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Supplemental Information

**A Hypothalamic Midbrain Pathway
Essential for Driving Maternal Behaviors**

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Figure S1

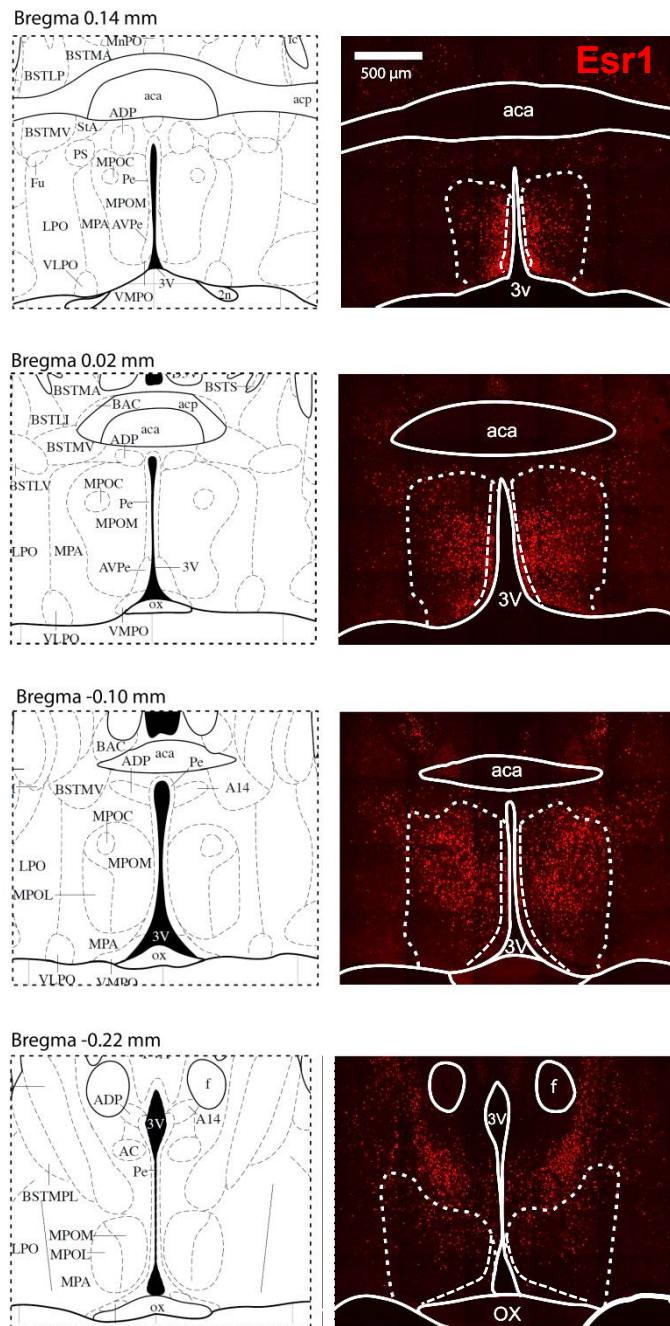


Figure S1. *Esr1* expression in the MPOA. Related to Figure 1.

Left shows the MPOA containing coronal sections in the reference atlas (The mouse brain atlas in stereotaxic coordinates 2nd edition, Paxinos and Franklin). Right shows the expression of *Esr1*. Scale bar: 500 μm . The dashed lines indicate the boundary of the MPOA that is used in the study. aca: anterior commissure. 3v: third ventricle. f: fornix. ox: optic chiasm.

Figure S2

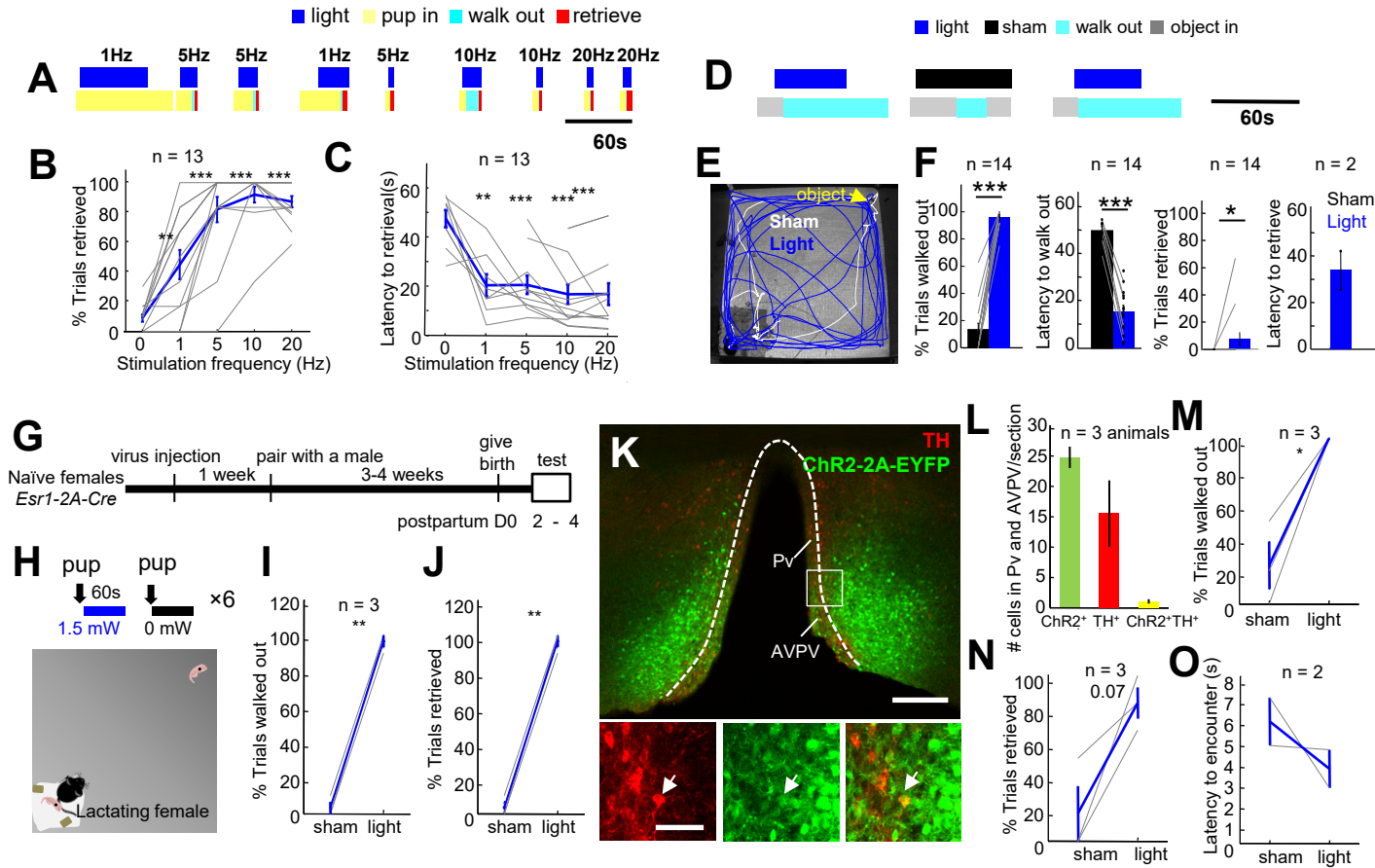


Figure S2. Additional optogenetic activation data. Related to Figure 2.

(A) Representative behaviors during light stimulation with different stimulation frequencies.

(B and C) Percentage of trials that animals retrieved (B) and the latency to retrieve under different stimulation frequencies. Blue lines show population average. For calculating the latency, only successful retrieval trials were included. One way ANOVA for (B) and (C). $p < 0.001$. Asterisks right above error bars indicate paired t-test results between 0 Hz and each of other frequencies.

(D) The behaviors during light and sham stimulation during object trials.

(E) Tracking results during three sham (white) and light trials (blue).

(F) The walk-out (n = 14) and object retrieval (n = 2) performance during light (blue) and sham (black) stimulation. During sham trials, the animal never retrieved the object.

(G) Experimental schedule for optogenetic activation of the MPOA^{Esr1+} cells in lactating females.

(H) Schematics of testing arena and stimulation protocol.

(I-J) The percentage of trials the animal walked out (I) and the percentage of trials with successful retrieval (J) during sham and light stimulation. n = 3 lactating animals. Paired t-test.

(K) A representative image showing the expression of TH and ChR2-2A-EYFP in the Pv, AVPV and MPOA. Dashed lines mark the Pv and AVPV. Right shows the enlarged view of the boxed area. Scale bars: 250 μ m (left) and 30 μ m (right). White arrow indicates a TH⁺ChR2⁺ cell.

(L) The average number of ChR2⁺, TH⁺ and ChR2⁺TH⁺ cells in the Pv and AVPV per section.

(M-O) The percentage of trials the animals walked out (M) and retrieved the pup (N), and the latency to encounter the pup after walking out the home base (O) during sham and real stimulation from three animals expressing ChR2-2A-EYFP. Paired t-test.

Error bars: \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Gray lines and black dots in B, C and F, I-J, M-O indicate data from individual animals.

Figure S3

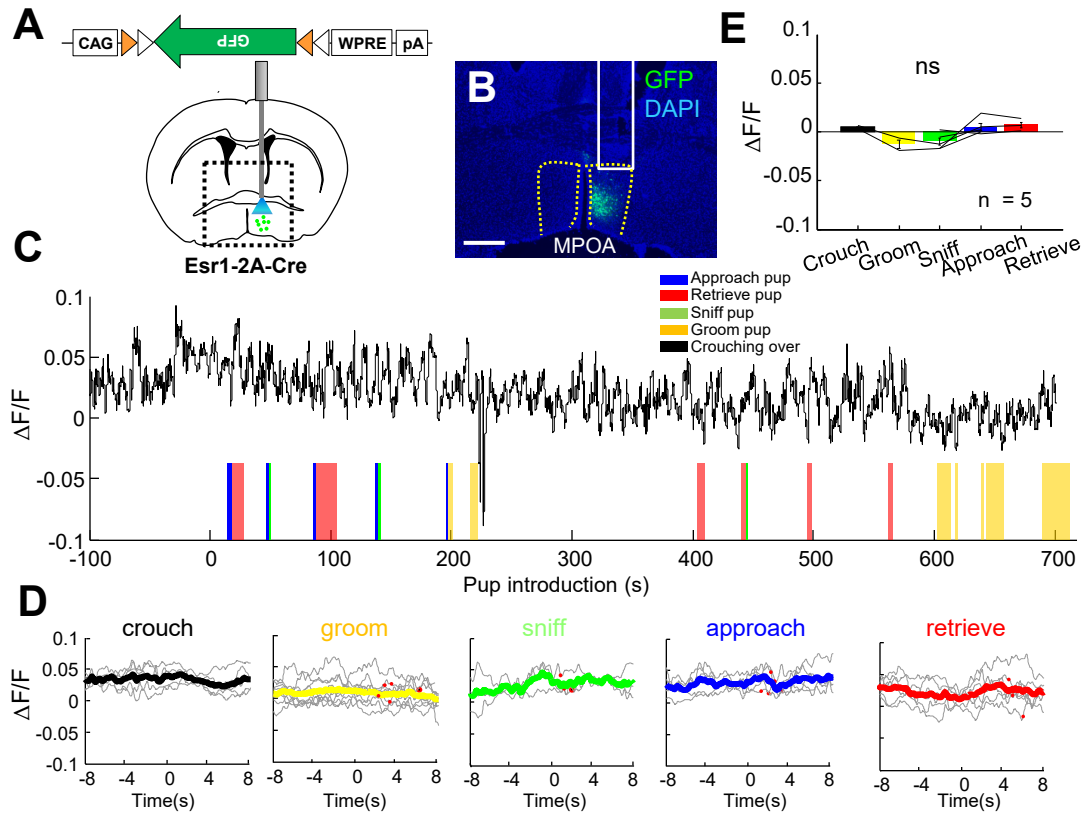


Figure S3. No change in fluorescence signal during pup-directed behaviors in control animals expressing GFP in the MPOA^{Esr1+} cells. Related to Figure 3.

(A) Experiment schematics.

(B) A representative histological image showing the GFP expression in the MPOA (yellow dashed lines) and fiber track (white lines). Scale bar: 500 μ m.

(C) A representative trace of $\Delta F/F$ during a pup session. Color shades indicate periods of behaviors.

(D) The PETH aligned to the onset of various maternal behaviors. Color and gray lines show the average signal and signal of individual trials, respectively. Red dots indicate the behavior offset.

(E) The averaged $\Delta F/F$ during pup directed behaviors of all animals. $n = 5$ animals.

Error bars: \pm SEM. Student t-test. $p > 0.05$ for all groups.

Figure S4

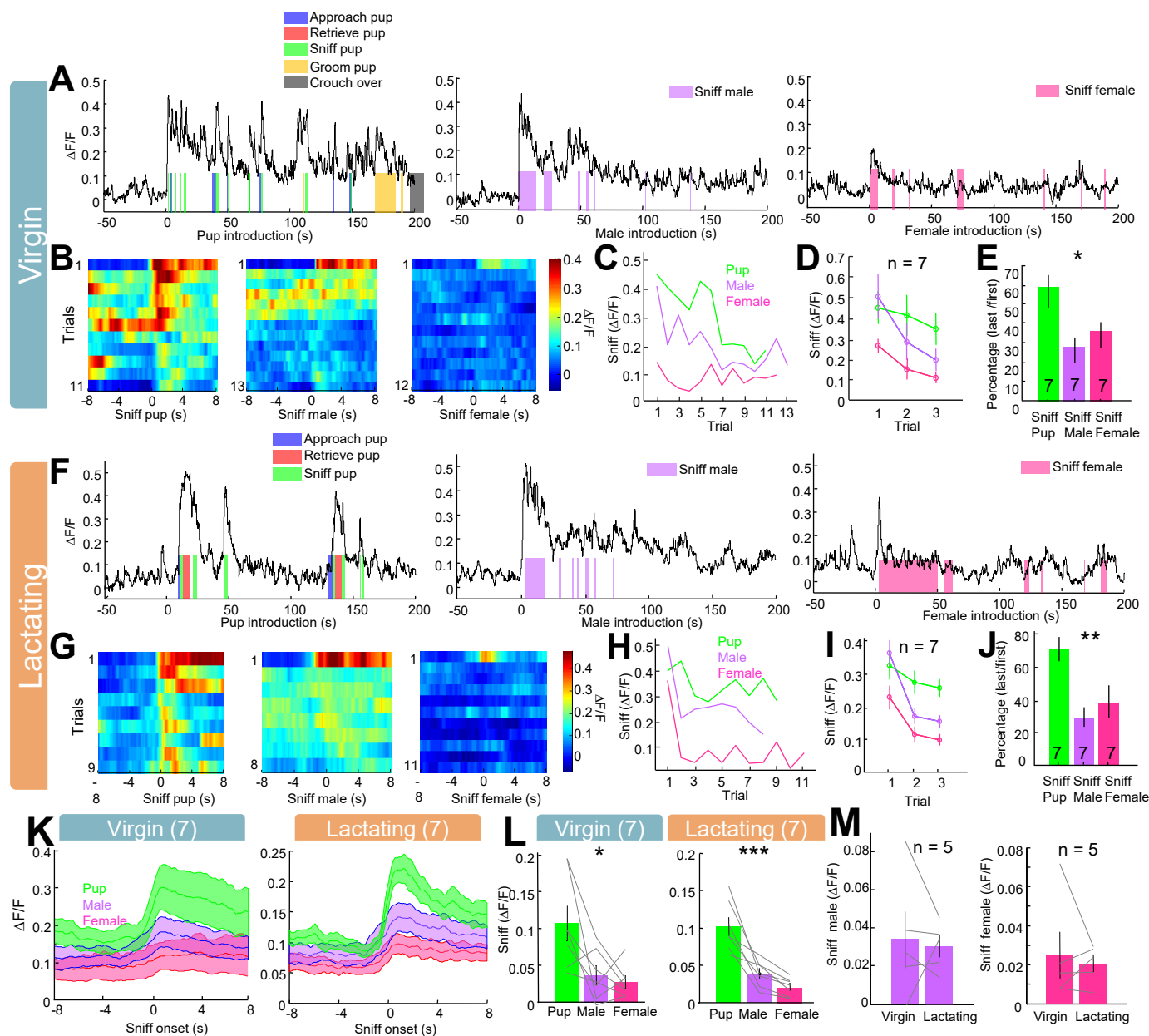


Figure S4. Comparison of MPOA Esr1+ cell responses to pups, male and female adult mice. Related to Figure 3.

(A and F) The GCaMP6 recording trace ($\Delta F/F$) when the naïve female (A) and the lactating female (F) interacted with pups (left), adult male (middle) and adult female mice (right).

(B and G) Heat maps showing the GCaMP6 signal over repeated sniffing trials in A and F.

(C and H) Quantification of the peak GCaMP6 signal over repeated sniffing trials in A and F.

(D and I) The average peak GCaMP6 responses during the first three trials of pup sniffing (green), male sniffing (blue) and female sniffing (red) of naïve (D) and lactating (I) females.

(E and J) The average ratio of GCaMP6 response during the last sniffing trial over that during the first sniffing in naïve (E) and lactating (J) females. $n = 7$ for each group. One way ANOVA.

(K) The average PETHs aligned to pup sniffing (green), male sniffing (blue) and female sniffing (red).

(L) The average GCaMP6 signal during pup, male and female sniffing. One way ANOVA with repeated measures.

(M) GCaMP6 responses during sniffing male (left) and female (right) do not differ in naïve and lactating animals. Lines in L and M indicate individual animals.

Error bars: \pm SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. See methods for detailed statistic results.

Figure S5

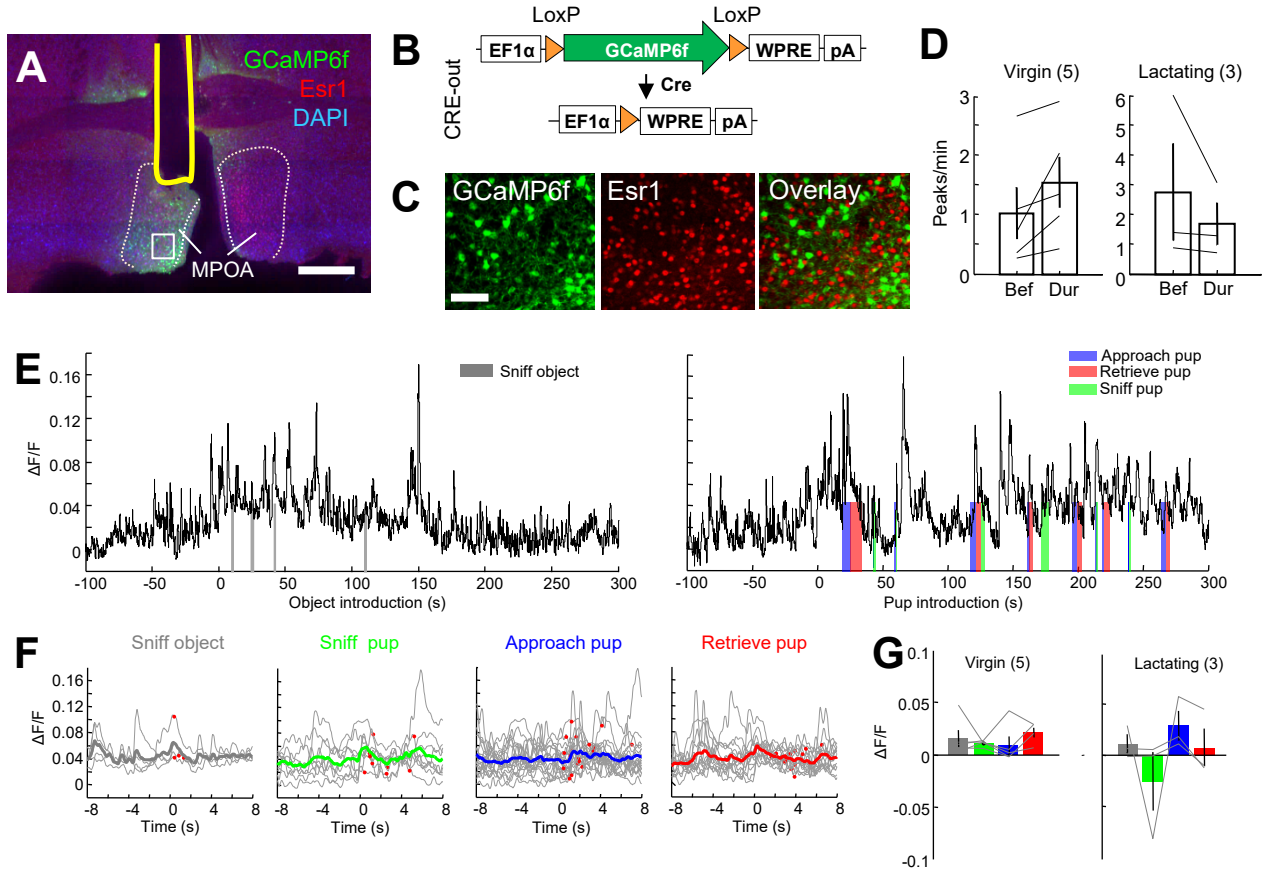


Figure S5. MPOA *Esr1*⁻ cells are minimally excited during maternal behaviors. Related to Figure 3.

(A) Histological image showing the expression of GCaMP6f (green) and *Esr1* (red) in the MPOA (white dashed lines) in a representative Cre-out animal. Yellow lines indicate the optic fiber track. Scale bar: 500 μ m.

(B) Cre-out construct.

(C) Zoomed in view of the boxed area in A. Scale bar: 50 μ m.

(D) GCaMP peak frequency before and during pup presentation in virgin (left) and lactating females (right). Paired t-test.

(E) $\Delta F/F$ of GCaMP6 signal during an object session (left) and a pup session (right). Color shades indicate annotated behaviors.

(F) PETHs aligned to the onset of various behaviors. Gray lines show individual trials and color lines show the average. Red dots indicate the end of trials.

(G) Average responses during sniffing object, sniffing pup, pup approach and pup retrieval. Color convention as in E and F. One-way ANOVA. All $p > 0.05$.

Error bars: \pm SEM.

Figure S6

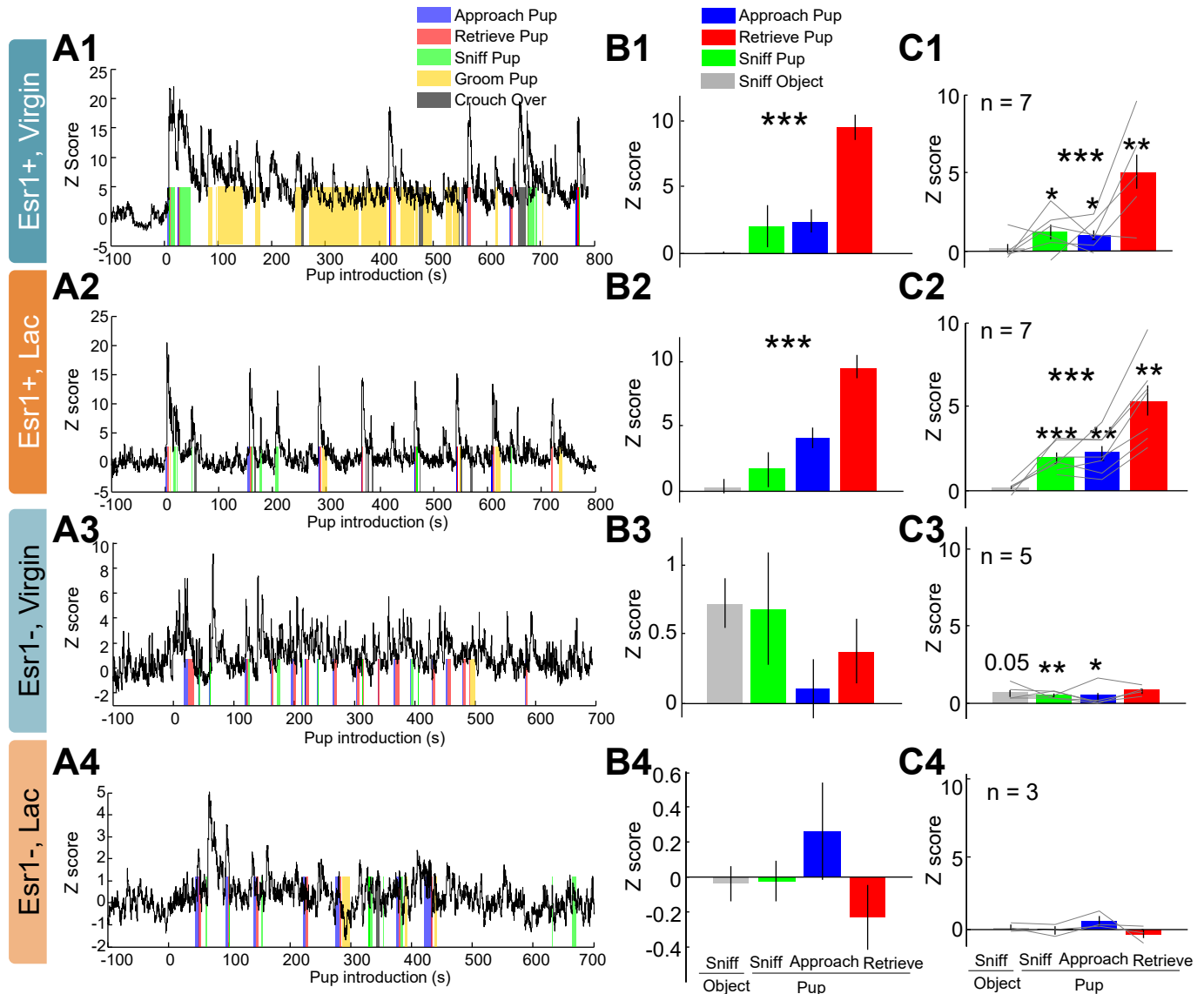


Figure S7

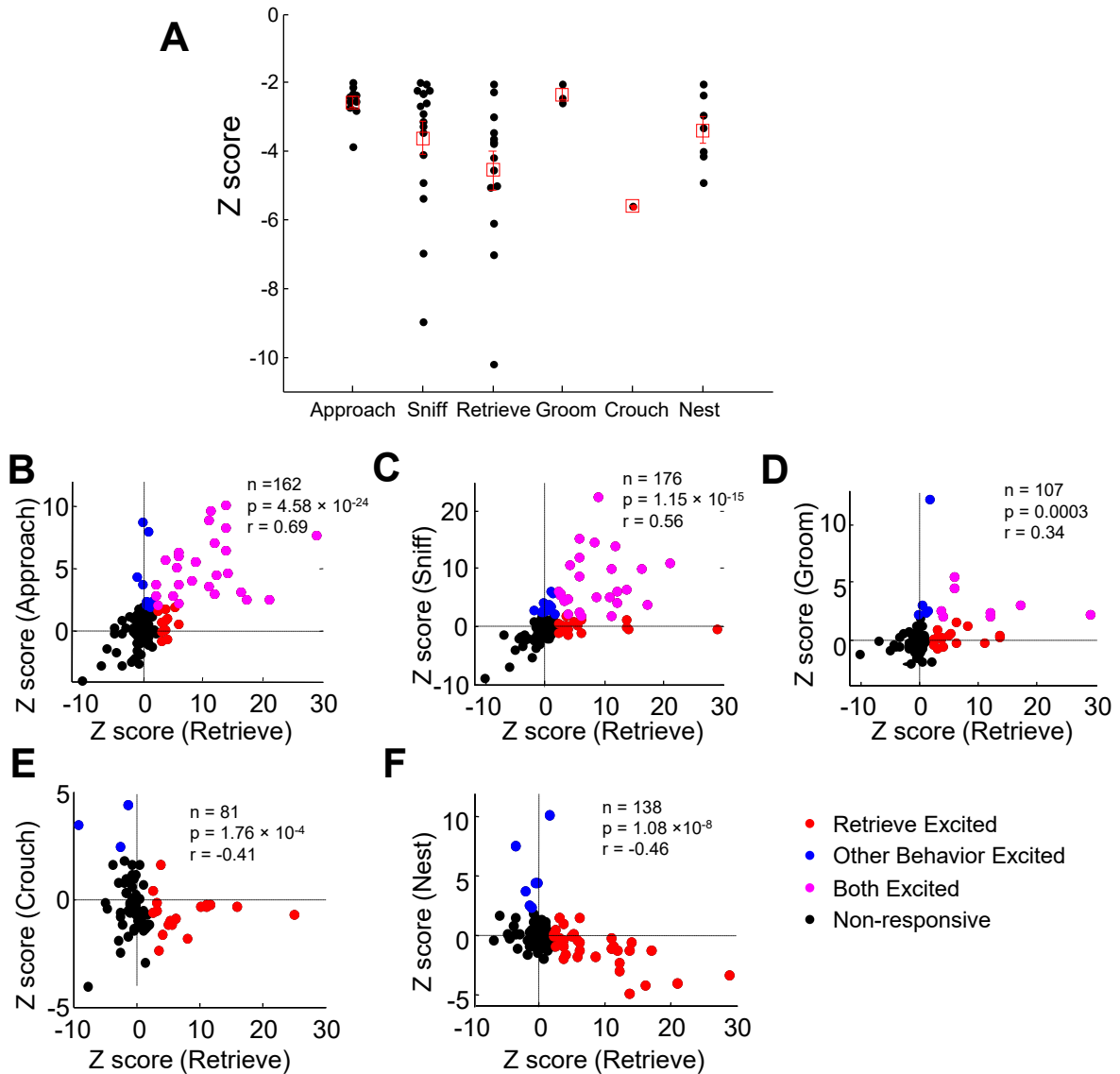
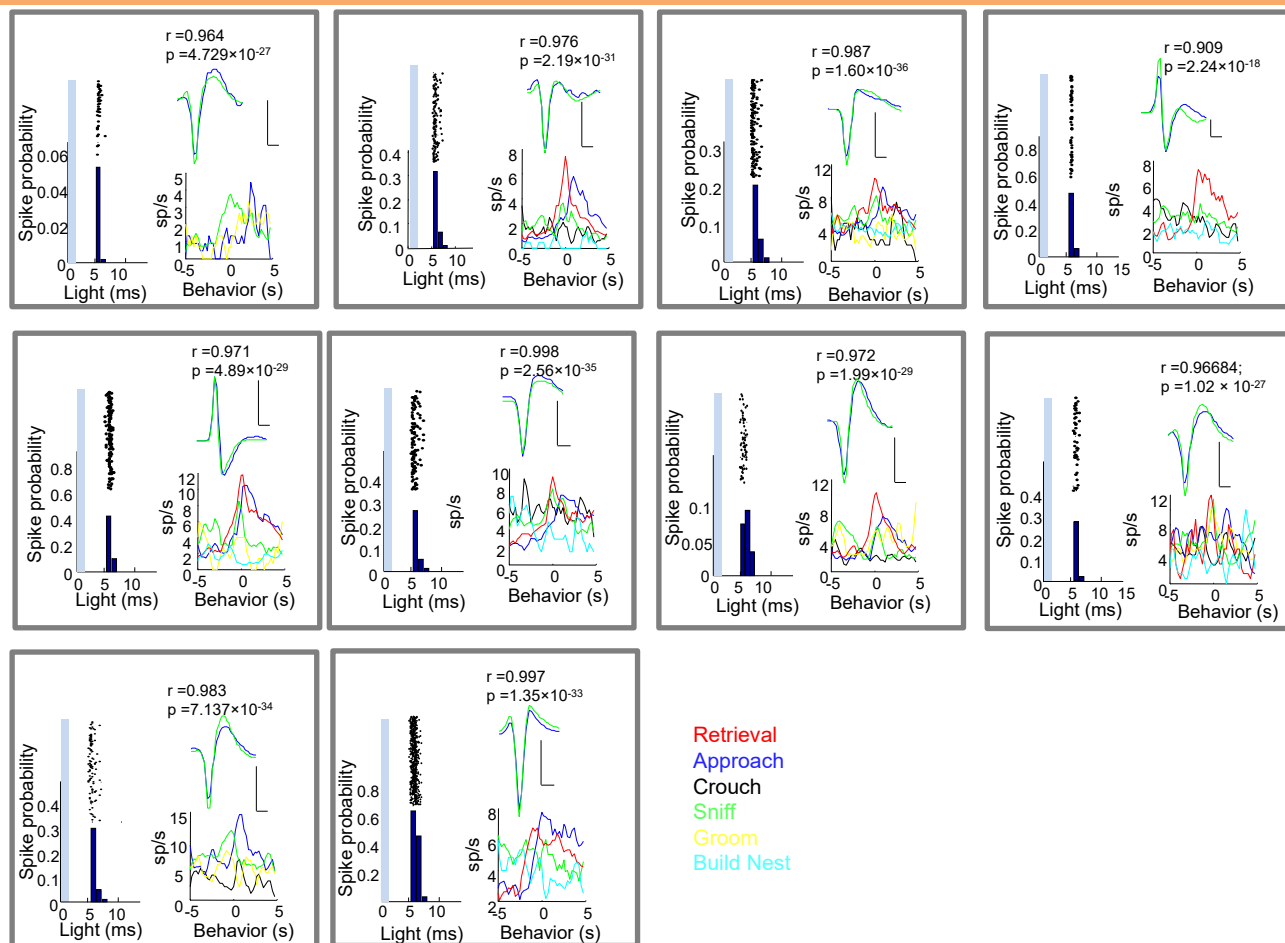


Figure S7. Additional data of the MPOA electrophysiological recording. Related to Figure 4.

(A) Responses of inhibited cells ($Z < -2$) during various maternal behaviors. One way ANOVA. $p > 0.05$. (B-F) Relationship between cell responses (Z score) during pup retrieval (x axis) and other maternal behaviors (y axis). Red dots: cells excited only during pup retrieval. Blue dots: cells excited during other maternal behaviors; magenta: cells excited during both behaviors. Error bars: \pm SEM. Pearson moment-product correlation.

Figure S8

Pup excited MPOA Esr1+ cells



Pup non-responsive MPOA Esr1+ cells

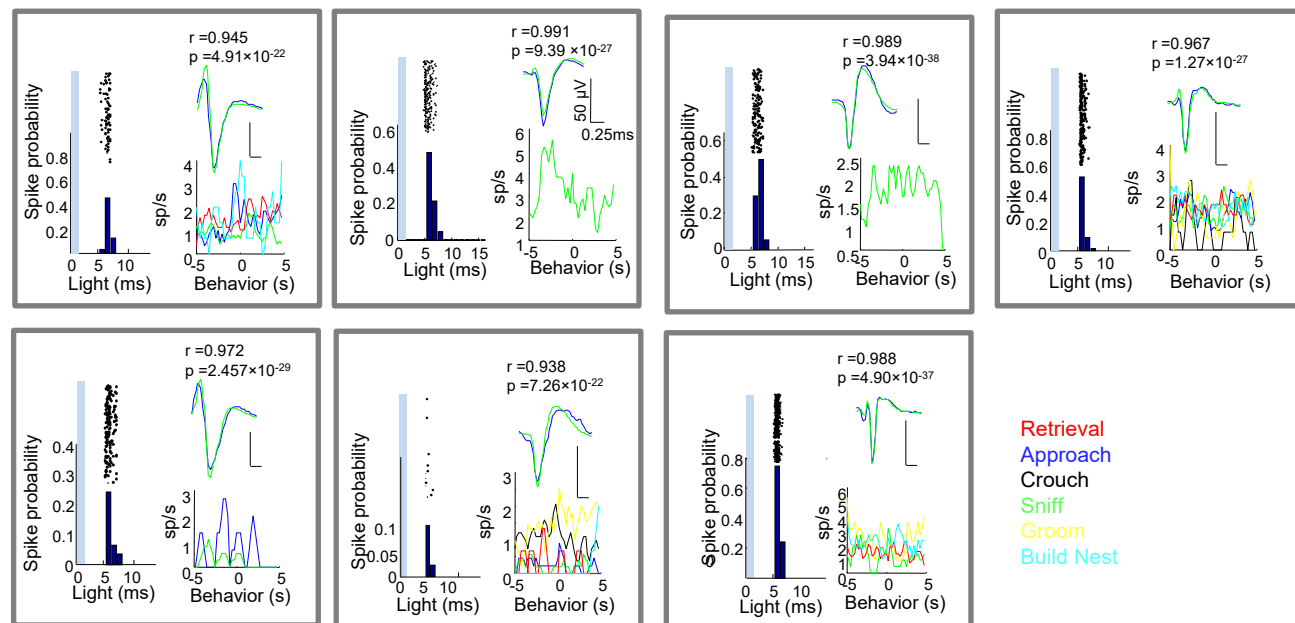


Figure S8. Responses of 17 putative MPOA^{Esr1+} cells. Related to Figure 5. Each box shows the response of one putative MPOA^{Esr1+} cells. Left side shows the raster plot and the spiking probability after 1-ms blue light pulse. Bin size: 1 ms. Shades represent light on period. Right top shows the average waveforms of spontaneous spiking (green) and light evoked spiking (blue). Scale bars: 50 μ V (vertical) and 0.25 ms (horizontal). Pearson moment-product correlation. Right bottom shows the average PETHs of spiking activity aligned to the behavioral onset. Green: sniff pup; Blue: approach pup; Red: retrieve pup; Yellow: groom pup; Black: Crouch over pups; Cyan: build nest. Bin size: 250 ms.

Figure S9

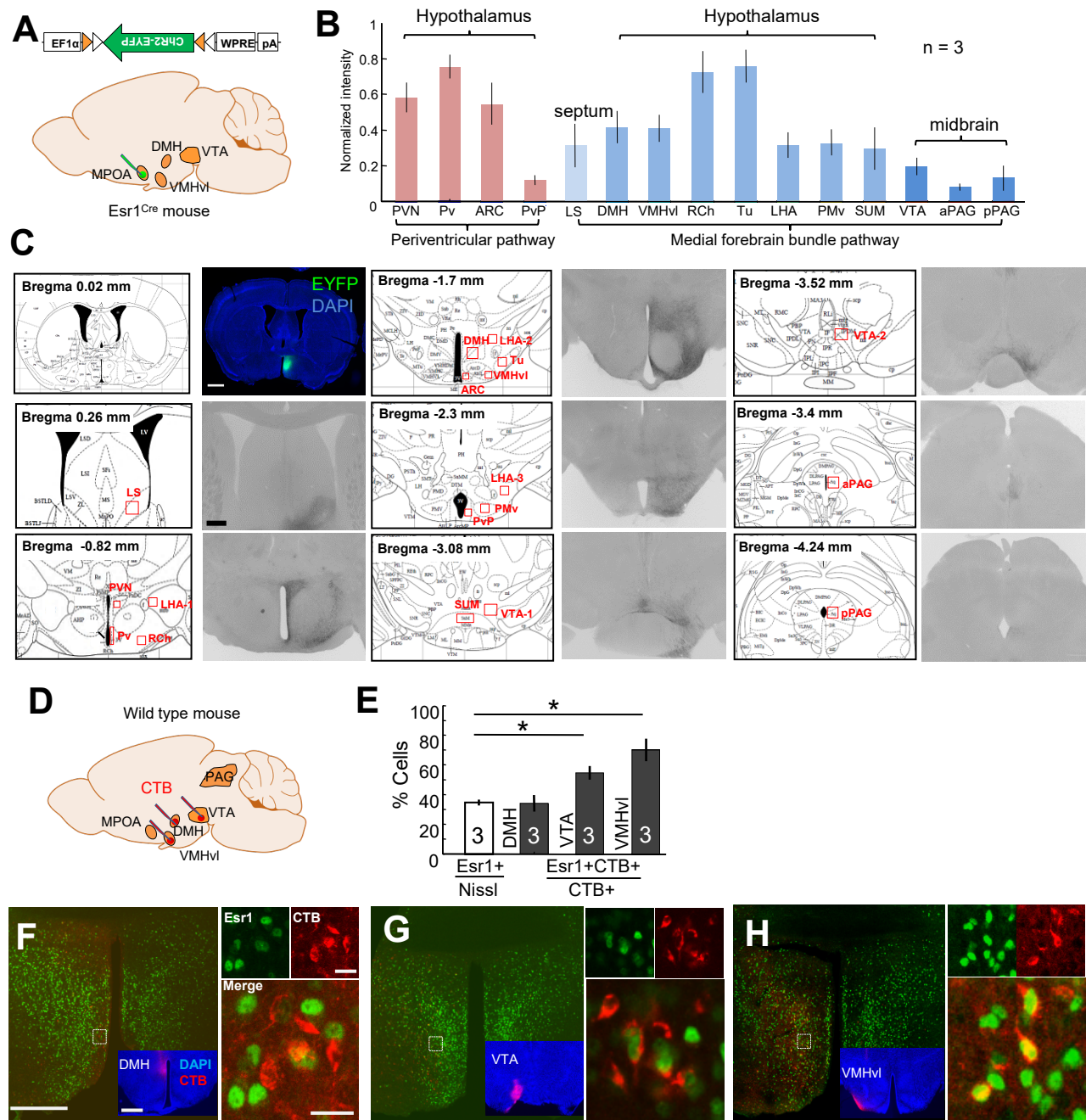


Figure S9. The overall projection pattern of the MPOA^{Esr1+} cells. Related to Figure 7.

(A) Experiment schematics.

(B) The density of the terminal fields is measured as the normalized average pixel intensity in the red boxed areas in (C). n =3 animals.

(C) The injection site in the MPOA and the terminal fields. Scales bars:1 mm (MPOA) and 500 μ m. The reference atlas is from the mouse brain atlas in stereotaxic coordinates 2nd edition (Paxinos and Franklin). The sizes of the red boxes are 220 x 220 μ m for lateral septum (LS), 120 x120 μ m paraventricular hypothalamic nucleus (PVN), 420 x 80 μ m for periventricular hypothalamic nucleus (Pv), 120 x 120 μ m for arcuate nucleus (ARC), 100 x 100 μ m for posterior periventricular nucleus (PvP), 250 x 250 μ m for retrochiasmatic area (RCh), 170 x 170 μ m for lateral hypothalamic area (LHA), 250 x 250 μ m for dorsomedial hypothalamus (DMH), 150 x 150 μ m for ventrolateral part of the ventromedial hypothalamus (VMHvl), 170 x 170 μ m for tuberal nucleus (Tu), 170 x 170 μ m for ventral premammillary nucleus (PMv), 190 x 400 μ m for supramammillary nucleus (SUM), 330 x 200 μ m for ventral tegmental area (VTA), 220 x 220 μ m for anterior periaqueductal gray (aPAG) and 220 x 220 μ m for posterior periaqueductal gray (pPAG).

(D) Schematics of retrograde labeling.

(E) The percentage of Esr1+ cells in the MPOA and the percentage of CTB+ cells that express Esr1 from DMH, VTA and VMHvl. Unpaired t-test. *p<0.05.

(F-H) Retrogradely labeled cells (red) and Esr1 staining (green) at the MPOA after injecting CTB-555 into the DMH (F), VTA (G) and VMHvl (H). Insets show the injection sites. Scale bars: 500 μ m. Right shows the zoomed in view of the boxed area. Scale bars: 20 μ m (top) and 40 μ m (bottom).

Error bars: \pm SEM.

Figure S10

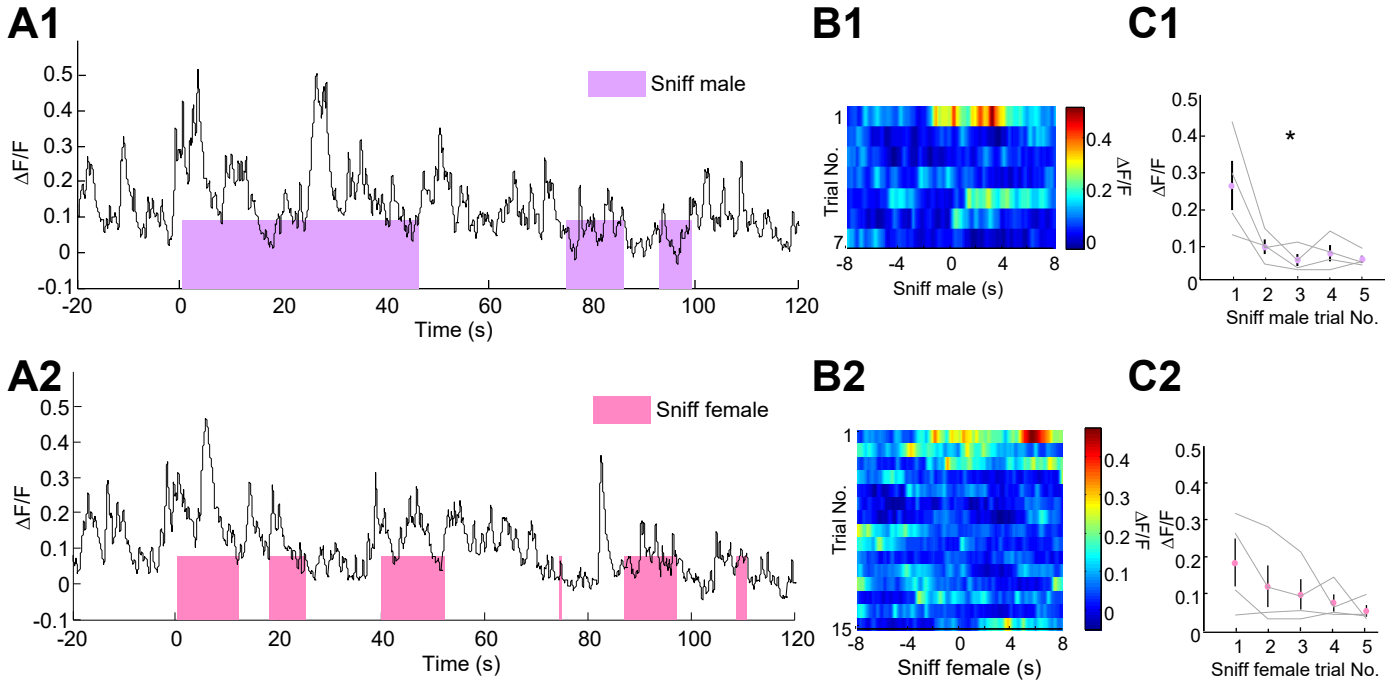


Figure S10. Response of female VAT^{DAT+} cells to conspecific adults adapted quickly with repeated sniffing. Related to Figure 7.

(A1 and A2) GCaMP6 traces during interaction with adult male (A1) and female (A2) intruder.

(B1 and B2) heat mapping showing the responses over repeated sniffing trials in (A1) and (A2) sessions.

(C1 and C2) The average $\Delta F/F$ over repeated male (C1) and female (C2) sniffing trials. Gray lines indicate individual animals. Error bar: \pm SEM. $n = 4$ animals. One way ANOVA with repeated measures. * $p < 0.05$.

Figure S11

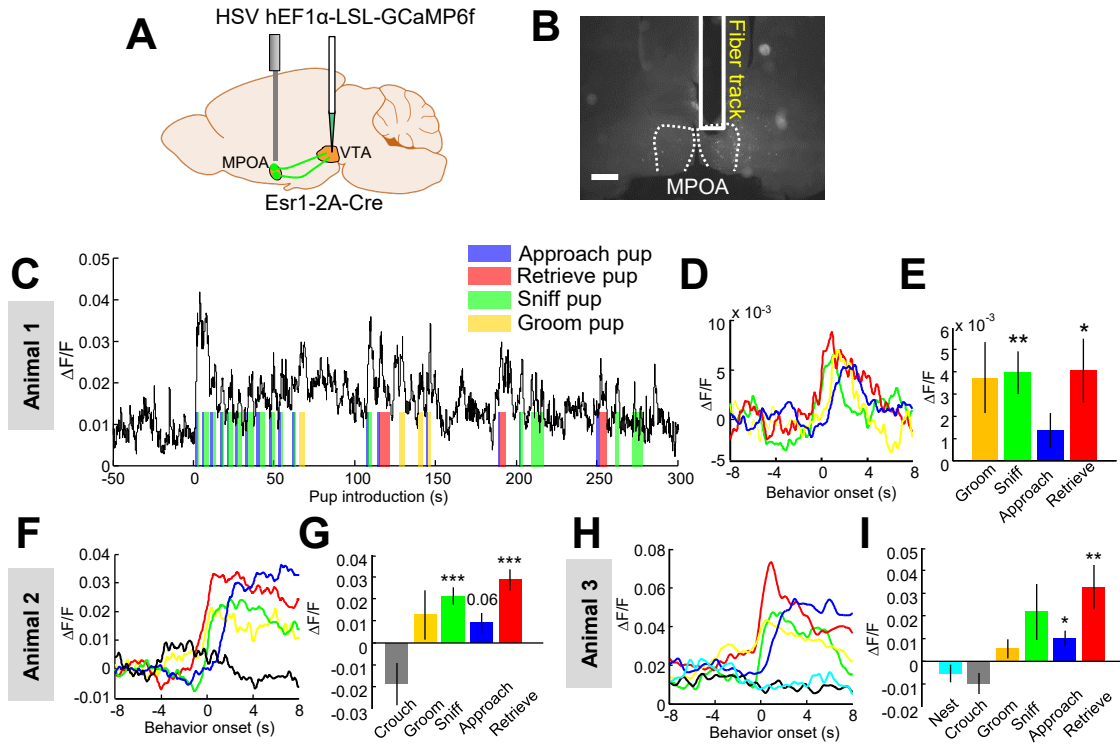


Figure S11. GCaMP6 responses of MPOA^{Esr1+} to VTA projectors. Related to Figure 7.

(A) Experimental schematics.

(B) A representative histological image showing the GCaMP6 expressing cells in the MPOA. Scale bar: 500 μ m.

(C) The GCaMP6 recording trace from Animal 1. Color shades indicate the annotated behaviors.

(D, F and H) Averaged PETs aligned to various maternal behaviors from animal 1 (D), animal 2 (F) and animal 3 (H).

(E, G and I). Average $\Delta F/F$ during various maternal behaviors in animal 1 (E), animal 2 (G) and animal 3 (I). Color convention in D-I follows that in C. All animals are spontaneous retrieving virgin females.

Error bar: \pm SEM. Student t-test for individual behaviors across trials. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure S12

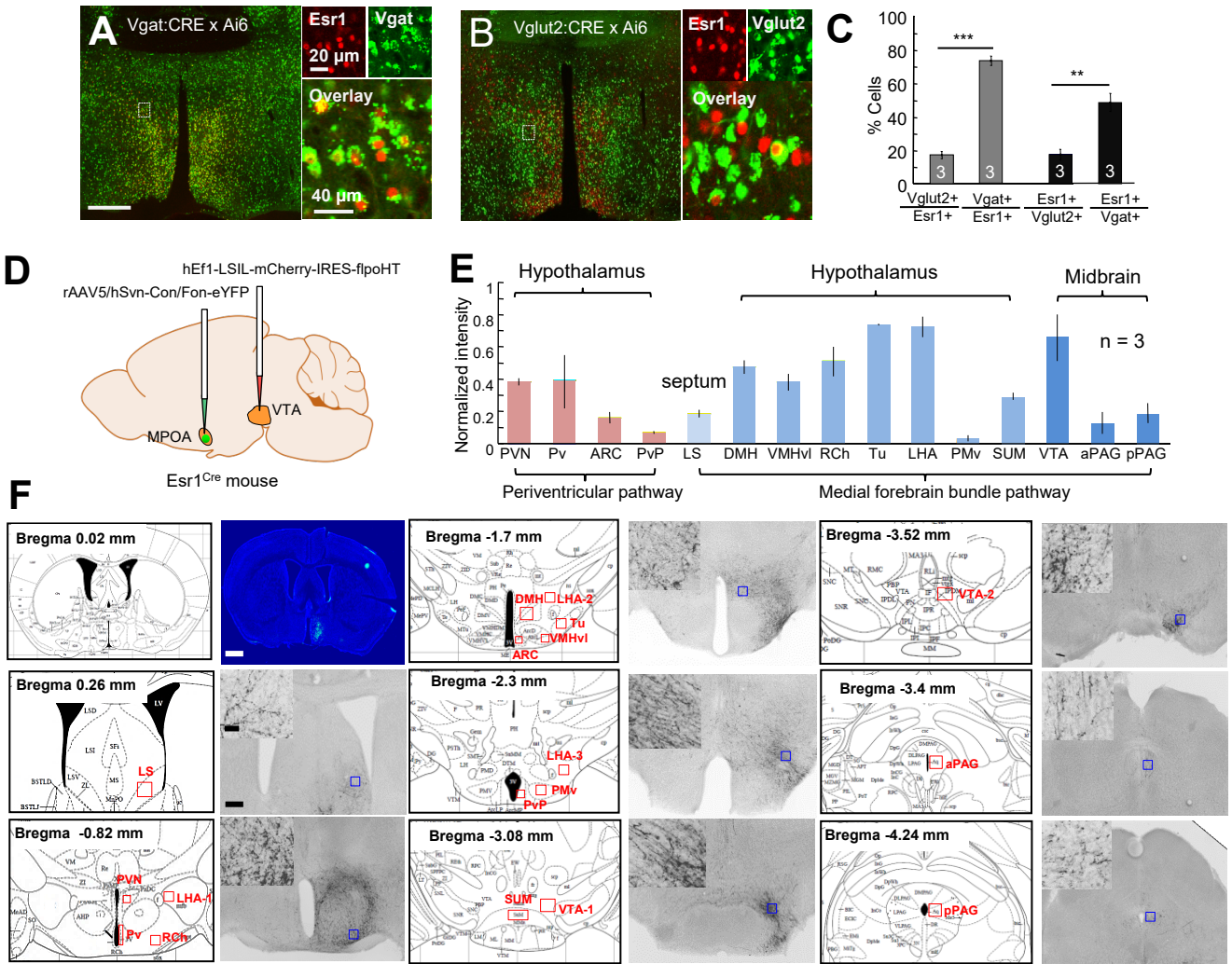


Figure S12. The neurotransmitter type of the MPOA^{Esr1+} cells and the overall innervation pattern of the MPOA^{Esr1+} to VTA projecting cells. Related to Figure 8.

(A and B) Overlay between Vgat (A) or Vglut2 (B) (green) and Esr1 (red) at the MPOA. Right images show the enlarged views of the boxed areas. Scale bars: 250 μ m (left), 20 μ m (top, right), and 40 μ m (bottom, right).

(C) The percentage of MPOA^{Esr1+} cells that are glutamatergic or GABAergic, and the percentage of glutamatergic cells expressing Esr1 and the percentage of GABAergic cells expressing Esr1. Unpaired t-test. ***p<0.001.

(D) Experiment schematics.

(E) The density of the terminal fields measured as the normalized average pixel intensity in the red boxed areas in (C). n = 3 animals.

(F) The injection site and the terminal fields. Scales bars: 1 mm (MPOA), 500 μ m and 50 μ m (insets). The reference atlas is from the mouse brain atlas in stereotaxic coordinates 2nd edition (Paxinos and Franklin). The sizes of the red boxes are 220 \times 220 μ m for lateral septum (LS), 120 \times 120 μ m paraventricular hypothalamic nucleus (PVN), 420 \times 80 μ m for periventricular hypothalamic nucleus (Pv), 120 \times 120 μ m for arcuate nucleus (ARC), 100 \times 100 μ m for posterior periventricular nucleus (PvP), 250 \times 250 μ m for retrochiasmatic area (RCh), 170 \times 170 μ m for lateral hypothalamic area (LHA), 250 \times 250 μ m for dorsomedial hypothalamus (DMH), 150 \times 150 μ m for ventrolateral part of the ventromedial hypothalamus (VMHv), 170 \times 170 μ m for tuberal nucleus (Tu), 170 \times 170 μ m for ventral premammillary nucleus (PMv), 190 \times 400 μ m for supramammillary nucleus (SUM), 330 \times 200 μ m for ventral tegmental area (VTA), 220 \times 220 μ m for anterior periaqueductal gray (aPAG) and 220 \times 220 μ m for posterior periaqueductal gray (pPAG). Blue box (170 \times 170 μ m) indicate the zoom-in view of the axon terminals.

Error bars: \pm SEM.