions lasted 100 minutes, during which the mice typically harvested at least 4 mL of water. The final session for water reinforcement behavior was a recall session designed to quantify the retained poke preferences without any water reward. The behavioral hardware was controlled by custom MATLAB programs and a Bpod and PulsePal[7].

Stereotactic surgeries

An adeno-associated virus (AAV) DJ serotype[9] (1.3×10^{13} vg/ml [genomic], 8×10^{8} IU/ml [infectious] titer, 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 ketamine-xylazine mixture (0.13 mg/g body weight ketamine and 0.01 mg/g xylazine). Small craniotomies were made above the posterior MeA (-2.0 mm AP and 2.3 mm ML from the bregma) or AOB (3.2 mm AP, 1 mm ML, and 0.8–1.5 mm DV). Virus was injected

515 with a glass micropipette using a Picospritzer (General Valve). For posterior MeA injections,
516 20–60 pulses of 10 ms duration were delivered at 0.2 Hz starting from a depth of 4.6 mm from
517 the brain surface up to 5.2 mm in 200 μm steps, waiting a minimum of 10 minutes per site to
518 allow diffusion of the virus. Following virus injection, fiber optic cannulas were implanted. The
519 animals received a supplemental dose of ketamine at 30- to 90-minute intervals to maintain the
520 depth of anesthesia. The cannula was positioned with the help of a stereotaxic arm (David Kopf
521 Instruments) and cannula holder (Doric Lenses) above the craniotomy. The optical cannula was
522 gradually lowered close to the viral injection depth (100 to 300 μm above the injection site). Two
523 miniature watch screws (Micro-Mark) were fixed into the parietal plates as anchors. The cannula
524 was secured to the skull with light-curable dental cement (Vitrebond Plus) followed by a layer of
525 black dental acrylic (Lang Dental Manufacturing Co.). For post-operative analgesia, ketoprofen
526 (5 mg/kg body weight) was administered subcutaneously. The animals were allowed to recover
527 for one week.
528
529 **Social cue exposure of Arc-CreER mice**
530 One week after stereotaxic viral infection and cannula surgery, 6- to 8-week-old Arc-CreER
531 mice were transferred to a reverse day/night cycle. They were individually housed unless
532 mentioned otherwise and estrus was synchronized through exposure to male-soiled bedding for
533 60 hours[3,8]. Mice were then injected with 2 mg of tamoxifen (Sigma T5648), which was
534 prepared as a 10 mg/ml stock solution dissolved in a mixture of ethanol and sunflower seed
535 oil (Sigma S5007). Five hours after tamoxifen injection, the mice were exposed to darcin,
536 MUP11, saline, cat salivary lipocalin (Fel-D4), ESP1 (exocrine-gland secreting peptide), male
537 urine with normal levels of darcin, female urine, and male urine with low levels of darcin on a

glass microfiber filter (10 mm diameter) placed through the roof of their home cage; 10 µL (equivalent to 11 µg of darcin, MUP11, equivalent to 3.3 µg of Fel-D4 and 25 µg of ESP1) was used[8]. The lactating females were separated from their pups 5 hours before tamoxifen injection and exposed to recombinant darcin between postpartum days 3 and 5. Recombinant cat Fel-D4 was produced using 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 cue exposure.

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

Immunohistochemistry

Once the behavioral criteria for each behavior assay were met, the mice were anaesthetized with a ketamine and xylazine mixture (0.30 mg/g body weight ketamine, 0.03 mg/g 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 X 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 minutes each), and incubated overnight at 4°C with primary antibodies diluted in blocking solution. The following primary antibodies were used: anti-GFP (rabbit polyclonal, 1:1000, Rockland), anti-GFP (chicken polyclonal 1:400, Aves Labs), anti-nNOS (rabbit polyclonal, 1:400, Invitrogen),

anti-mCherry (rat monoclonal, 1:800, Thermo Scientific) and anti-c-fos (goat and rabbit polyclonal, 1:500, Santa Cruz Biotechnology, guinea pig polyclonal, 1:5000, with RRID #: AB_2814707, generated by Susan Brenner-Morton, at ZMBBI, Columbia University). The following day, the sections were washed in 0.1% PBST (3 washes, 15 minutes each) and incubated for 2 hours 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 1X PBS for 15 minutes 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 conducted using ImageJ (NIH) software.

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 test. Port bias for left port over right port was computed by taking the difference in total poke count between the left and right port for each animal. Comparisons were across each session (habituation day 2, habituation day 13, cue exposure, and recall) by 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 so we used non-parametric tests. Call counts were compared across independent animal cohorts by Mann-Whitney test and across different sessions of the same animal cohort by Wilcoxon Sign Rank test. Adjusted p-values were reported where multiple comparisons were

584 made on the same sample set by using the Holm's sequential Bonferroni correction method. The
585 probabilities of urinary scent marking behavior were compared across sessions by McNemar test.
586 Exact tests were performed for all comparisons, including where the sample sizes were small
587 (the discordant pairs in some of our comparisons were less than 25). The mean latencies to first
588 urinary scent marking were compared by paired t-test. The latency data approximated to
589 normality as confirmed by Shapiro-Wilk, Lilliefors, Kolmogorov-Smirnov, Anderson Darling,
590 D'Agostino-K squared, and Chen Shapiro tests. All analyses were done using R, OriginLab, and
591 MATLAB.
592
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616

617 **Data Availability:**

618 The datasets generated and/or analyzed during the current study are available from the
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620

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Author contributions

E.D., R.J.B., J.L.H., A.K., and R.A. discussed the design of experiments, results and wrote the manuscript. J.I.S. designed the custom behavior and stimulation systems. E.D. performed all 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 financial interests.

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Supplementary Video 1 Legend | Darcin reinforces recall of ultrasonic vocalization and scent marking behaviors. A female mouse, previously exposed to darcin, emits nearly synchronous ultrasonic vocalization and scent marking by the prior darcin exposure port.

Supplementary Video 2 Legend | Activation of darcin-responsive neurons in the medial amygdala reinforces recall of ultrasonic vocalization and scent marking behaviors. A female mouse, previously experienced photo-stimulation of neurons expressing ChR2-eYFP

induced by darcin exposure, emits nearly synchronous ultrasonic vocalization and scent marking by the prior photo-stimulation port.

Extended Data Figure and Table Legends

Extended Figure 1 | Darcin and photo-activation of posterior MeA neurons condition scent marking place-preference. a, (1-3) Representative frames from videos of the pheromone (1) and photo-activation sessions (2), and free-range behaviors (3). **b,** Distance from urinary drop to each of the poke ports during various sessions. Individual frames are analyzed using Adobe Photoshop CC to quantify the distance from the center of a urinary drop to the base of each poke port. Units are scaled from pixels to centimeters. Distances are compared with two-sided Wilcoxon Signed Rank test (*** $p<0.0005$, and * $p=0.01$, (1) n=24, (2) n=12, (3) n=20). **c,** Area of urinary drops for various conditions. Individual frames are analyzed using Adobe Photoshop CC to quantify the area of the urinary marks. Units are scaled from pixels-squared to centimeters-squared. Mean scent mark area for darcin ± sem= 5 ± 0.05, n= 24, recall of darcin = 5 ± 0.09, n=14, photo-activation = 4 ± 0.4, n= 12, recall of photo-activation= 4 ± 0.5, n=8 and free-urination = 13 ± 2 cm2, n=20). Areas are compared with two-sided Mann-Whitney test (*** $p<0.0005$), adjusted for multiple comparisons. The bounds in boxplots (b-c) 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.

675

676 **Extended Figure 2 | Darcin and photo-activation of posterior MeA neurons reinforce recall**
677 **of vocalization and scent marking behaviors. a-c,** Individual animal data for all unique
678 sessions across the study were pooled. **a,** Mean (horizontal line, n=43 (darcin group), n=24
679 (photo-stimulation group)) and total calls by individual mice (diamonds) detected during various
680 sessions. Call counts are compared with two-sided Wilcoxon Signed Rank test within the
681 respective groups (*** $p < 0.0005$), adjusted for multiple comparisons. **b,** Latency from session
682 start to urinary marking and vocalization behavior during exposure to darcin (with mean±sem of
683 3160±311 seconds, n=24), recall of darcin exposure (mean±sem, latency, 956±217, n=14),
684 photo-stimulation (mean±sem latency, 4195±372, n=12), and subsequent recall (mean±sem
685 latency, 1315±418, n=8) sessions. Latencies are compared within groups with matched-pair two-
686 sided t-test (*** p= 0.00009 and * p=0.005). The bounds in boxplots are defined by the 25th and
687 75th percentile of the distribution. The line represents the median and the upper and lower
688 whiskers represent 75th percentile + 1.5*IQR and 25th percentile - 1.5*IQR, respectively. **c,**
689 Probability of urinary scent marking and vocalization behaviors. Mean probabilities for darcin
690 session (0.6, n=43), and recall of darcin session (0.3, n=43), photostimulation-evoked urinary
691 marking and vocalization (0.5, n=24), and recall of photostimulation-evoked behaviors (0.3,
692 n=24). Probabilities are compared with two-sided McNemar test (* $p < 0.05$). **d,** Probability and
693 mean latency to first urinary scent marking in the session (n=9). Data from 100-minute sessions
694 of habituation before and after exposure to male soiled-bedding in the home-cage (mean±sem
695 latency for urination, 1411±126 seconds), low darcin BALB/C urine (1116±232), recall of
696 BALB/C urine (1607±268), normal level darcin C57BL6/6J urine (2666±337), and recall of
697 C57BL6/6J urine (1032±198) are shown. Probabilities are compared with two-sided McNemar

698 test (*p=0.02), adjusted for multiple comparisons. Latencies are compared within groups with
699 matched-pair two-sided t-test and across groups with unpaired two-sided t-test (** p= 0.0008, *
700 p= 0.02), adjusted for multiple comparisons. Scent marking behaviors in response to low darcin
701 urine during the subsequent recall sessions are compared (habituation to recall, p=1, cue to recall
702 session comparison, p=0.1, two-sided McNemar test).

703 **Extended Figure 3 | Activation of darcin responsive neurons in the posterior MeA**
704 **recapitulates darcin-induced behaviors. a,** Heat map showing percent occupancy time during a
705 habituation, photostimulation, and recall session. **b,** Occupancy plot showing percent time spent
706 in the photostimulation room. Arc-CreER animals were exposed to darcin (magenta), saline
707 (green) or MUP11 (blue). Mean±sem (n=5 per group, total n=15) percent time spent in
708 stimulation room during habituation, photostimulation, and recall sessions are shown. For
709 occupancy time, pairwise comparisons were done with two-sided Mann-Whitney (* $p < 0.05$)
710 and three-way comparisons were done with Kruskal Wallis Tests (habituation p=0.6, light
711 stimulation p= 0.009 and recall sessions p=0.008). **c-f,** Activation of nNOS neurons in the
712 posterior MeA recapitulates darcin-induced behaviors. **c-d,** Cumulative poke counts during
713 habituation (laser off) (1), light stimulation (laser on) (2), and recall (laser off) (3) sessions in
714 mice expressing eYFP (c) or ChR2 (d) in nNOS neurons. Light stimulation was performed in one
715 port (red) and not in the second port (blue). During habituation (1) and recall (3) sessions, no
716 light stimulation was given and red and blue reflect right and left ports, respectively. Mean (bold,
717 n=11 for each group) and individual (fine) cumulative poke counts are shown. The time-stamps
718 for USV and scent marking behaviors are indicated as arrowheads (d2,3). Poke counts are
719 compared with two-sided Wilcoxon Signed Rank test (*** p= 0.0001). (c) Control group (eYFP)
720 port entries are contrasted to (d) the ChR2 group during light stimulation (red entries for (d2)

ChR2 compared to (c2) eYFP, p=0.0002) and recall sessions (red entries for (d3) ChR2 compared to (c3) eYFP, p=0.0002, two-sided Mann-Whitney test, adjusted for multiple comparisons. **e,** Occupancy plot showing percent time spent in photostimulation room. nNOS-ires-Cre animals were injected with the virus encoding either eYFP (green) or ChR2-eYFP (purple). Mean percent time spent in stimulation room (n=6 per group, n=12 total) by all animals during various sessions. Plots are color-coded to their respective groups. Occupancy percent times are compared with two-sided Mann-Whitney test (* $p < 0.05$). **f,** Mean (horizontal line, n=11 per group, n=22 total) and total calls by individual mice (diamonds) detected during the photostimulation (2) sessions in mice expressing eYFP (c2) or ChR2 (d2) in nNOS neurons. Call counts are compared with two-sided Mann-Whitney test (* p= 0.007).

Extended Figure 4 | In lactating females, darcin activates mitral cells in AOB, but it fails to activate MeA neurons. a-c, Representative images showing c-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) (scale bar 200 μm, D = Dorsal, P = Posterior, n=6 per group). **d,** Bar plots are shown quantifying c-fos expressing cells in AOB, n=6 per group: (a) Saline, mean±sem: c-fos counts, 153±38, (b) darcin in virgin, 378±35, and (c) in lactating females, 358±45. Cell-counts are compared with two-sided Mann-Whitney test (* p= 0.02), adjusted for multiple comparisons. **e,** Bar plots quantifying the mitral/tufted cells in AOB. (a) Saline, mean±sem: 1188±167, (b) darcin, in virgin 1129±93, and (c) in lactating females, 1210±163, n=6 per group. Cell-counts are compared with two-sided Mann-Whitney test, **f-g,** Representative images showing eYFP expression in coronal sections of the MeAp of Arc-CreER

744 animals following exposure to darcin in virgin (f) and lactating females (g) (scale bar 200 μm, V
745 = Ventral, L = Lateral, n=13 in (f) and n=4 in (g). h, Bar plots quantifying eYFP cell-counts in
746 MeApd and MeApv. Cell counts are compared with a two-sided Mann-Whitney test, adjusted for
747 multiple comparisons (* p= 0.008, ** p= 0.0006, *** p < 0.0005, n=13 saline exposures
748 (mean±sem eYFP counts, 16±5 in MeApd and 23±7 in MeApv), n=13 darcin exposures in virgin
749 (251±29 in MeApd and 115±16 in MeApv), n=4 in lactating females (23±11 in MeApd and
750 15±12 in MeApv).

751 **Extended Figure 5 | Identification of neurons in the posterior MeA that respond to**
752 **vomeronasal stimuli and their overlap with genetic marker nNOS. a,** Representative images
753 showing the stimulus-responsive (eYFP, orange) and nNOS-expressing neurons (cyan) in the
754 posterior MeA of Arc-CreER mice exposed to cat salivary lipocalin Fel-D4 (n=5), saline (n=8),
755 ESP1 (n=5), MUP11 (n=5), female urine (n=5), male urine with low levels of darcin (n=4), male
756 urine with normal levels of darcin (n=9), and darcin (n=7). **b,** Corresponding box-plots
757 quantifying the percentage overlaps between the stimulus-responsive (eYFP) and nNOS+
758 neurons in the posterior MeA of mice exposed to the various stimuli. Orange plots represent the
759 percentage of YFP cells that overlap with nNOS. Cyan plots represent the percentage of nNOS
760 cells that overlap with YFP. The bounds in boxplots are defined by the 25th and 75th percentile
761 of the distribution. The line represents the median and the upper and lower whiskers represent
762 75th percentile + 1.5*IQR and 25th percentile - 1.5*IQR, respectively. Number of mice used for
763 cat salivary lipocalin Fel-D4 (n=5), saline (n=8), ESP1 (n=5), MUP11 (n=5), female urine (n=5),
764 male urine with low levels of darcin (n=4), male urine with normal levels of darcin (n=9), and
765 darcin (n=7).
766

Extended Figure 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 hours in their home cage (2), followed by one additional day of habituation before male urine (with normal levels of darcin (1 μg/μl)) was presented in one of the two ports (3). Urine was removed for 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), male urine (3), and recall (4) sessions for animals expressing eNpHR-eYFP (n=10) with (b) and without optical silencing (c). Poke counts are shown from days indicated by arrows. 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) and individual (fine) cumulative poke counts are shown. Poke counts are compared with two-sided Wilcoxon Signed Rank (*** p= 0.0002). The effect of silencing nNOS neurons is quantified with matched pair differences (male urine session comparisons, b3 to c3, p=0.002) and recall of male urine with darcin (recall session comparisons, b4 to c4, p=0.002) with two-sided Wilcoxon Signed Rank test, adjusted for multiple comparisons. **d-e,** Optical silencing of nNOS 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 (d1-4) and with optical silencing only during recall sessions (e4). Poke counts are shown from days indicated by 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) and individual (fine) cumulative poke counts are shown.

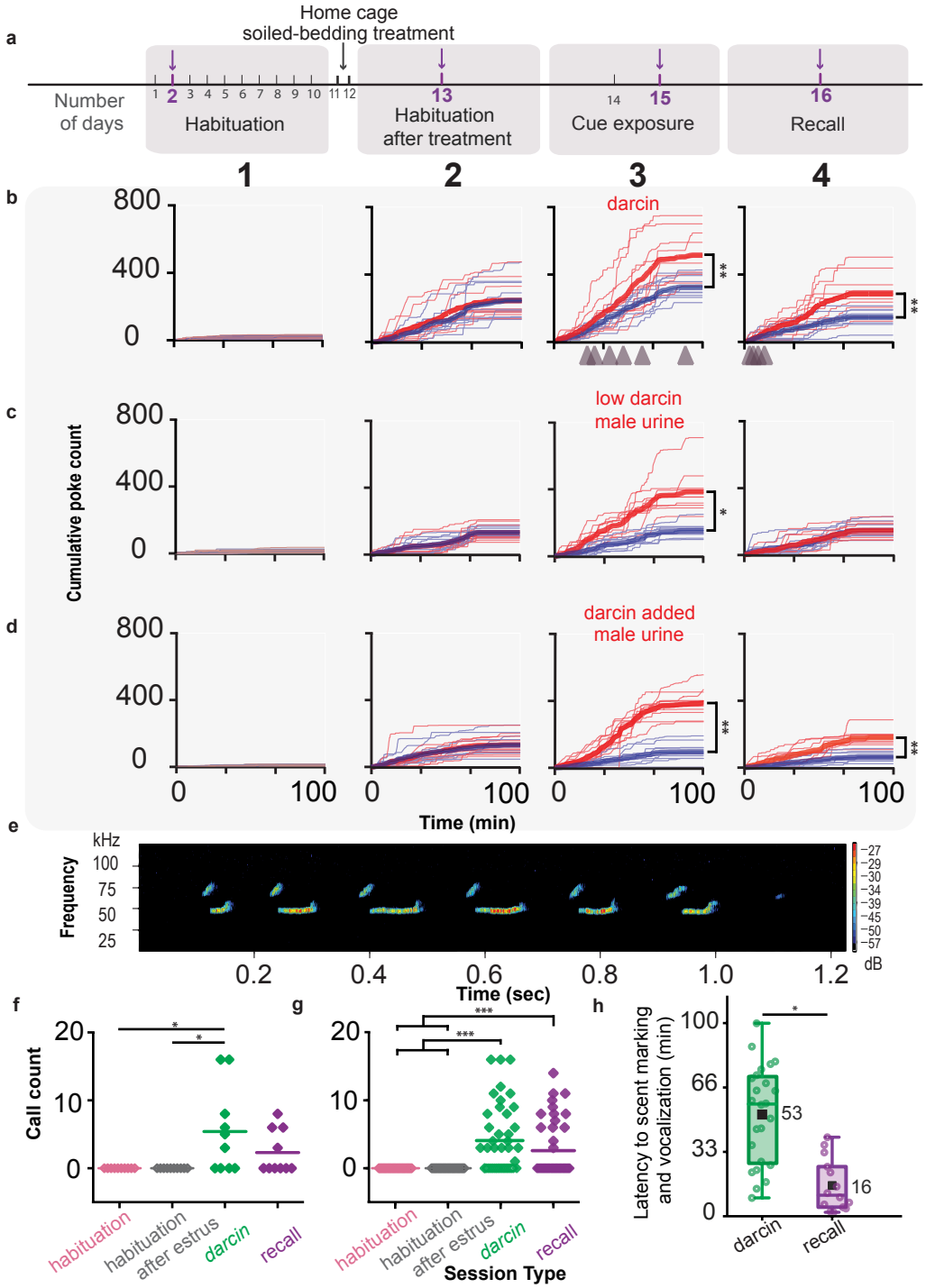
790 Poke counts are compared with two-sided Wilcoxon Signed Rank test (** p= 0.001). The effect
791 of silencing nNOS neurons during recall sessions is tested with matched pair differences (c, cue
792 (e3) to recall (e4) comparisons, laser off (e3) and on (e4), p=0.1, with two-sided Wilcoxon
793 Signed Rank test, adjusted for multiple comparisons.
794
795 **Extended Figure 7 | Animals subjected to optical silencing of nNOS neurons retained a**
796 **motivation to poke.** In order to establish the primacy of the MeA in mediating darcin evoked
797 behaviors rather than altering general motivation, animals expressing eNpHR in nNOS neurons
798 were additionally tested. **a,** Timeline of the preference assay. **b-d,** Cumulative poke counts
799 during habituation (1), habituation following exposure to male soiled-bedding in the home cage
800 (2), darcin exposure (3), and recall (4) sessions with (b), without optical silencing (c), and with
801 optical silencing again after 4 weeks (d) (n=11). Poke counts are shown from days indicated by
802 arrows. Mice were exposed to darcin in one port (red) and a blank filter (blue) in the second port.
803 During habituation (1,2) and recall (3) sessions both ports contained a blank filter. Mean (bold)
804 and individual (fine) cumulative poke counts are shown. Poke counts are compared with two-
805 sided Wilcoxon Signed Rank test (** p= 0.001). The effect of silencing nNOS neurons after a
806 learning experience is quantified during habituation sessions following soiled bedding exposure
807 in the home cage (port entries to the same port (red) with blank filters are compared during
808 habituation after home-cage treatment sessions in b2 and c2, laser on and off, p=0.002, in b2 and
809 d2, laser on, p=0.001, and c2 and d2, laser off and on, p=0.5). The paired count differences (red-
810 blue port) are compared across darcin sessions (b3 to d3, laser on, p=0.5, and c3 to d3, laser off
811 and on, p=0.0001) and recall of darcin (recall session comparison b4 to d4, p=0.9, and c4 to d4,
812 p=0.0001) with two-sided Wilcoxon Signed Rank test, adjusted for multiple comparisons. **e,**

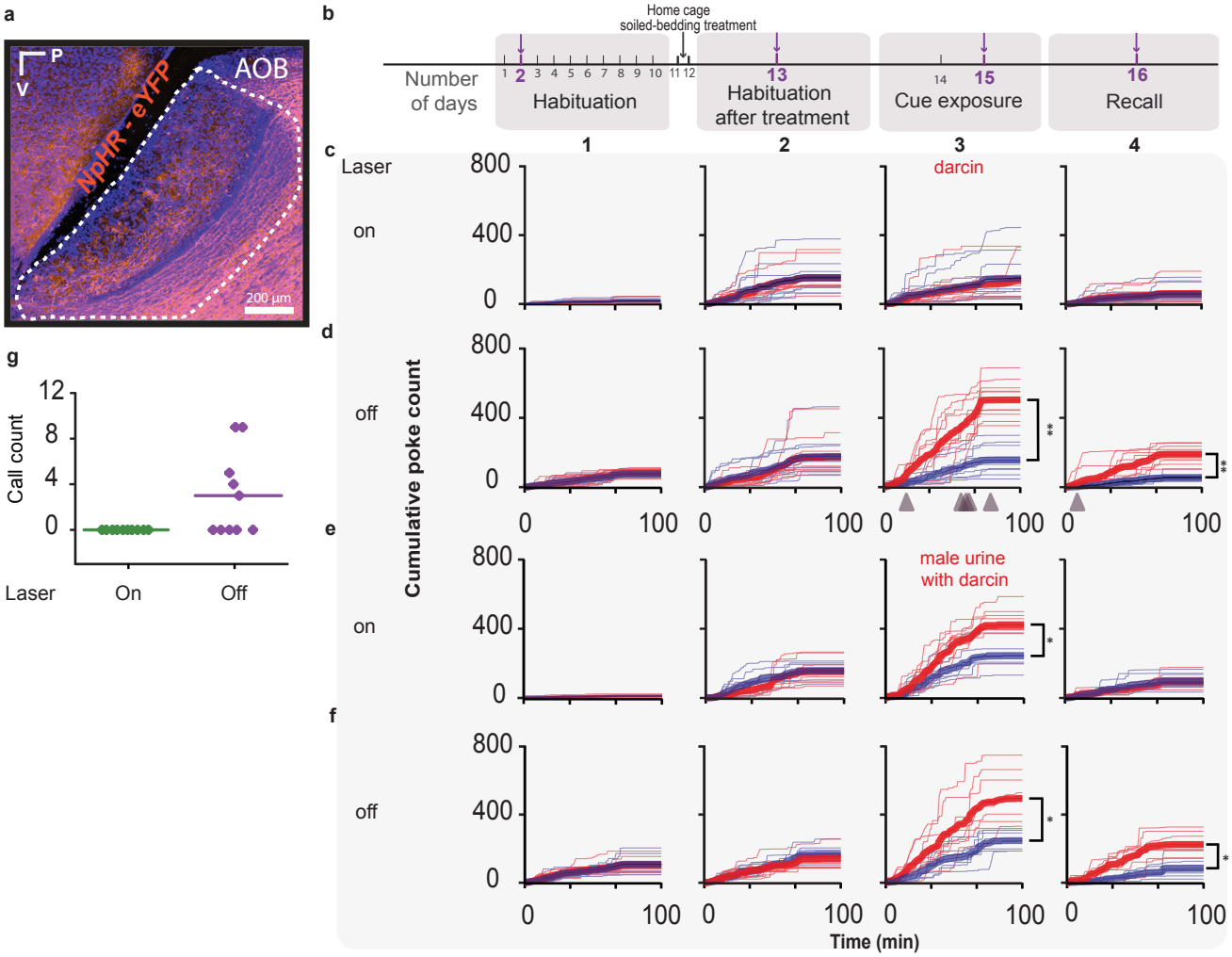
Optical silencing of nNOS in the MeA does not affect non-social reinforcement behavior. Cumulative poke counts during habituation (1), habituation after treatment (2), and water (3), sessions in mice expressing eNpHR (n=12) in nNOS neurons in MeA with silencing. Poke counts are shown from days indicated by arrows in (a). Water-restricted mice were rewarded with a drop of water (5µl) 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) and individual (fine) cumulative poke counts are shown. Poke counts are compared with two-sided Wilcoxon Signed Rank (** p= 0.0005).

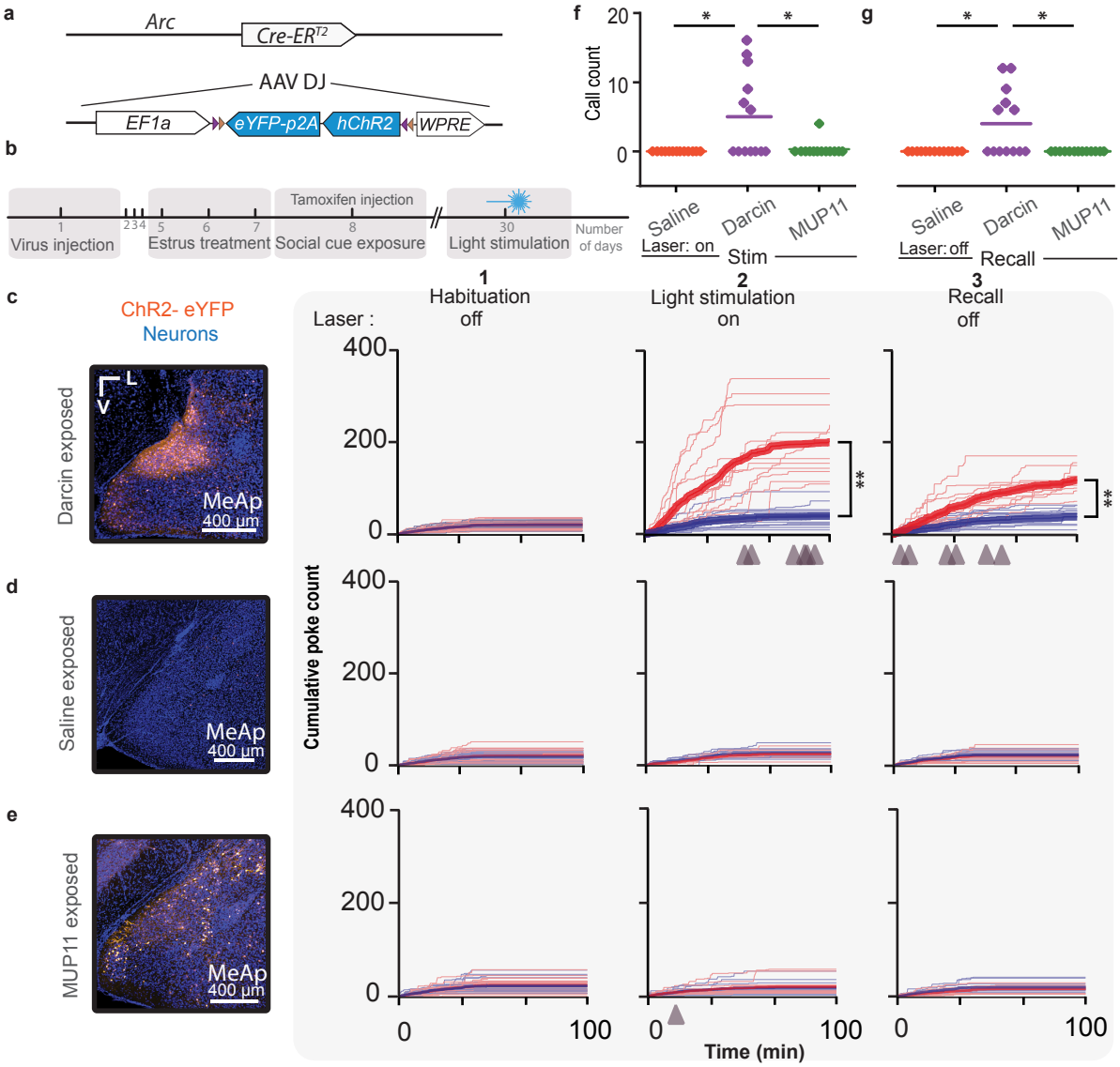
Extended Figure 8 | Ultrasonic vocalizations that are emitted by animals exposed to darcin or stimulated optogenetically consist of seven unique syllable categories. a, Representative spectrograms of ultrasonic vocalizations classified into seven categories of calls. Heat map showing the vocalization intensities. Descriptive statistics (mean±standard deviation) for frequencies are given at locations indicated with the corresponding letters on the spectrograms (See Extended Table 2). **b,** Percentages of different call categories emitted by animals exposed to darcin (n=24, in green) and optogenetically stimulated (n=12, in blue).

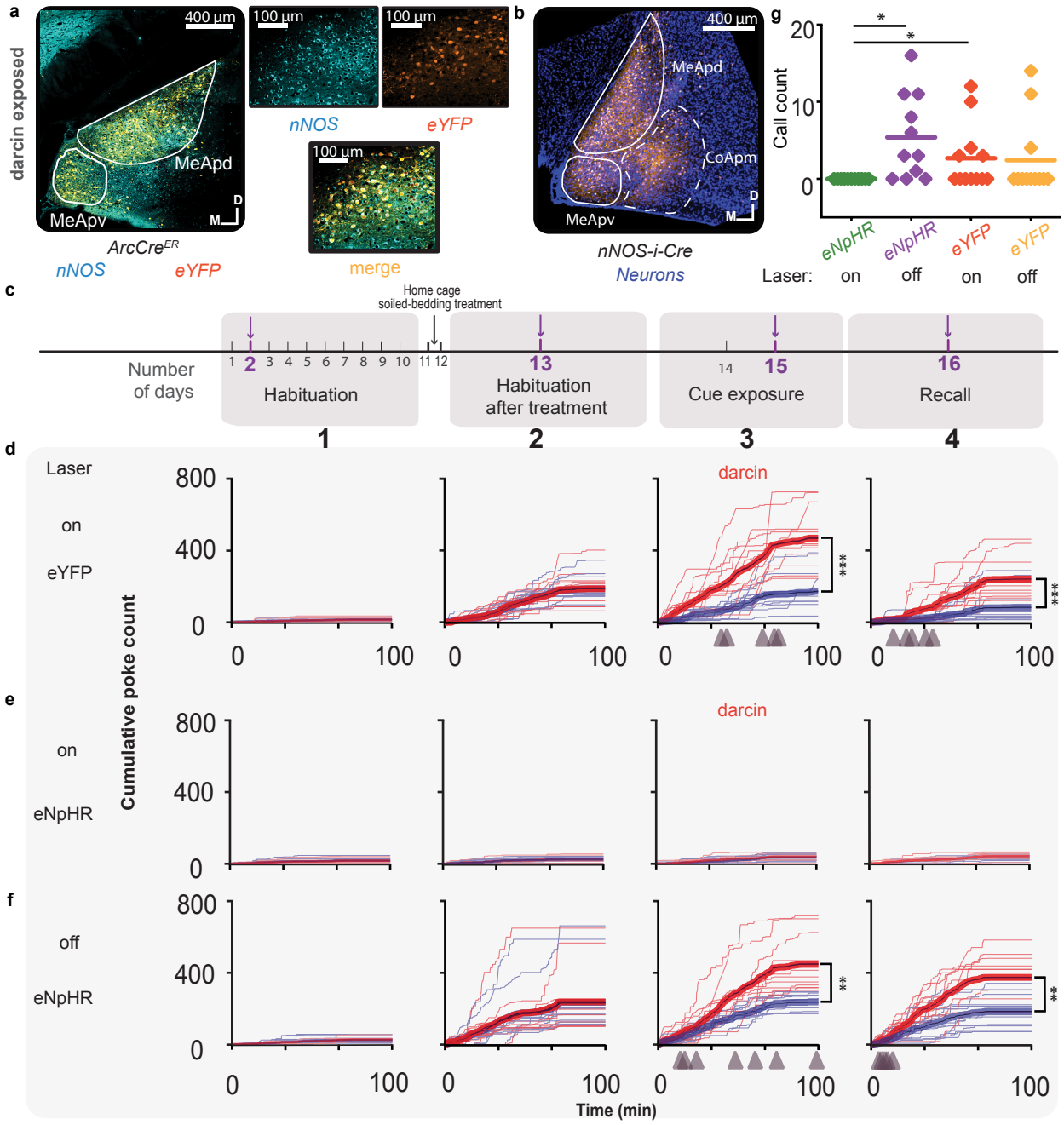
Extended Data Table 1 | Cell counts for exposure to different cue-types, nNOS expression and the overlaps in the posterior MeA. Counts (mean ± sem cell counts) quantifying region-specific and overlapping expression of cue-responsive (eYFP positive neurons) and nNOS-expressing neurons in the MeApd and 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, and saline. The percentage of overlaps (mean ± sem) are quantified between total

836 eYFP and nNOS expressing neurons in posterior MeA. Comparisons are made pair-wise
837 between darcin and all the other cue-types for YFP+ counts and the overlaps using two-sided
838 Mann-Whitney test (* $p < 0.05$). Comparisons are made pair-wise between darcin and all the
839 other cue-types for percent nNOS overlapping with YFP using two-sided Mann-Whitney test (*
840 $p < 0.05$).
841
842 **Extended Data Table 2 | Syllable categories for darcin and light evoked ultrasonic**
843 **vocalizations.** Different call categories emitted by animals exposed to darcin (n=24, in green)
844 and optogenetically stimulated (n=12, in blue). Frequencies and durations are compared with
845 unpaired two-sided t-test (* $p<0.05$, ** $p<0.005$, *** $p<0.0006$).


a
Home cage
soiled-bedding treatment
Number
of days
1 2 3 4 5 6 7 8 9 10 11 12
Habituation
13
Habituation
after treatment
14 15
Cue exposure
16
Recall
1
2
3
4
b
darcin
c
low darcin
male urine
d
darcin added
male urine
Cumulative poke count
800
400
0
100
Time (min)
e
kHz
100
75
50
25
Frequency
0.2
0.4
0.6
0.8
1.0
1.2
Time (sec)
dB
f
Call count
20
10
0
habituation
habituation
after estrus
darcin
recall
g
Session Type
h
Latency to scent marking
and vocalization (min)
100
66
33
53
16
darcin
recall


a
P
V
AOB
NpHR - eYFP
200 µm
b
Home cage
soiled-bedding treatment
Number
of days
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Habituation
Habituation
after treatment
Cue exposure
Recall
1
2
3
4
c
Laser
on
d
off
e
on
f
off
darcin
male urine
with darcin
Cumulative poke count
800
400
0
0
100
Time (min)
*
**
g
Call count
12
8
4
0
Laser
On
Off


a
Arc
Cre-ER^{T2}
AAV DJ
EF1a
eYFP-p2A
hChR2
WPRE
b
Tamoxifen injection
1
Virus injection
2 3 4
5
Estrus treatment
6
7
8
Social cue exposure
30
Light stimulation
Number of days
f
Call count
20
10
0
Saline
Darcin
MUP11
Laser: on
Stim
1
2
g
Laser: off
Recall
3
c
ChR2- eYFP Neurons
Darcin exposed
L
V
MeAp
400 µm
d
Saline exposed
e
MUP11 exposed
Laser :
Habituation
off
Light stimulation
on
Recall
off
Cumulative poke count
400
200
0
100
Time (min)
**


a
darcin exposed
400 µm
MeApd
MeApv
D
M
ArcCre^ER
nNOS
eYFP
100 µm
nNOS
100 µm
eYFP
100 µm
merge
b
400 µm
MeApd
CoApm
MeApv
nNOS-i-Cre
Neurons
g
Call count
20
10
0
*
*
eNpHR
eNpHR
eYFP
eYFP
Laser:
on
off
on
off
c
Home cage
soiled-bedding treatment
Number
of days
1 2 3 4 5 6 7 8 9 10 11 12
Habituation
13
Habituation
after treatment
14 15
Cue exposure
16
Recall
1
2
3
4
d
Laser
on
eYFP
Cumulative poke count
800
400
0
0
100
darcin
***
e
on
eNpHR
f
off
eNpHR
**
Time (min)