MATERIALS AND METHODS

Histology, immunohistochemistry and microscopy. We processed brains with immunohistochemistry to detect cells expressing Avp, and GFP and, in separate tissue, the immediate early gene product Arc. For Arc analysis, Avp-Cre mice received optical stimulation, as described above, then 90 min later were deeply anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde solution. Brains were stored in 20% sucrose in phosphate buffer (PB) at 4°C, and then cut into 15 µm coronal sections using a cryostat and mounted on slides. For Avp, and GFP analysis, mice were anesthetized and perfusion, and brains were sectioned, as decribed above.

A 1:6 series through the PVN and hippocampus of each brain was processed for single-labeling for Arc or double-labeling for GFP-ir/Avp-ir, respectively. For Arc staining, mounted sections were rinsed in 0.1 M PB for 5 min (3x) and again after each of the following steps: incubation in 1% $NaHB_4$ in PB for 10 min; incubation for 10 min in 1X Powerblock (Universal Blocking Reagent, Biogenex) to block endogenous peroxidase activity and 24 h in 1:100 anti-Arc (Rabbit, sc-15325, Santa Cruz); and blocked for 10 min with Peroxidase Blocking Agent (Dako). Sections were then rinsed in 0.1 M Tris (ph 8.0) for 5 min (3x) and again after each of the following steps: 30 min incubation in a humidified chamber with Super Picture HRP Polymer Conjugate Rabbit antibody (Invitrogen); 20 min incubation in a dark humidified chamber with 1:50 TSA Plus FITC tyramide (Perkin Elmer); and a 5 min incubation in 1:10K DAPI. For GFP/Avp staining, mounted sections were processed as described above, with substitution of the primary antibody for 1:200 anti-GFP (Rabbit, A6455, Molecular Probes) and repeated the protocol for Avp staining using a Avp antibody (1:100, Mouse, gifted from Dr. Harold Gainer, NIMH) and TSA Plus Cyanine 3 (Perkin Elmer).

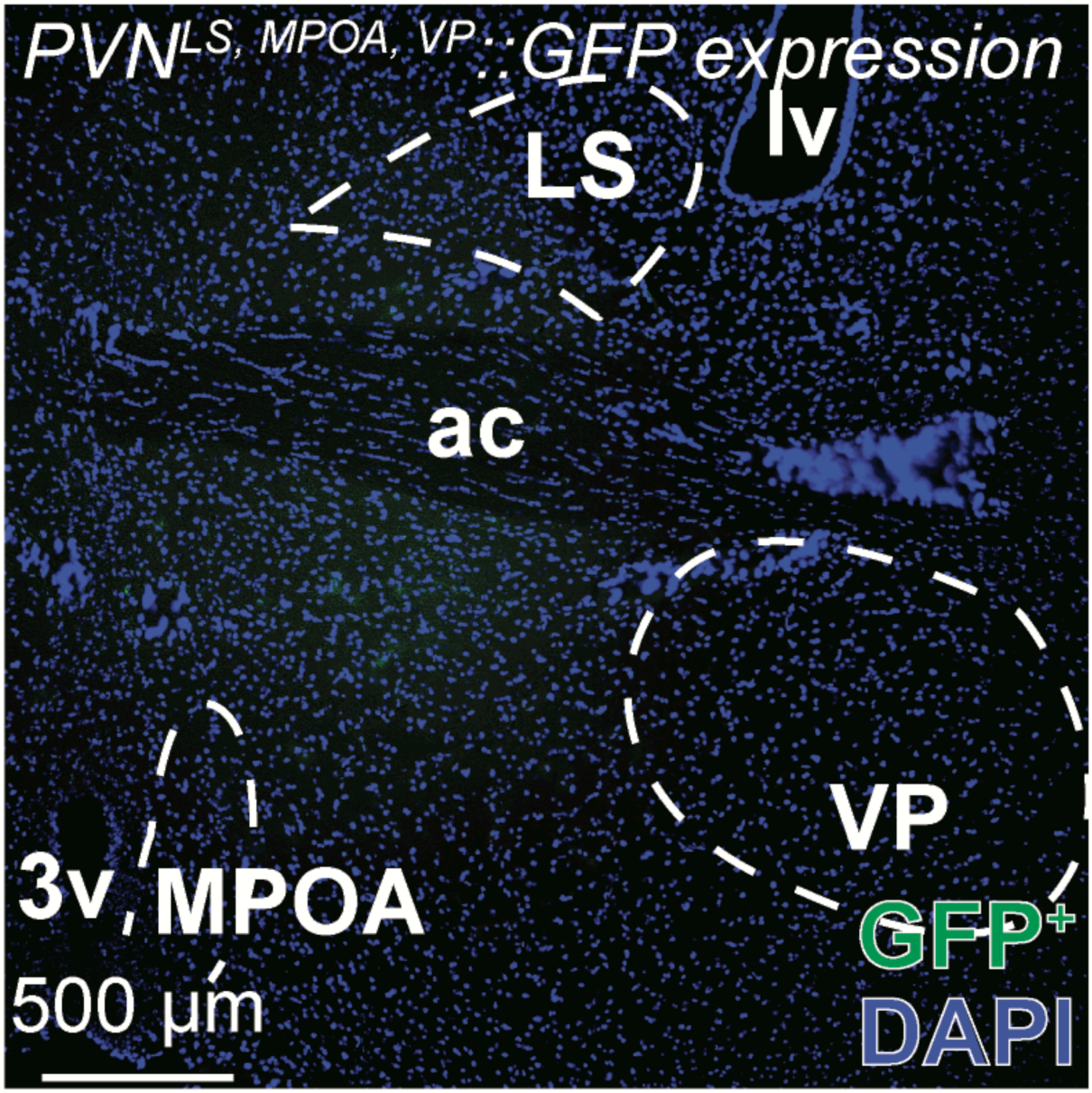
Images were acquired by using a fluorescent microscope (Nikon 50i) with a 4',6-diamidino-2-phenylindole (DAPI) filter for DAPI-stained sections, a fluorescein isothiocyanante (FITC) filter for Arc- and GFP-stained sections, and a tetramethylrhodamine isothiocyanate (TRITC) filter for Avp-stained sections and mCherry fluorescence. iVision (BioVision Technologies) was used to process images, with between 100-500 msec exposure times. Staining measurements were conducted using ImageJ (U.S. National Institutes of Health). Densities of single-labeled (Avp, GFP, and DAPI) and double-labeled cells (GFP cells expressing Avp) in the PVN were counted in every sixth section (which occurred at 90 µm intervals), resulting in three sections counted per mouse. The average of the counted single-labeled Arc cells was then divided by the area of the PVN (averaging 0.225 mm^2 per hemisphere) to yield the number of single-labeled Arc cells per 0.1 mm^2. Percentages of labeled neurons expressing Avp and GFP were calculated by dividing the number of these cells the total number of DAPI-labeled cells in the PVN. GFP-labeled cells expressing Avp were also divided by total GFP-labeled cells in the PVN.

ViewRNA *in situ* hybridization (ISH) tissue assay

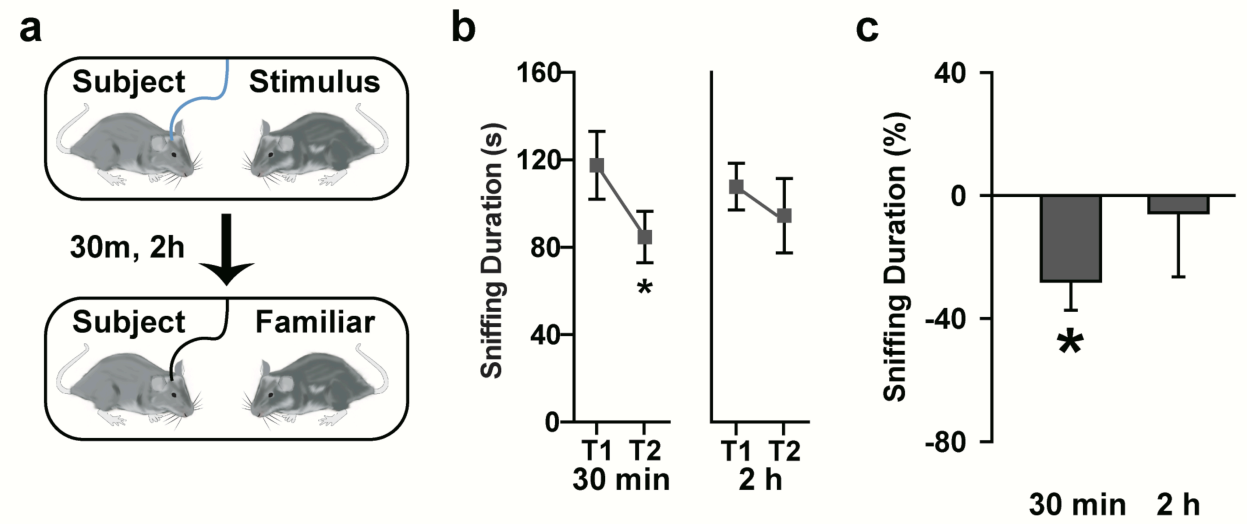
The ViewRNA ISH Tissue Assay for RNA *in situ* hybridization histochemistry was used to visualize transcripts of the Avpr1a, Avpr1b, and Oxtr genes according to the manufacturer's instructions (Panomics-Affymetrix, Santa Clara, CA). The proprietary probes were designed and synthesized by Affymetrix. Briefly, 16µ sections were cut from fresh-frozen brains and mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). The slides were incubated at 4°C

in 4% formaldehyde in phosphate-buffered saline (PBS) overnight. The following day, slides were dehydrated through graded ethanol washes (50%, 70%, 100%) at room temperature for 10 min each, and then baked at 60°C for 30 min. A working protease dilution was then made at 1:100 in 40°C PBS, and slides were incubated at 40°C for 10 min. After a brief wash in PBS, the sections fixed again in 4% formaldehyde for 5 min at room temperature. The working probe set solutions were prepared at a 1:40 dilution in 40°C probe set diluent, and hybridization took place for 2 hr at 40°C. Following hybridization, slides were washed three times for 2 min each in wash buffer with constant and vigorous agitation. Next, signal amplification was performed with the Preamplifier Mix QT (25 min, 40°C) followed by the Amplifier Mix QT(15 min, 40°C) with wash steps in between. Next, the slides were placed in Label Probe 1-AP kit solution at a 1:1000 dilution in 40°C Label Probe Diluent QF for 15 min at 40°C. A fast red substrate was then applied and slides were incubated for 30 min in the dark at 40°C. Immediately following development, the tissues were briefly washed in PBS and counterstained with DAPI.

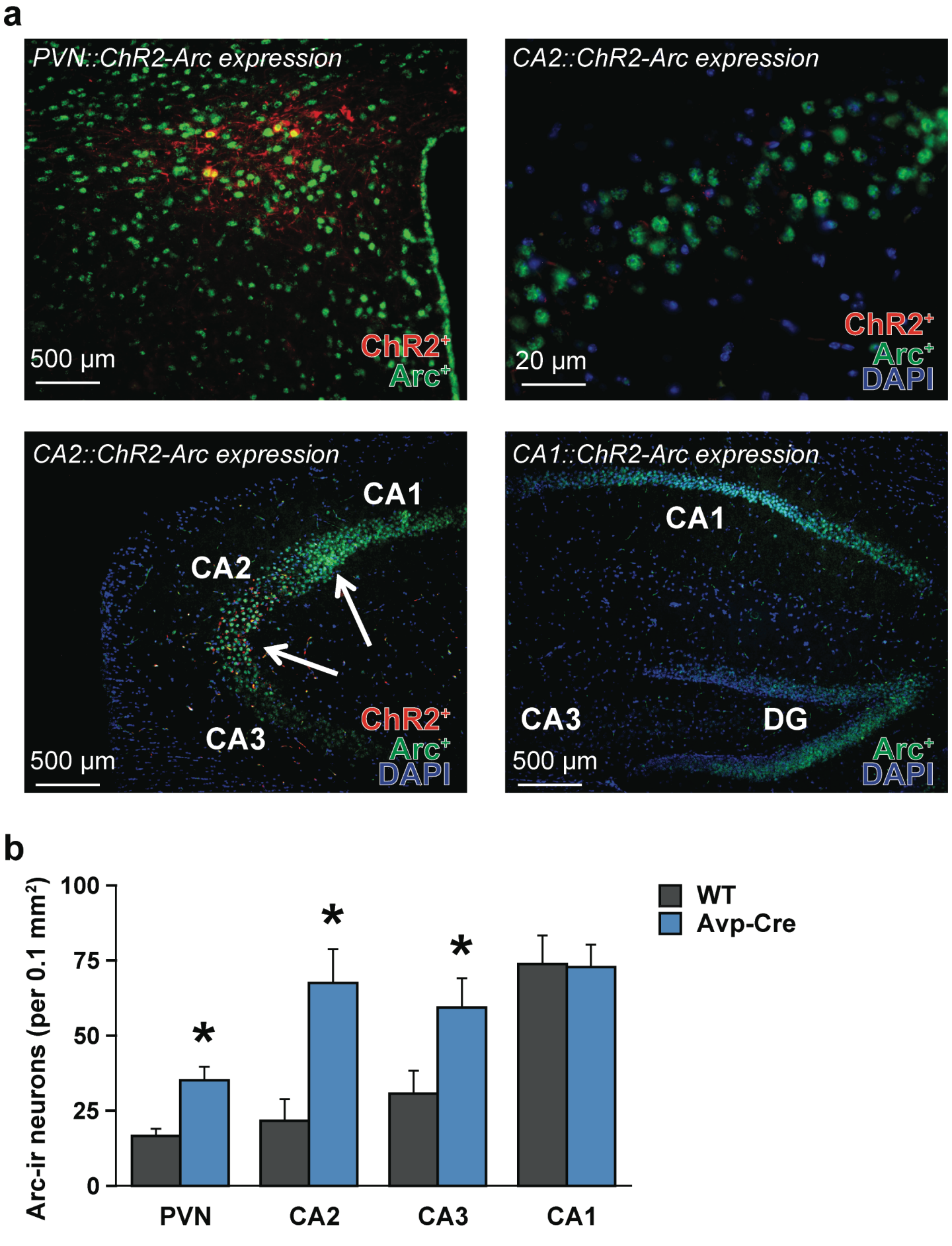
FIGURES



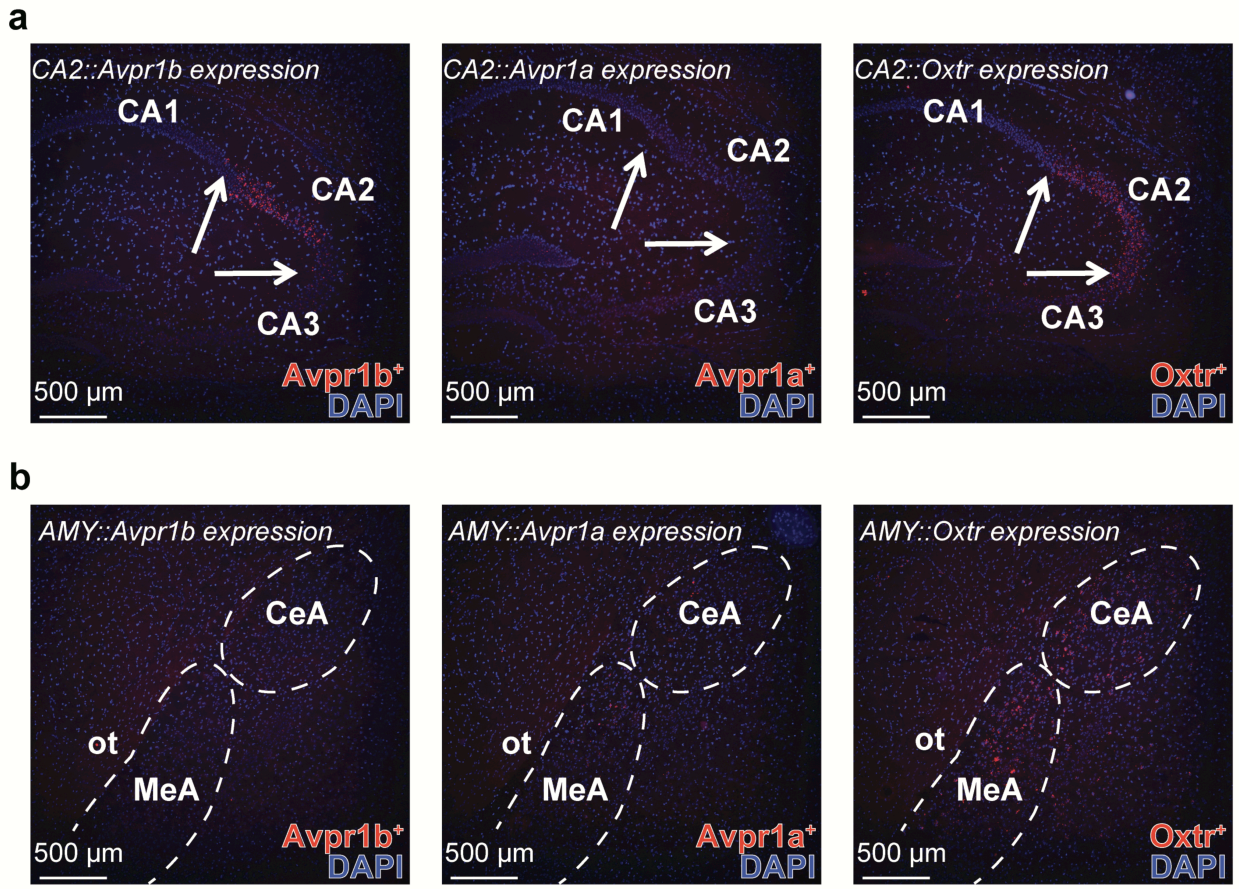
Supplemental Figure 1. The Avp$^{PVN\rightarrow CA2}$ pathway does not bifurcate to other regions. This is a representative image of coronal sections showing the absence of green fluorescent protein (GFP^{+}, green) expression in the lateral septum (LS), ventral pallidum (VP), and medial preoptic area (MPOA), regions hypothalamic paraventricular nucleus (PVN) Avp neurons are known to innervate, following a Cre-inducible HSV-GFP injection into the CA2 of AVP promoter-driven Cre (Avp-Cre) mice. The lateral ventricle (lv), anterior commissure (ac), and third ventricle (3v) are labeled for anatomical reference points. Cell nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI, blue). The scale bar is 500 µm.



Supplemental Figure 2. Retention of social memory in WT mice is limited. a, Wild-type (WT) mice were exposed to the same female during two 5-minute trials (T1 and T2) with a retention interval of 30 min or 2 hr (h). **b,** WT mice decrease investigation of a mouse encountered in trial 1 if trial 2 occurs 30 min (n = 12, $t(11) = 2.24$, $P < 0.05$) later but not 2 hr (n = 13, $t(12) = 1.44$, $P = 0.18$). **c,** The percent change score for investigation was significantly in the 30-min retention paradigm (n = 16, $t(15) = -2.93$, $P < 0.01$) but not the 2-hr retention paradigm (n = 17, $t(16) = -0.27$, $P = 0.79$).


a
PVN::ChR2-Arc expression
500 μm
ChR2+
Arc+
CA2::ChR2-Arc expression
20 μm
ChR2+
Arc+
DAPI
CA2::ChR2-Arc expression
CA1
CA2
CA3
500 μm
ChR2+
Arc+
DAPI
CA1::ChR2-Arc expression
CA1
CA3
DG
500 μm
Arc+
DAPI
b
Arc-ir neurons (per 0.1 mm²)
100
75
50
25
0
*
*
*
PVN
CA2
CA3
CA1
WT
Avp-Cre

Supplemental Figure 3. Optical stimulation increases Arc-immunoreactivity (-ir) density in the PVN and CA2. a, Images of coronal sections showing expression of ChR2 fused to mCherry ($ChR2^+$, red) and Arc (Arc^+, green) staining in neurons in the PVN of Avp-Cre mice (top left). Arc^+ staining is observed throughout the hippocampus. However, $ChR2^+$ fibers, originating from the PVN Avp neurons, are observed in the CA2 (top right) and immediately adjacent CA3 (bottom left) hippocampal areas, but not the dentate gyrus (DG) or CA1 (bottom right). Arrows are used to identify anatomical boundaries for the CA areas. Cell nuclei are stained with DAPI (blue). Scale bars are labeled for each image. **b,** Optical stimulation increased Arc-ir density in the PVN ($t(13) = 3.77$, $P < 0.005$), CA2 ($t(13) = 3.59$, $P < 0.005$), and CA3 ($t(13) = 2.34$, $P < 0.05$) of Avp-Cre mice (blue, n = 7) compared to wild-type (WT) mice (grey, n = 8). Arc-ir density in the CA1 was not affected ($t(13) = 0.08$, $P = 0.93$).



Supplemental Figure 4. Expression of Avp and Oxt receptors in the hippocampus and amygdala. a, These are images of coronal sections showing expression of vasopressin 1b receptor ($Avpr1b^+$, left), vasopressin 1a receptor ($Avpr1a^+$, middle), and oxytocin receptor ($Oxtr^+$, right) mRNA in different subregions of the hippocampus (CA1, CA2, and CA3). Arrows are used to identify anatomical boundaries for the CA areas. Avpr1b expression in the hippocampus is restricted, nearly exclusively, to the CA2 with some expression in the CA3. By comparison, Avpr1a is all but absent in the CA areas, while Oxtr expression is widely seen in both CA2 and CA3. **b,** These are images of coronal sections showing expression of $Avpr1b^+$ (left), $Avpr1a^+$ (middle), and $Oxtr^+$ (right) mRNA in different subregions of the amygdala or AMY (medial amygdala, MeA; central amygdala, CeA). In the MeA and CeA (outlined by

dashed lines), Oxtr is expressed most robustly, Avpr1a is expressed more sparingly, and Avpr1b is absent. The optic tract is labeled for as an anatomical reference point. Cell nuclei are stained with DAPI (blue). The scale bars are 500 μm.