Title

Oxytocin activity in the Paraventricular and Supramammillary Nuclei of the Hypothalamus is Essential for Social Recognition Memory in Rats

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SUMMARY

Oxytocin plays an important role in modulating social recognition memory. However, the direct implication of oxytocin neurons of the paraventricular nucleus of the hypothalamus (PVH) and their downstream hypothalamic targets in regulating the short- and long-term forms of social recognition memory is not fully understood. In this study, we employed a chemogenetic approach to specifically target the activity of PVH oxytocin neurons in rats and found that silencing these neurons impaired both long and short-term social recognition memory. We combined viral mediated fluorescent labeling of oxytocin neurons with immunohistochemical techniques and identified the supramammillary nucleus (SuM) of the hypothalamus, as a novel target of PVH oxytocinergic axonal projections. Furthermore, we used multiplex fluorescence in-situ hybridization and found that oxytocin receptors in the SuM are predominantly in excitatory neurons. Finally, we examined the role of the SuM in social recognition memory, by using a highly selective antagonist to block oxytocin receptors in the SuM. We found that oxytocin activity in the SuM is necessary for the formation of long- and short-term social recognition memory. This study discovered a previously undescribed role for the SuM in regulating social recognition memory via oxytocin signaling and reinforces the specific role of PVH oxytocin neurons in regulating social recognition memory.

INTRODUCTION

Social recognition memory (SRM) is a fundamental component of social behavior, which sub-serves everyday life interactions and is conserved across several species including rodents[1,2]. A key feature of SRM is the ability of a species to acquire, remember and recall identities of conspecifics, which serve to maintain and facilitate social organizational structures among conspecifics[3]. Several studies have identified the neuropeptide oxytocin (OXT) as a major modulator of SRM. For example, mice that lack either the OXT or OXT receptor (OXTR) coding gene display deficits in SRM and rats injected with OXT display enhanced SRM[4-6]. Furthermore, inactivation of OXT signaling by blocking OXTRs in the lateral septum in mice and rats or the hippocampus in mice, impairs SRM[7-9].

OXT is produced by three hypothalamic nuclei, the paraventricular, the supraoptic, and the accessory nuclei of the hypothalamus (PVH, SON, and AN, respectively). All three nuclei project predominantly to the posterior pituitary gland, where it is released into the blood stream to modulate peripheral activities such as milk ejection during breast feeding and uterus contraction during parturition[10]. PVH-OXT neurons project to a wide range of cortical and limbic structures including the medial amygdala, lateral septum, and nucleus accumbens, all of which are characterized by a high level of OXTR receptor expression[11] and are part of what is known as the “SRM circuit”[12-15]. Furthermore, efferent projections from the medial septum to the hippocampus[16], as well as afferent projections from the hippocampus to the medial prefrontal cortex (mPFC), have been shown to regulate SRM in mice[17]. Although, the role of OXT in social recognition memory is widely acknowledged[4-6], no studies have directly investigated the contribution of individual OXT synthesizing nuclei in regulating SRM. Similarly, OXT activity within extra hypothalamic structures such as the lateral septum and medial amygdala have been shown to modulate SRM[13,14,18] however it is unclear if additional brain regions are involved in regulating this form of memory.

A recent study in mice demonstrated that the supramammillary nucleus (SuM), which is a caudal hypothalamic nucleus that is positioned superior to the mammillary body, is also involved in processing social novelty information[19]. The authors showed that the SuM relays contextual and socially salient information to the hippocampus through anatomically segregated populations of projection neurons; SuM to hippocampal CA2 (SuM->CA2) projecting neurons relay socially salient information, whereas SuM to dentate gyrus (SuM->DG) projecting neurons carry context specific information. The same study also demonstrated that these two populations of SuM neurons receive significantly distinct inputs, with the SuM->DG projecting neurons receiving inputs from the ventral tegmental area and the nucleus accumbens shell whereas the SuM->CA2 projecting neurons receive inputs from the PVH. The identity of these inputs, however, has not yet been determined. One of the major functions of the SuM is to control the frequency of theta activity in the hippocampus[20]. Theta activity emerge from synchronous firing of hippocampal neurons and play an important part in regulating learning and memory[21].The SuM is also known to fire synchronously with theta rhythms generated in the hippocampus, which in turn are necessary for processing mnemonic information[22]. In the present study, we hypothesized that the PVH-OXT neurons project to the SuM and that OXT modulation of SuM neurons is necessary for SRM.

To address this hypothesis, we first sought to determine if the activity of OXT neurons in the PVH is specifically necessary for SRM, given that PVH-OXT neurons constitute a major source of OXT axonal projections to several forebrain regions[23]. Next, we sought to identify if the SuM receives specific inputs from OXT neurons of the PVH, and lastly to examine if OXT signaling within the SuM is crucial for SRM.

METHOD DETAILS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Male Sprague Dawley (SD) rats (Charles River, Wilmington, MA, USA) were used as test subjects for all experiments. Wistar and Wistar Hannover rats (Charles River, Wilmington, MA, USA) were used as stimuli strain for the social recognition memory experiments. All stereotaxic viral injections and cannula implantations were performed at the age of 8 weeks. Animals were housed in groups of 2 under a 12h light/dark cycle at 22 ± 2°C with food and water available *ad libitum.* All animal procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

Experimental Design

To take advantage of the designer receptors activated by designer drugs (DREADD) system, we used a cross-over design wherein the same rat received either 0.9% saline or clozapine N Oxide (CNO)/OXTR antagonist across the testing paradigm (**Fig. 1a**). The behavioral task is detailed here: Half of the experimental rats in the cohort received saline and the remaining received CNO/OXTR antagonist and were tested on the short-term social discrimination task to assess short-term SRM. A week later, the rats that previously received saline now received CNO/OXTR antagonist and vice versa and were tested on the short-term social discrimination task to assess short-term SRM. A week after assessing short-term SRM, the same experimental design was repeated but this time, the rats were tested on the long-term social discrimination task to assess long-term SRM. A week later the rats that previously received saline now received CNO/OXTR antagonist and vice versa and were tested on the long-term social discrimination task to assess long-term SRM. For the DREADD experiments, behaviors were performed across 3 cohorts (n=8, n=8, n=6) and for oxytocin receptor (OXTR) antagonist experiments behaviors were performed across 2 cohorts (n=8 each). In all experiments, the order of the discrimination tests (i.e. short-term and long-term social recognition memory), was randomized between cohorts. Since the same animals were being used as within subject controls (saline vs CNO or saline vs OXTR antagonist), a priori condition was used wherein animals that failed to exhibit short or long-term

SRM on saline treatment were not considered for further analysis. This was determined based on a threshold of 0.05 on the ratio of duration of investigation (RDI) index calculated as (Investigation Time^{Novel} - Investigation Time^{Familiar}) / (Investigation Time^{Novel} + Investigation Time^{Familiar}).

Viral Vectors

For specific silencing of OXT neurons, we used a previously validated AAV1/2-OXTp-hM4DGi-mCherry virus, which has been shown to reduce mean frequency and input resistance of OXT neurons[24]. A control virus (AAV1/2-OXTp-mcherry) that lacks the DREADD backbone was used to account for the non-specific effects of CNO. To identify OXT neuron projection fibers from the PVH or SON, we used an anterograde virus driven by an OXT promoter (AAV1/2-OXTp-Venus)[23]. All OXTp viruses were produced and validated by Dr. Valery Grinevich's laboratory at the Central Institute of Mental Health, University of Heidelberg, Germany.

Stereotaxic Surgery

Animals were anesthetized with 4% isoflurane for induction of anesthesia and maintained at 2% isoflurane and 2% oxygen using a tabletop vaporizer. The surgical area was shaved and aseptically cleaned. An incision was made along the dorsal midline of the skull, bregma and lambda were identified, the region of injection was marked, and a small burr hole (50um) was drilled. The virus (AAV1/2-OXTp-hM4DGi-mCherry or AAV1/2-OXTp-Venus or AAV1/2-OXTp-mCherry) was loaded into a 20µl NanoFil syringe fitted with a 33gauge needle (cat no. NF33BL, World Precision Instruments Inc, Sarasota, FL, USA). A final volume of 270nl was injected into the PVH (A-P -1.7mm, M-L ±0.3mm, D-V 8.0mm) at a 10° angle. Following injection, the syringe was left in place for 10 min and withdrawn at a rate of 0.2 mm/min. Incision wound was closed using wound clips (EZ Clips, Stoelting Inc, Wood Dale, IL, USA). Rats received intraoperative subcutaneous fluids for hydration (Lactated Ringer Solution, Thermo Fisher Scientific, Waltham, MA, USA) and buprenorphine (0.05mg/kg) for analgesia. Additional analgesia (buprenorphine,

0.05mg/kg) was administered subcutaneously every 12h for 72h post-operatively. For cannulation, a guide cannula (7mm, P1 Technologies Inc, Roanoke, VA, USA) was implanted at a 15° angle (A-P -4.65mm, M-L – 0mm). Two additional bone screws (Stoelting Inc, Wood Dale, IL, USA) were implanted on the skull surface for anchoring, and the guide cannula was secured using dental cement (Stoelting Inc, Wood Dale, IL, USA). A dummy cannula (7mm) was placed inside the guide cannula and was left in place until the day of experiment when OXTR antagonist/saline was delivered using an infusion cannula (9mm). Animals were allowed to recover for 3 days before experimentation.

Drugs

For DREADD experiments, clozapine N oxide was dissolved in 0.05% DMSO and 0.9% saline and injected intraperitoneally (i.p) using a 1ml BD Luer-Lok syringe (cat. no. 309328, BD Biosciences, Mississauga, Ontario, CA). For OXTR antagonist experiments, a stock (1mg/ml) of OXTR antagonist (desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT was prepared by diluting the drug in 0.9% saline. A working stock of 0.25ug/ul was prepared on the day of the experiment and was injected using a syringe pump (Amuza Inc, San Diego, CA, USA). A 5ul Hamilton syringe (Hamilton Company, Reno, NV, USA) was connected to a plastic tubing on one end and an infusion cannula on the other. Saline/OXTR antagonist (0.25ug/ul) was loaded into the infusion cannula and a volume of 0.3ul (75ng total) was injected at a rate of 0.1ul/min. The infusion cannula was left in place for 1min before being withdrawn.

Short and long-term discrimination task

Short and Long-term SRM were assessed using previously published paradigms, known as social discrimination tasks[25]. Briefly, the paradigms involve an initial encounter with a social stimuli followed by a short (30min) or long (24hour) inter-trial interval after which the test animal is simultaneously exposed to the same stimulus (“Familiar”) as before and a novel stimuli (“Novel”).

Social recognition is considered to have occurred when the test rat shows greater preference for the novel stimuli over the previously encountered stimuli[26]. For all behaviors, test and stimulus rats were habituated to handling and to the testing arena for 4 days before testing. To assess short-term SRM, test rats were placed in the testing arena (50x50x40cm) at the start of the experiment. 30min later, they received an i.p injection of either saline or CNO (8mg/kg) and 30min following the injection, a juvenile rat of a different strain (3-5 week old, Wistar or Wistar Hannover strain) was placed in an enclosure and introduced into the testing arena for the test rat to investigate for 5min (1st encounter). Following an inter-trial interval of 30min during which time the test rat remained in the testing arena, the juvenile rat from the 1st encounter ("Familiar") and a new juvenile rat ("Novel") were placed in two small enclosures and introduced in two opposing corners of the testing arena for the test animal to investigate for 10min ("2nd encounter"). The strain of the juvenile rats used for the first encounter were randomized such that each test rat interacted with a different strain across treatment sessions. Additionally, the position of the juvenile rat within the testing arena during the first encounter was always different than the one during the second encounter. To assess long-term-SRM, test rats received an i.p injection of either saline or CNO (8mg/kg) and were placed in the testing arena. 15min later, a juvenile rat of a different strain (3-5 week old, Wistar or Wistar Hannover strain) was placed in the testing arena for the test rat to freely interact and investigate for 1h (1st encounter). After a 24h inter trial interval, the test rat was placed in the testing arena for 1h for habituation followed by introduction of the "Familiar" and a "Novel" rat that were placed in two small enclosures and introduced in two opposing corners of the testing arena for the test animal to investigate for 10min (2nd encounter).

Novel Object Recognition Memory

Novel object recognition task was performed based on a previously established protocol[27]. Test rats were injected with 0.9% saline or CNO (8mg/kg). 15 min later, test rats were allowed to interact for 3 min with two identical objects (lego or cone), which were placed on one side of the

arena ("1st encounter"). After an inter-trial interval of 30min during which the test rat remained in the testing arena, test rats were introduced to one of the objects from the first encounter ("Familiar") and a novel object ("Novel") and was allowed to interact with both for 3min. The choice of objects was randomized across treatment groups.

Behavioral Analysis

All behaviors were scored and quantified using TrackRodent, an open source Matlab based automated tracking system that uses a body-based algorithm [28,29]. The traces and heat-maps were also obtained using the same system. The source code can be accessed on GitHub (https://github.com/shainetser/TrackRodent).

Histology

Rats were anesthetized with an i.p injection of Ketamine (100mg/kg) and Xylazine (13mg/kg). Once a surgical plane of anesthesia was achieved, rats were peristaltically perfused at a rate of 30ml/min with 0.2M Sodium phosphate buffer for 6 min followed by 4% paraformaldehyde (PFA) for at 40ml/min for 6 min. Brains were removed, immersed in 4% PFA overnight at 4°C, then placed in a 30% sucrose in 1xPBS for 48h. Brains were flash frozen in a slurry of dry ice and isopentane and sectioned on a cryostat (Leica CM 1860 Leica Biosystems, Buffalo Grove, IL, USA).

Immunohistochemistry

To visualize overlap between the DREADD virus and OXT neurons a total of 12 sections, spanning the entire PVH were used. Briefly, sections were washed (3x10 min each in 1xPBS, 0.05% Triton X-100), blocked and permeabilized for 1h in 5% donkey serum in 1XPBS-0.5% triton-X-100 for 1h at room temperature (RT). They were then co-stained with anti-OXT (1:1000) and anti-DsRed (1:1000) antibodies for 24h at 4°C in 1xPBS-0.5% triton-X-100. Sections were then washed and incubated in donkey anti-mouse IgG 488 (1:1000) and donkey anti-rabbit IgG

594 (1:1000) in 0.5% Triton X-100 in PBS for 2h at RT. Sections were washed and mounted with antifade mounting Medium with DAPI.

For immunofluorescence experiments, PVH and SuM sections from an 8 week male SD rat were blocked in 5% donkey serum and co-stained with OXT (1:1000) and calretinin (1:2000) or parvalbumin (1:2000) for 24h at 4°C. Sections were then washed and incubated in donkey anti-mouse IgG 488 (1:1000) and donkey anti-rabbit 594 (1:1000) or donkey-anti-goat 594 in for 2h at RT. Similarly, PVH and SuM sections from an OXTp-Venus injected rat were co-stained with anti-GFP (1:1000) and anti-calretinin (1:2000) or anti-parvalbumin (1:2000) antibodies and incubated for 24h at 4°C. This was followed by incubation in donkey anti-chicken IgG 488 and donkey anti-rabbit 594 or donkey anti-goat IgG 594 for 2h at RT.

To visualize OXT fibers using enzymatic staining, brain sections (40um) representing the PVH, SON or SuM from an 8 week male SD rat were used. Sections were treated with 3% hydrogen peroxide (H_2O_2) followed by 1h incubation in 5% goat serum. Next, sections were incubated with anti-OXT antibody (1:1000) for 40h at 4°C, washed and incubated with goat anti mouse HRP 2h at RT, and developed using an ImmPACT diaminobenzidine (DAB) peroxidase substrate. Alternate SuM sections from the same animal were used for staining tissue with 1% cresyl violet. Similarly, to visualize OXTp-Venus fibers, OXTp-Venus was injected into the PVH or SON.. PVH, SON or SuM sections were treated with 3% H_2O_2 to block endogenous peroxidase activity and incubated in 5% goat serum followed by incubation with anti-GFP antibody for 40h at 4°C. Sections were washed and incubated in the goat anti chicken HRP for 2h at RT and developed using DAB.

RNAscope

Rat *Oxtr*[30], *vglut2 (slc17a6)*[31] and *vgat1 (slc32a1)*[31] probes were purchased from ACDBio. Fresh brains were collected by cervical decapitation and flash frozen in a slurry of isopentane and dry

ice. Tissue was immediately sectioned at 15um, mounted on glass slides (SuperFrost Plus Microscope Slides, Fisher Scientific) and frozen at -80°C until the day of experiment. RNAscope was performed using the following the manufacturer's protocol (RNAscope Multiplex Fluorescent Reagent Kit, ACDBio, Newark, CA). Briefly, tissue sections were thawed at RT for 10min, fixed with 4%PFA for 15min at 4°C, and then dehydrated with ethanol at RT. Sections were then incubated in H_2O_2 for 10min and a mix of *Oxtr, vglut2 and vgat1* probes (50:1:1) was added to the sections and left to incubate for 2h in a 40°C oven (HybEZ II Hybridization System, ACDBio, Newark, CA). This was followed by an amplification step that involves amplification probes (Amp1, Amp2, and Amp3), provided with the kit, and then an incubation step with opal dyes (Akoya Biosciences, Marlborough, MA) 520, 570, and 690 to visualize the RNA transcripts.

Microscopy and Image Analysis

PVH sections (10-12) from OXT-hM4DGi-mcherry injected rats were imaged on a confocal microscope (Leica SP5 DMI, Leica Micro-Systems, Buffalo Grove, IL, USA)) at the Microscopy CoRE at the Icahn School of Medicine at Mount Sinai. Sections were imaged at 20x and Z stacks were acquired at step size of 1.0um and stacked images were exported to FIJI (ImageJ) and single plane images were generated using Z project (maximum intensity projection)[32]. mCherry overlap with OXT neurons was manually counted from 3 animals (12 PVH sections each) and presented as % $mCherry^+/OXT^+$ (**Fig. 1c**). Fluorescent in situ hybridization (RNAscope) images were acquired on a Zeiss AxioImager Z2M with ApoTome.2 at 10x, 40x and 63x magnification. Images were imported into FIJI and a grid drawn over the acquired image. Individual neurons were counted grid by grid using the cell counter plugin on FIJI (1 section per animal, 3 animals). DAB stained sections were acquired using a bright field microscope (EVOS, Thermo Fisher Scientific, Waltham, MA).

Statistical analysis

Statistical analysis was performed using GraphPad prism 9.0 software (GraphPad Prism, San Diego, CA). Total investigation time between Familiar and Novel social stimuli were evaluated using a two-way repeated measures Analysis of Variance (RM-ANOVA) to compare main effects of treatment (saline vs. CNO or saline vs. OXTR antagonist) and social preference (Familiar vs. Novel). Sidak's multiple comparison test was then used for post-hoc testing.

RESULTS

Chemogenetic silencing of PVH-OXT neurons impairs short-term SRM

In order to test the direct role of PVH-OXT neurons in SRM, we utilized inhibitory DREADDs that are specifically designed to express in OXT neurons (AAV1/2-OXTp-hM4DGi-mCherry) and examined the impact of PVH-OXT neural inhibition in SD rats on the short- and long-term social discrimination tasks, which assess short- and long-term social SRM, respectively (Experimental design, **Fig. 1a**). We first confirmed and validated previous findings, demonstrating the specific expression of the AAV1/2-OXTp-hM4DGi-mCherry virus in PVH-OXT neurons[24] by finding a 70% overlap between mCherry, expression and OXT neurons (**Fig. 1b-c**).

Next, we tested the impact of chemogenetic silencing of PVH-OXT neurons on short-term SRM (**Fig. 2a**). We found that rats that were injected with the inhibitory DREADDs showed a significant preference for the novel over the familiar social stimuli on the short-term discrimination task after saline (control) injection. However, the same rats failed to show a similar preference following CNO injection (**Fig. 2b & 2c**).

The temporal dynamics of social novelty preference are significantly influenced by the quality of social interaction, as measured by the duration of each interaction (bout) during the task. SD rats engage in different lengths of bouts during the social recognition task and show specific temporal dynamics that are distinct from other outbred rats and mice[33]. Additionally, bouts that are shorter than 6 secs typically produce no clear separation of preference for the novel vs. familiar social

stimuli, while bouts that are longer than 6 sec reflect more meaningful interactions in both mice[28] and rats[33]. Therefore, we further analyzed the data based on bout lengths. As expected, we found that during short bouts (≤6sec) rats did not show preference to the novel stimuli, regardless of the treatment (saline or CNO) (**Fig. 2d**). During long bouts (≥6sec), however, there was a significant preference for the novel over the familiar social stimuli following saline but not CNO injection (**Fig. 2e**). In order to rule out a non-specific effect of CNO on short-term SRM, and therefore, confirm that the decrease in social preference is due to an effect of the inhibitory DREADD, we injected an independent group of rats with a control virus that has the same backbone as the inhibitory DREADD virus, but lacks the hM4DGi receptor (AAV1/2-OXTp-mCherry). We first confirmed its overlap with OXT neurons (**Supplemental data** Fig.1a). Next we followed the same experimental design as described above and found that following saline or CNO injection rats showed a significant preference for the novel over the familiar social stimuli (**Fig. 1b**). These findings rule out a non-specific effect of CNO on SRM. Next, in order to examine if the effects of PVH-OXT neural inhibition on social preference is consistent across the length of the social discrimination task, we also examined social preference as a function of time. We found that following saline injection, rats maintained their preference for the novel stimuli across time whereas following CNO injection, they showed no clear preference for either stimuli at any of the time points (**Supplemental data, Fig.1a-b**). In order to examine if CNO has an impact on the investigation time during the 1st encounter (time when the test rat interacts with the social stimuli for the first time) (**Fig. 2a**) we compared the total investigation time following CNO and saline injection and found no difference between the two treatment conditions (**Supplemental data, Fig. 1c**).

Finally, in order to confirm that the effect of PVH-OXT neuronal inhibition is specific for SRM and not to other aspects of non-social memory, we assessed a separate cohort of rats for their object recognition memory, using the novel object recognition memory task (**Supplemental data, Fig. 1d**). We found that OXTp-hM4DGi injected rats showed a clear preference for the novel

over the familiar object, following saline or CNO injection (**Supplemental data, Fig. 1e**). Furthermore, there was no difference in the total investigation time following saline or CNO injection during the 1st encounter (time during which the test rats are exposed to the object for the 1st time) (**Supplemental data, Fig. 1f**) and we also ruled out the possibility that test rats could have an innate preference for one object over the other, as we found they spent similar times investigating the cone or the lego (1st encounter) (**Supplemental data, Fig. 1g**). Taken together, these results demonstrate that the activity of PVH-OXT neurons is necessary for short-term social recognition memory.

Chemogenetic silencing of PVH-OXT neurons impairs long-term SRM

To determine if OXT neurons in the PVH are also necessary for long-term SRM, we tested the same cohort of rats which were tested on the short-term social discrimination task on the long-term social discrimination task (**Fig. 3a**). We found that following saline injection, inhibitory DREADDs-injected rats showed significant preference for the novel over the familiar social stimuli. However, the same rats failed to show such a preference following CNO injection (**Fig. 3b & 3c**). By classifying the stimuli interaction time into short (≤6sec) and long (≥6sec) bouts, we found that rats do not display any social preference when analyzing the short bout interactions, regardless of treatment (saline or CNO) (**Fig. 3d**). However, preference to the novel stimuli over the familiar social stimuli was clearly observed when analyzing long bouts following saline, but not CNO injection (**Fig. 3e**). When we analyzed the social preference data across time, we found that preference to the novel stimuli was sustained throughout the duration of the testing period following saline but no preference was observed following CNO injection (**Supplemental data, Fig. 2a & 2b**). Importantly, no significant differences between treatments was observed in the investigation time during the first 10 minutes of the 1st encounter (when the test rats are exposed to one of the social stimuli for the 1st time) (**Supplemental data, Fig. 2c**). Altogether, these

findings demonstrated that PVH-OXT neurons also play a critical role in mediating long-term social recognition memory.

Supramammillary nucleus is a target for OXT innervation that originates in the PVH but not SON

The SuM is a caudal hypothalamic nuclei that is juxtaposed immediately over the mammillary bodies[20]. Acute inactivation for the supramammillary area has been shown to impair spatial memory and is involved in regulating in spatial navigation[34]. However, it was unclear until recently, if the SuM also responds to socially salient stimuli and its subsequent role in social memory[19]. Having identified that the PVH-OXT neurons to be necessary for social recognition memory (**Fig. 2b & c and Fig. 3b & c**), we sought to determine if PVH-OXT neurons project to the SuM. For this purpose, we used immunohistochemistry with anti-OXT antibodies, to visualize OXT fibers in the SuM and found that they are broadly distributed across the rostro-caudal parts of the SuM with fibers identified in both, the medial (SuMm) and lateral part of the SuM (SuMl) (**Fig. 4a**). In order to determine the origin of these OXT fibers, we unilaterally injected, into the PVH or SON, an anterograde virus, which expresses specifically in OXT neurons (AAV1/2-OXTp-Venus) and, therefore, allows for the specific labelling of OXT neurons and their projections[23]. We found that the PVH is a major source for OXT fibers in both the SuMm and SuMl (**Fig. 4b**) and were able to visualize axonal varicosities, using a higher microscopic magnification (**Fig. 4a & 4b;** Right insets). We also found that the SON does not send any OXT projections to the SuMm or the SuMl, as no OXT fibers were detected in either region when the virus was injected into the SON (**Fig. 4c**). We also confirmed the presence of OXT fibers by co-labeling SuM sections with anti-OXT and anti-calretinin or anti-parvalbumin antibodies (**Fig. 4d**). Calretinin and parvalbumin are used as markers help distinguish the mammillary body, which is caudal to the SuM and is parvalbumin positive but calretinin negative, from the supramammillary nucleus, which is calretinin positive but parvalbumin negative[20]. Additionally, we co-labeled SuM sections from rats injected with AAV1/2-

OXTp-Venus with anti-calretinin or anti-parvalbumin antibodies to confirm the presence of the PVH-OXT fibers within the SuM (**Fig. 4e**).These experiments demonstrate that PVH-OXT neurons are the major source for OXT projection fibers in the SuM.

OXT receptors are expressed by specific population of SuM neurons

We then aimed to reveal if SuM neurons express OXT receptors. SuM neurons are predominantly glutamatergic with very few GABAergic neurons[35]. It is also one of few regions in the rat brain where neurons co-express both glutamate and GABA[31]. Additionally, SuM neurons that project to the hippocampal CA2 region and modulate social memory are predominantly glutamatergic[19]. Here, we used RNAscope, an *in situ* RNA hybridization (ISHr) technology, to identify OXTR transcripts and examine their overlap with GABAergic and glutamatergic neural populations. We used vesicular GABA transporter (*vgat1)* probe as a marker for inhibitory neurons, and the vesicular glutamate transporter (*vglut2)*, as a marker for excitatory neurons, respectively.

First, we examined the proportion of SuM neurons that are GABAergic, glutamatergic or both and then then we determined if OXTR differentially segregate across these neural populations. We found that neurons within the SuM are primarily positive for *vlgut2* (83±0.7%), with a small fraction being positive for both, *vglut2* and *vgat1* (10.7±0.7%)*,* and an even smaller fraction of neurons positive only for *vgat1* (6±0.8%) (**Fig. 5a-c**). In order to determine how *Oxtr* expression segregates into these populations, we quantified its expression across the three populations. We found that nearly 60% of *vglut2*$^{+}$ neurons are also *Oxtr*$^{+}$, whereas 48% of *vglut2*$^{+}$:*vgat1*$^{+}$ neurons are *Oxtr*$^{+}$, and only 13% of *vgat1*$^{+}$ are *Oxtr*$^{+}$ (**Fig. 5c**). These results indicate that not only are OXTRs expressed in the SuM but they also distribute predominantly within glutamatergic neurons and neurons that co-express glutamate and GABA.

Blocking OXTR in the SuM affects both short and long social recognition memory

To follow up on our findings, which demonstrate that the SuM is heavily innervated by PVH-OXT fibers and expresses OXTR, we asked if OXT downstream signaling within the SuM is necessary for SRM. To address this question, we implanted a cannula within the SuM and infused a selective OXTR antagonist (**Supplemental data**, **Fig. 3a**) 10 min before testing the rats on the short or long-term social discrimination tasks, while following the same cross over design(**Fig. 6a & 6b**)., as described previously for the DREADD experiments We found that following saline infusion, rats showed a clear preference for the novel over the familiar social stimuli, whereas infusion of OXTR antagonist led to a decrease in preference (Fam vs Nov) with a trend toward significance (**Fig. 6d),** which was sustained across time (**Supplemental data**, **Fig. 3b & 3c**). As before, when the short bouts were assessed, neither saline nor OXTR antagonist produced any preference for the novel stimuli (**Fig. 6e**). When we focused our analysis on the long interaction bouts, we found that rats showed a significant preference for the novel over the familiar stimuli, and that OXTR infusion led to a decrease in preference that was statistically significant (**Fig. 6e**). In order to examine if OXTR blockade impacted the investigation time during the 1st encounter (when the test rats are exposed to one of the social stimuli for the 1st time) we compared the investigation time between saline or OXTR antagonist-infused rats and found no significant differences between the two treatment conditions (**Supplemental data**, **Fig. 3d**). Overall, these results suggest that OXT signaling in the SuM is necessary for modulating short-term SRM.

Similarly, when we examined the impact of the OXTR antagonist on long-term SRM (**Fig. 7a**), using the same cohort of rats, we found that blocking OXTR in the SuM produced a robust impairment in long-term SRM, wherein saline infused rats showed a clear preference for the novel over the familiar stimuli. However, OXTR antagonist infusion resulted in no clear preference for either stimuli (**Fig. 7b & 7c**), which was sustained across time (**Supplemental data Fig. 3e & 3f**). As expected, short interaction bouts showed no significant differences in the preference for the novel over the familiar social stimuli, regardless of whether rats were infused with saline or OXTR

antagonist.(**Fig. 7d**). When long interaction bouts were assessed, saline infused rats showed a clear preference for the novel over the familiar stimuli, whereas OXTR antagonist infused rats failed to show a preference for either stimuli (**Fig. 8e**). There was also no significant difference between the saline and OXTR infused groups when the investigation time during the 1st encounter (**Supplemental data, Fig. 3g**). Together, these findings demonstrate that OXTR signaling within the SuM is required for both short- and long- term SRM.

DISCUSSION

Social recognition memory is a key component of social behavior that is essential for distinguishing between familiar and novel conspecifics[36,37] and is regulated by a defined brain circuit[2,38], referred to as the "SRM circuit". This circuit engages several neural substrates including the lateral and medial septum, pre frontal cortex, medial amygdala and hippocampus[7,13,16,17]. Information processing within neural circuits is not hard-wired but rather adaptive to the surrounding environment, in part due to the activity of neuromodulators such as OXT[39-41]. OXT has been repeatedly shown to modify neural and synaptic activity in various brain regions of the SRM circuit[42-49]. Furthermore, deletion of the OXT or OXTR gene in mice [5,6] cause impairment in SRM, which in OXT-KO mice can be rescued by OXT infusion[14].

In this study, we demonstrated that activity of PVH-OXT neurons in rats is critical for mediating both the short- and long-term forms of SRM, which directly implicate, PVH-OXT neurons in SRM. Importantly, we found that the effect of acute silencing of PVH-OXT neural activity, as well as OXTRs blockade in the SuM was specific to the domain of SRM as the rats' overall social interaction following these manipulations remained unaffected. This is consistent with previous studies in OXT-KO mice, as WT and KO littermates spent similar amount of time investigating a conspecific mouse when presented for the first time. However, OXT KO mice showed specific deficits in SRM as reflected by the constant level of investigation (habituation) exhibited toward a repeatedly presented social stimulus as measured on the habitation-dishabituation task.[6]

Similarly, animals that lack OXTR also did not show deficits in social interaction, yet they failed to discriminate a novel from a familiar conspecific, when tested on the social discrimination task[5]. These findings suggest that during the social discrimination task in males, OXT may not be essential for the act of social interaction per se, but rather necessary for the formation of the social recognition memory. Moreover, although we had identified a role of PVH-OXT neurons in short and long-term forms of social memory, we did not discriminate its role between the acquisition, maintenance and consolidation processes of social memory. To be able to discriminate between the different phases of memory, future studies should be aimed at using tools such as optogenetics, which offer a much greater temporal control**.** Our findings that inhibition of PVH-OXT neurons leads to an impairment in both the short and long-term SRM also align with the previously established role of OXT in these two forms of memory[2,4,6,7,9,14,25] yet, it attributes, for the first time, a specific role for PVH-OXT, suggesting that PVH-OXT neurons are likely to be a common substrate for both. The direct implication of PVH-OXT neurons in modulating social memory is of translational significance as several studies in rodent models with mutations in high-risk genes for autism spectrum disorder (ASD), have shown changes in the overall number of PVH-OXT neurons and/or reduced OXT levels, thus suggesting that modified OXT activity could underlie some of the social behavioral phenotype reported in these models[50,51]. Our own work in a rat model that harbors a mutation in a high-risk gene for ASD, *Shank3*, identified long- but not short-term SRM deficits that could be ameliorated with exogenous administration of OXT[25]. These results raise the possibility that *Shank3* mutation has an impact on the OXT system.

SuM activity is important for synchronizing the frequency of theta activity in the hippocampus, modulating spike-time coordination during spatial navigation and also in regulation of spatial memory [20,52,53]. However, it was unclear until recently, if the SuM also responds to socially salient stimuli and its subsequent role in social memory. A recent study on the role of the SuM in modulating hippocampal memory had shown that specific neuronal sub-populations within the

SuM are engaged in processing social novelty[19]. They also demonstrated that SuM->hippocampal CA2 projections are primarily glutamatergic and that their activation regulates the excitation vs. inhibition (E/I) ratio within the hippocampal CA2, which in turn may play a role in tuning the response to novel social stimuli. Importantly, using projection-specific tracing techniques, the same study showed that SuM-> hippocampal CA2 projecting neurons receive neural inputs from the PVH[19].

Our findings demonstrate, for the first time, that PVH-OXT neurons project to the SuM, which expresses OXTRs predominantly in glutamatergic neurons, and OXT signaling in the SuM is necessary for short and long-term SRM. Altogether these results suggest that SuM is another important element in the neural circuitry underlying SRM. At the same time, our findings also raise several unaddressed questions. For instance, although we showed that OXTRs are expressed by glutamatergic neurons in the SuM, it is unclear whether the same OXTR positive neurons also project to the hippocampal CA2 region. It is also not clear how OXTR signaling affects the neural responses of SuM glutamatergic neurons and subsequently their hippocampal CA2 target neurons or if enhancing OXTR signaling within the SuM is sufficient to promote SRM? These questions can be addressed by combining retrograde viral tracing with *in situ* fluorescent hybridization techniques or with the recently generated OTR-IRES-Cre rats[54] to determine the identity and projection sites of OXTR expressing SuM-CA2 projection neurons.

Taken together, these findings led us to propose a working model where PVH, SuM and the hippocampal CA2 work together to modulate SRM. Specifically, we propose that the PVH-OXT->SuM pathway acts to amplify the salience of the social stimulus via OXTR signaling within the SuM, while the SuM-hippocampal CA2 pathway routes the social information to the CA2 to facilitate social memory. To summarize, our findings uncover a new node within the SRM circuit-the SuM, a novel role for OXT signaling within this node and provide the basis for future studies to investigate the role of the PVH-SuM-hippocampal CA2 pathway in SRM.

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AUTHOR CONTRIBUTIONS

K.T.R and H.H.N conceptualized and designed the experiments. K.T.R and M.B performed the experiments and analysis. K.N and N.C performed the analysis. A.F cloned and packaged the viruses. S.N performed the analysis. K.T.R and H.H.N wrote the manuscript and H.H.N, S.W and V.G provided additional comments.

DECLARATION OF INTERESTS

The authors report no completing interests.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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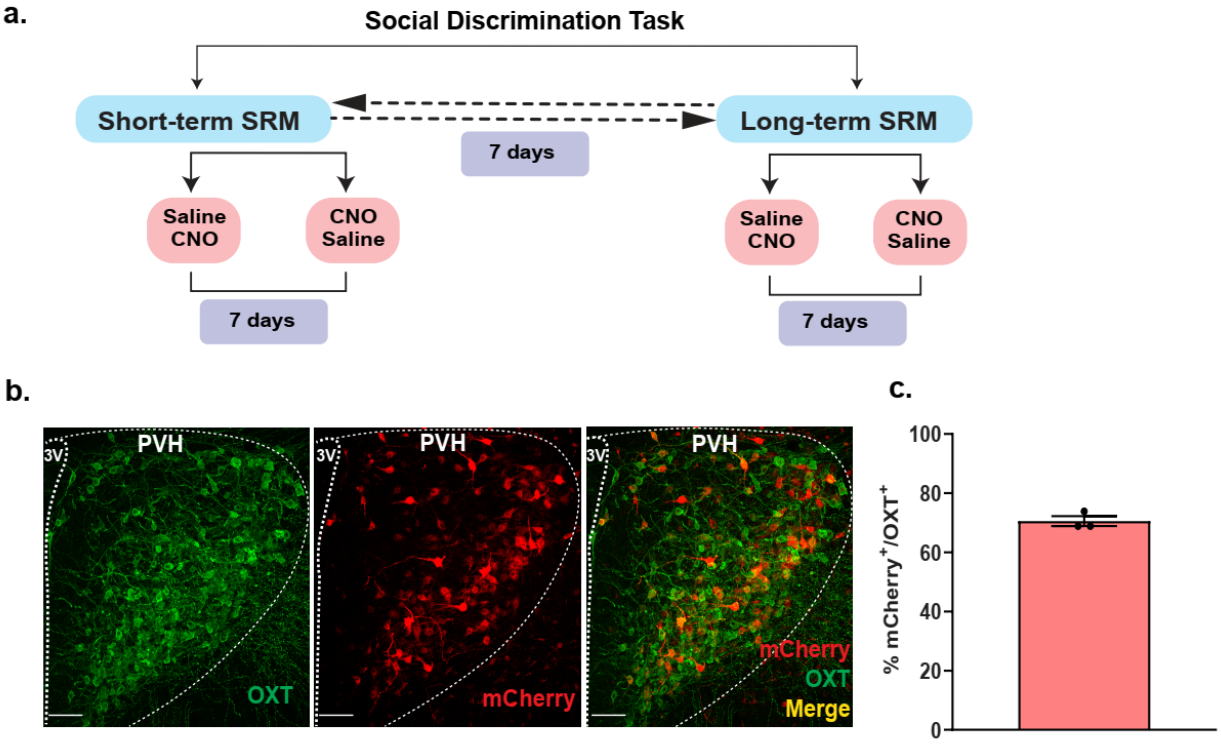
STATISTICAL ANALYSIS

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Primary Antibodies		
Mouse monoclonal anti-Oxytocin	A gift from Dr. Harold Gainer	PS 38
Rabbit polyclonal DsRed	Takara Bio	632496
Chicken polyclonal anti-GFP	Thermofisher Scientific	A10262
Goat polyclonal anti -Parvalbumin	Swant	PVG213
Rabbit polyclonal anti-Calretinin	Swant	CR7697
Serum		
Donkey serum	Jackson Immunoresearch	017-000-121
Goat serum	Jackson Immunoresearch	005-000-121
Secondary Antibodies		
Donkey anti-Mouse IgG Alexa Fluor 488	Thermofisher scientific	A21202

Donkey anti-Rabbit IgG Alexa Fluor 594	Thermofisher scientific	A21207
Donkey anti-Goat IgG Alexa Fluor 594	Thermofisher scientific	A11058
Donkey anti-Chicken IgG Alexa Fluor 488	Jackson Immunoresearch	703-545-155
Goat anti-Mouse HRP	Jackson Immunoresearch	115-035-003
Goat anti-Chicken HRP	Jackson Immunoresearch	103-035-155
Reagents		
Vectashield anti fade mounting medium with DAPI	Vector Laboratories	H-1200
Diaminobenzidine substrate peroxidase kit	Vector Laboratories	SK-4105
Bacterial and Virus Strains		
AAV1/2-OXTp-mCherry	Published work	N/A
AAV1/2-OXTp-hM4DGi-mCherry	Published work	N/A
AAV1/2-OXTp-Venus	Published work	N/A
Chemicals		
Clozapine N-Oxide	Cayman chemicals	16882
OXT receptor antagonist (desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT	A gift from by Dr. Mario Manning	N/A
Rodent strains		
Rattus Norvegicus (Sprague dawley strain)	Charles River Laboratories	N/A
Rattus Norvegicus (Wistar strain)	Charles River Laboratories	N/A

Rattus Norvegicus (Wistar hannover strain)	Charles River Laboratories	N/A
Fluorescent in-situ hybridization (RNAscope)		
RNAscope Multiplex Fluorescent Reagent Kit	ACDBio	323137
Rat *Oxtr* probe	ACDBio	483671
Rat *vglut2* (Slc17a6) probe	ACDBio	317011
Rat *vgat1* (Slc32a1) probe	ACDBio	424541
Opal 520	Akoya Biosciences	FP1487001KT
Opal 570	Akoya Biosciences	FP1488001KT
Opal 690	Akoya Biosciences	FP1497001KT
Software and algorithms		
GraphPad Prism 9.0	GraphPad	https://www.graphpad.com
Fiji	ImageJ	https://imagej.net/software/fiji/
Adobe Illustrator	Adobe Inc	https://www.adobe.com/products/illustrator.html
Matlab	Mathworks Inc	https://www.mathworks.com/products/matlab.html
TrackRodent	Custom code	https://github.com/shainetser/TrackRodent

Figure 1.



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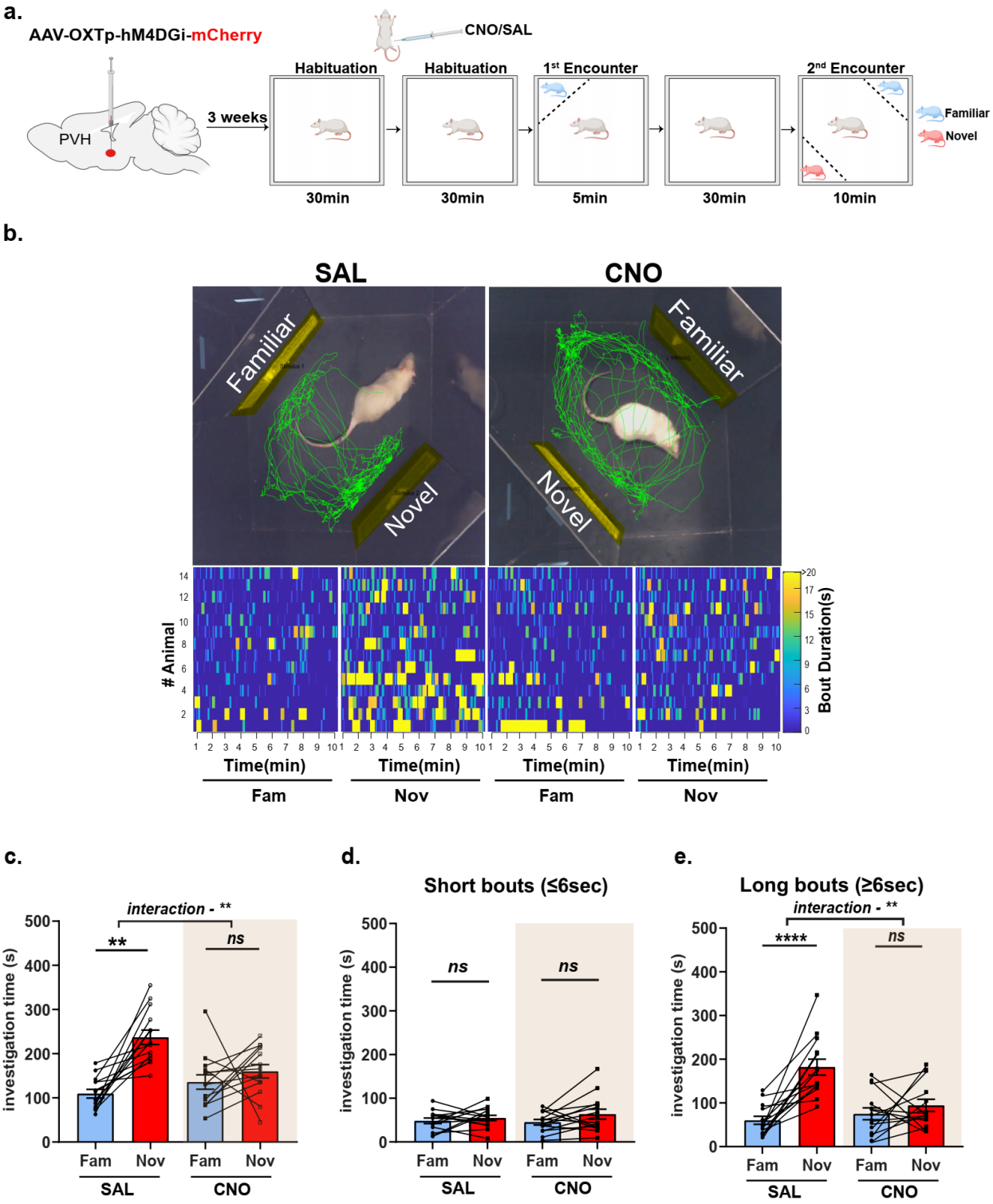

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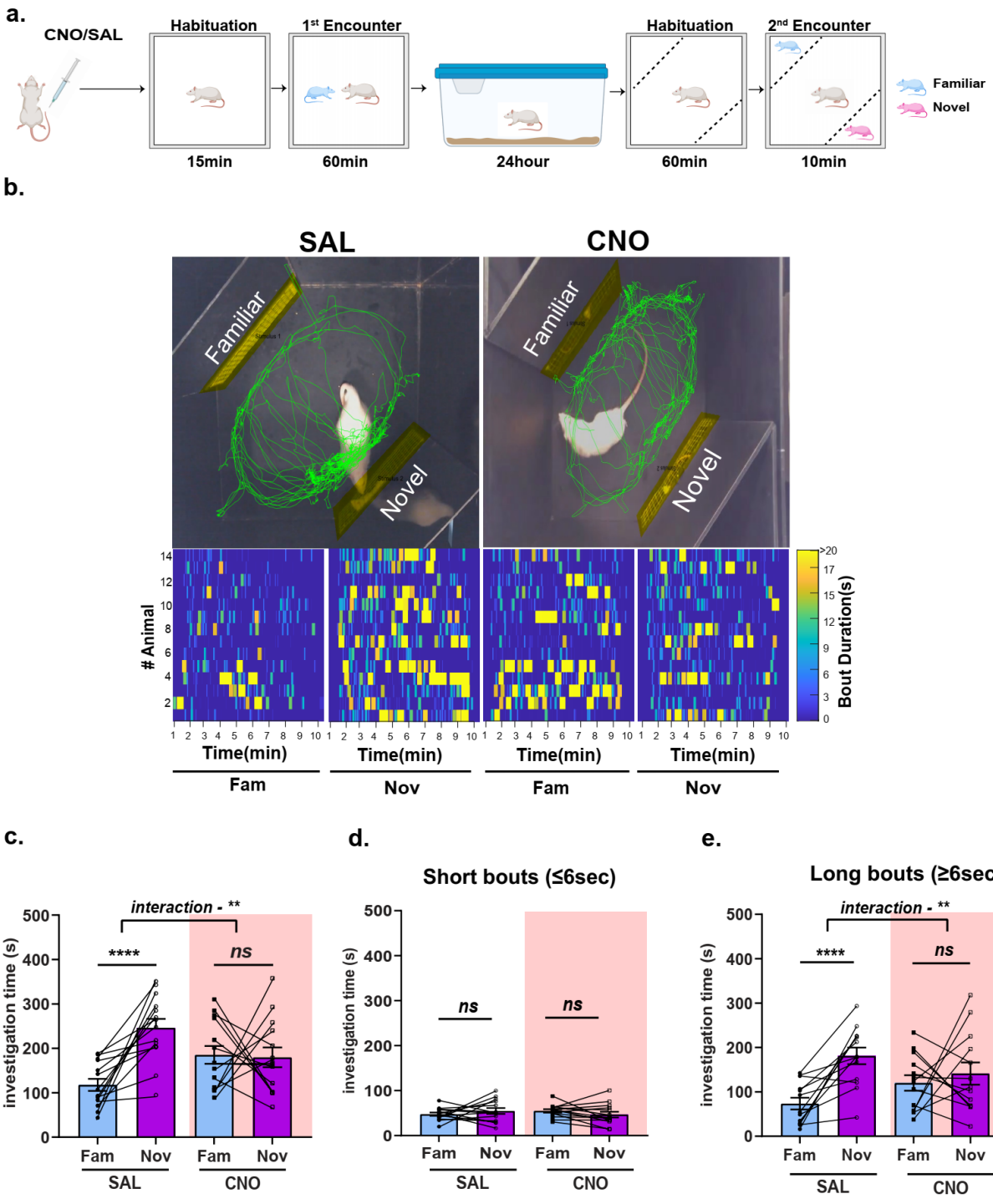

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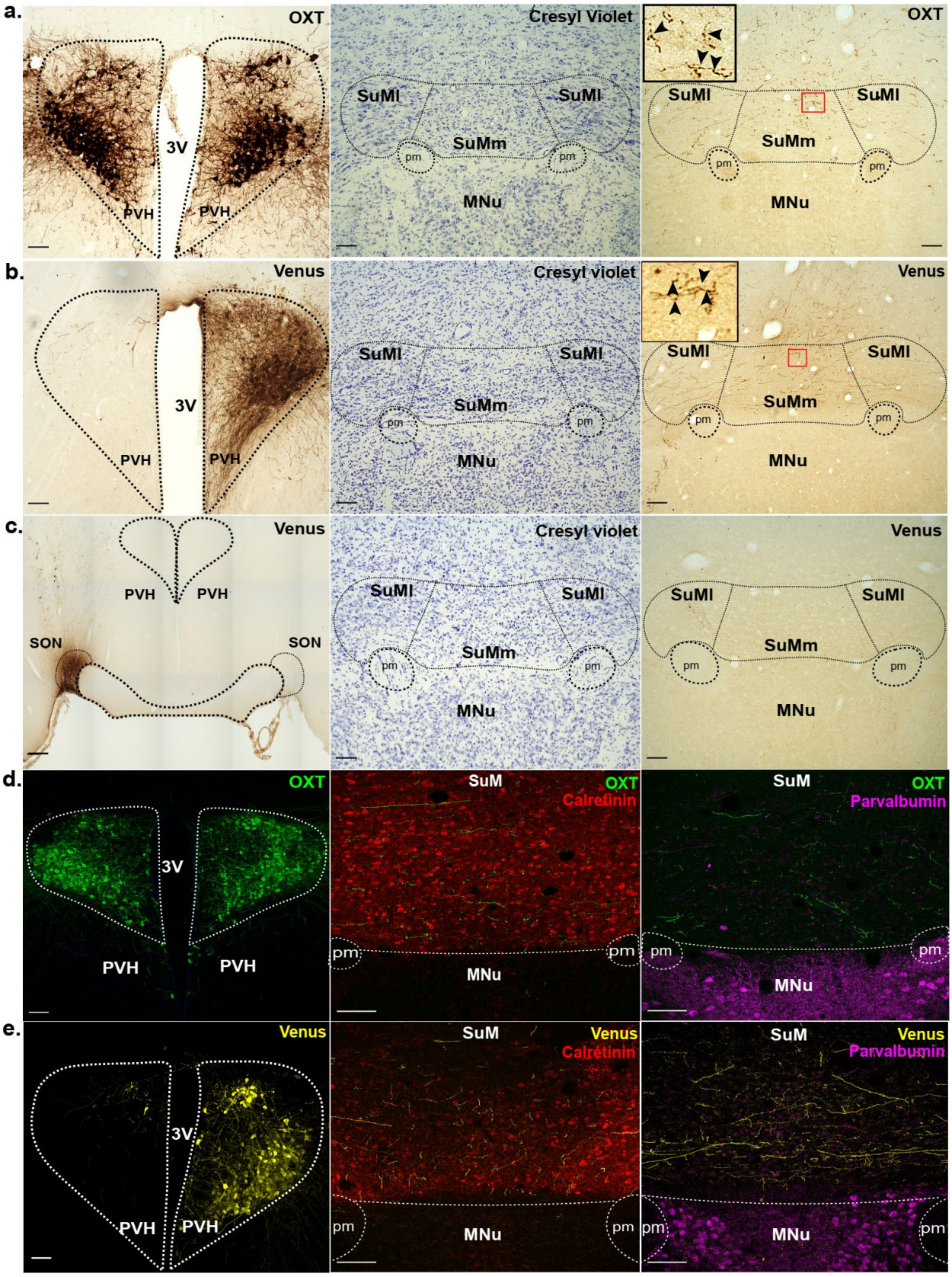

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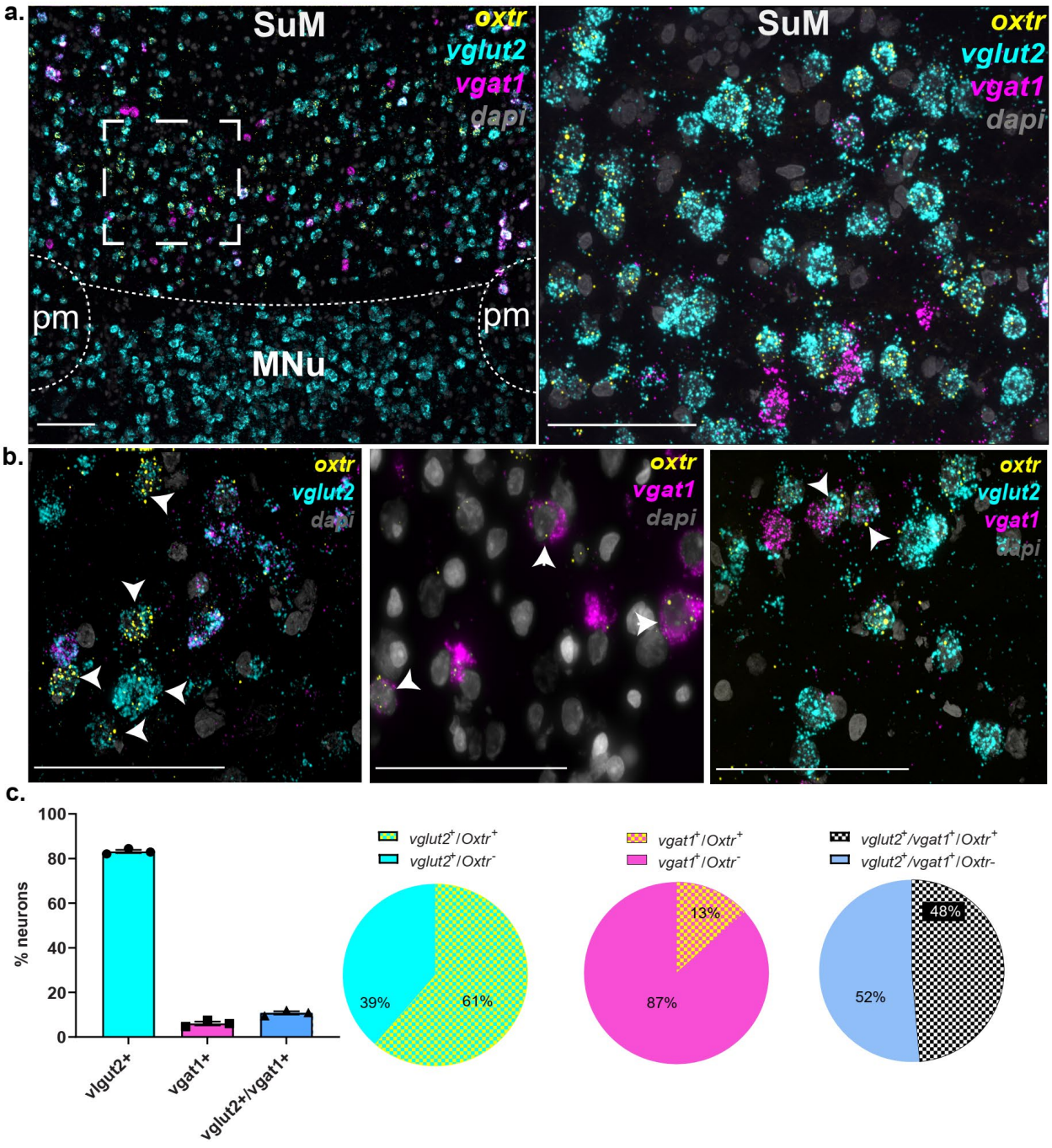

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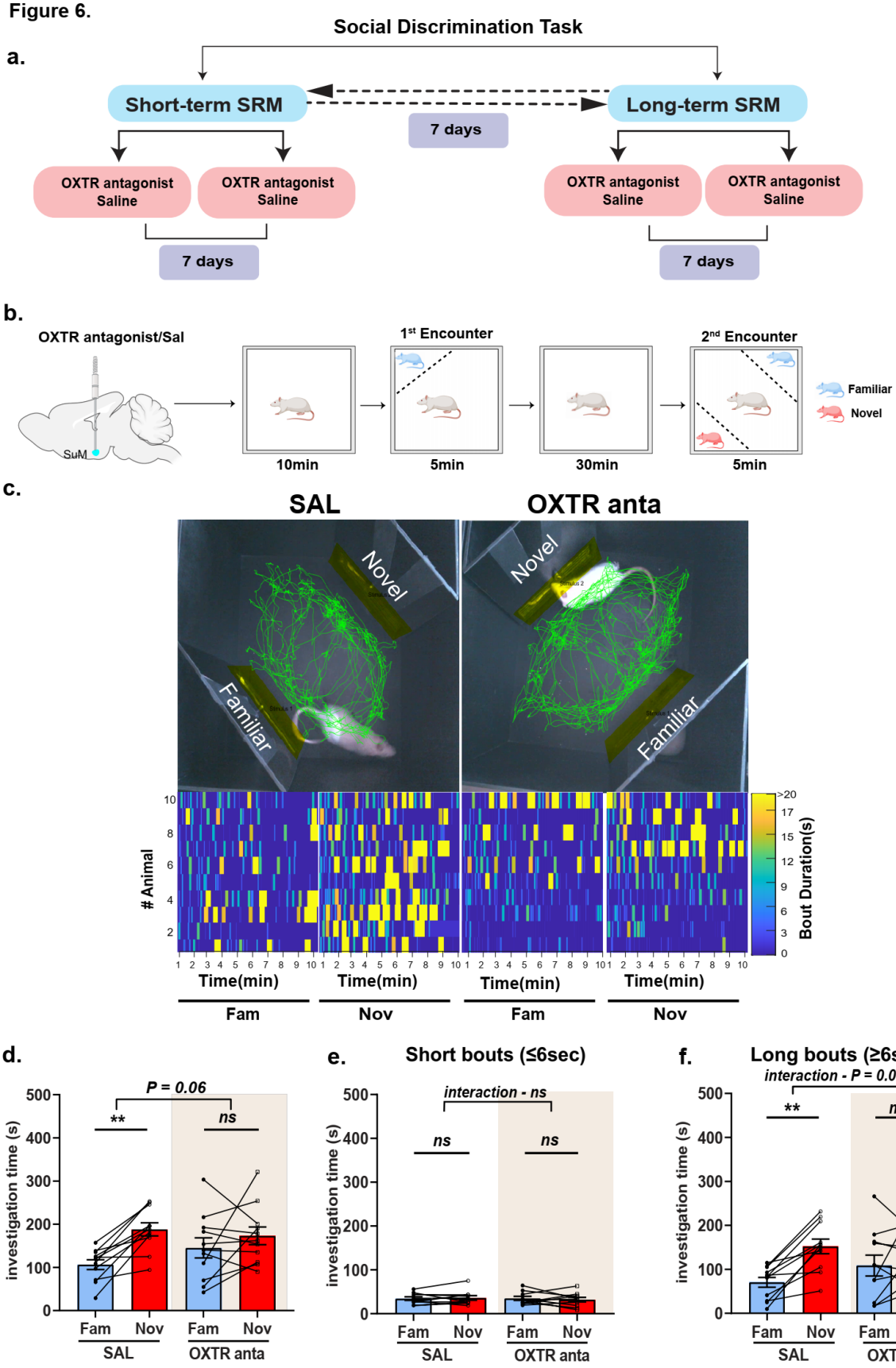

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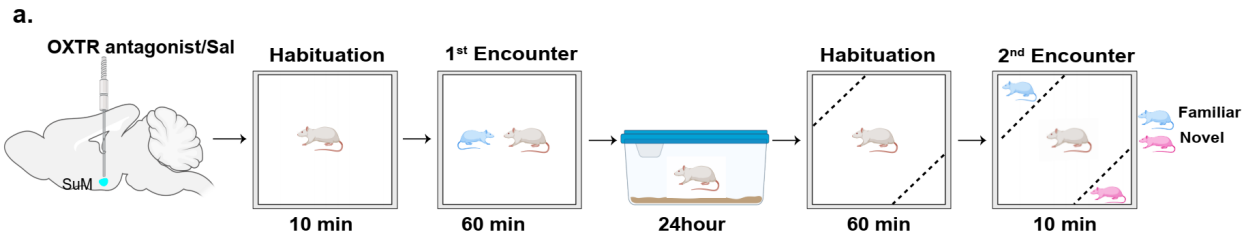

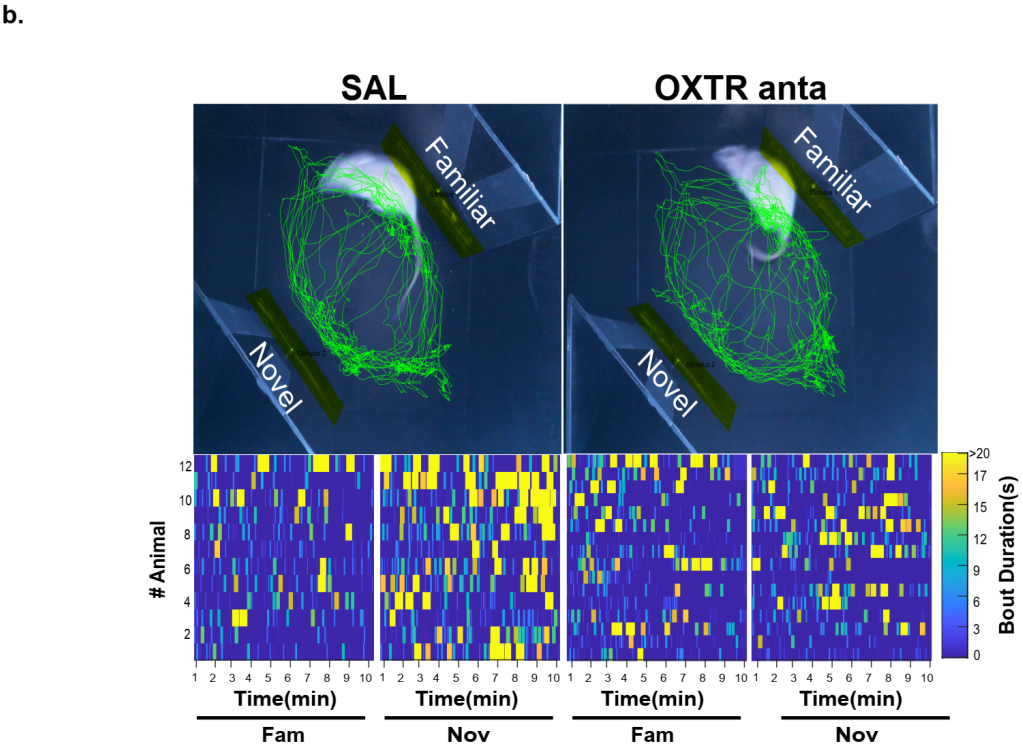

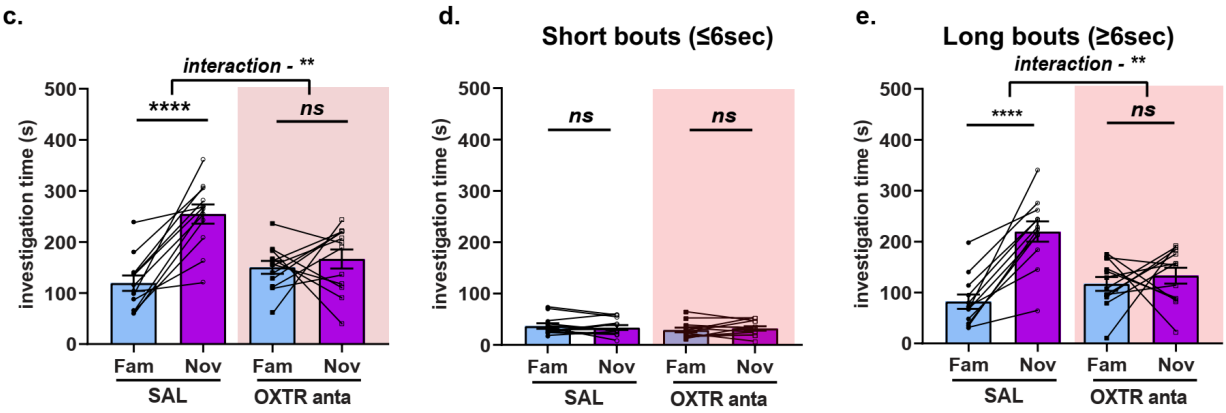

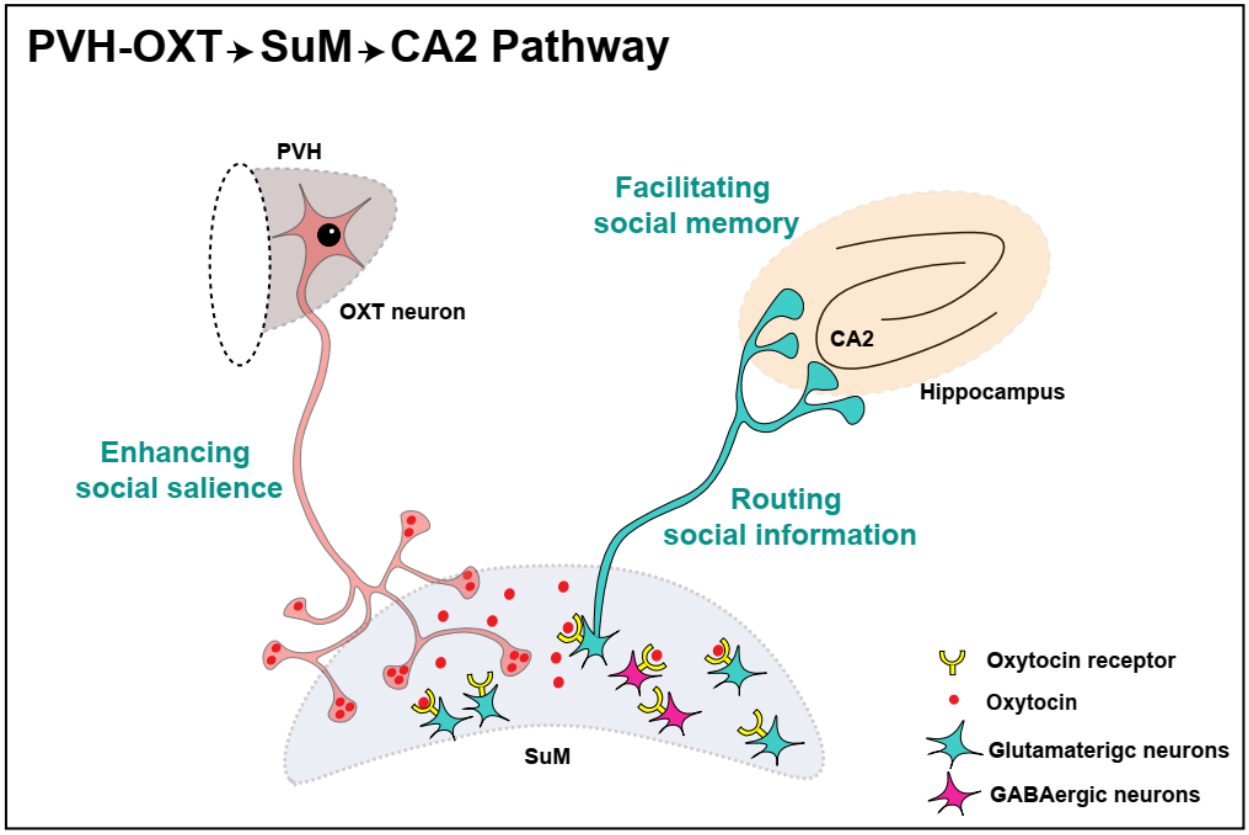

Figure Legends

Figure 1

Targeted expression of the inhibitory hM4DGi DREADDs in PVH-OXT neurons. **a.** A schematic showing the behavioral experimental design for the SRM experiments. Saline and CNO treatments were counterbalanced between test days, and short and long-term SRM were counterbalanced between cohorts. **b.** A representative image of PVH-OXT neurons showing AAV1/2-OXT-hM4DGi-mcherry and OXT co-expression in the PVH. **c.** Percentage of PVH-OXT neurons that express AAV1/2-hM4DGi-mcherry ($OXT^+/hM4DGi^+$) (70.5±1.6%, n=3, 10-12 bilateral PVH sections, OXT, 980±167.3, $OXT^+/hM4DGi^+$, 686.3±105.3). SRM, Social recognition memory, PVH, paraventricular hypothalamus, OXT, oxytocin CNO, clozapine-N-oxide. 3V, 3rd ventricle. Data represented as mean ± SEM. Scale bar (100um).

Figure 2

Chemogenetic silencing of PVH-OXT neurons impairs short-term SRM. a. A schematic of the experimental design. Saline or CNO was injected 30min prior the 1st encounter. **b. Top:** A representative trace (top) from one rat per treatment during the 2nd encounter following SAL or CNO injection. **Bottom:** Heat maps representing investigation time across all animals during Nov or Fam investigation following SAL or CNO. Each row represents one rat. **c.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter. SAL treated animals showed a clear preference for Nov over Fam stimuli, whereas the same animals showed no clear preference for the Nov or Fam stimuli after CNO injection (two-way repeated measures (RM) ANOVA, social preference (Fam v Nov) x treatment (SAL v CNO) interaction ($F_{1,26}$=9.11, $^{**}P$=0.0056, n=14), effect of social preference ($F_{1,26}$ = 35.07, $^{**}P$<0.0001) effect of treatment ($F_{1,26}$ = 1.7, P=0.203), post-hoc, Sidak multiple comparison test, SAL (Fam v Nov) $^{****}P$<0.0001, CNO (Fam v Nov, P=0.38, *non-significant* (*ns*). **d.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter

for short bouts of interaction (≤6sec). No significant differences in the preference for Nov over Fam stimuli following SAL or CNO injection (two-way, RM ANOVA, effect of treatment (SAL v CNO) x social preference (Fam v Nov), $F_{1,26}$ = 0.330, *P*=0.570, *ns),* effect of treatment ($F_{1,26}$ = 0.418, *P*=523, *ns*) or effect of social preference ($F_{1,26}$ = 2.074, *P*=0.161, *ns*). **e.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter for long bouts of interaction (≥6sec). There was a significant difference in the preference for Nov over Fam stimuli following SAL, whereas the same animals showed no clear preference for Nov or Fam stimuli CNO injection (treatment x social preference, $F_{1,26}$ = 10.51, ***P*=0.0032), effect of treatment ($F_{1,26}$ = 5.05, **P*=0.026), effect of social preference, ($F_{1,26}$ = 26.75, *****P*<0.0001), post-hoc Sidak multiple comparison test, SAL (Fam v Nov, *****P*<0.0001), CNO (Fam v Nov, *P*=0.424*, ns*). Data represented as Mean ± SEM. Fam, Familiar, Nov, Novel, SAL, Saline, CNO, Clozapine N Oxide. PVH, Paraventricular nucleus of hypothalamus, 3V, 3rd Ventricle.

Figure 3

Chemogenetic silencing of PVH-OXT neurons impairs long-term SRM. a. A schematic of the experimental design. Saline or CNO was injected 30min prior the 1st encounter. **b. Top:** A representative trace (top) from one rat per treatment during the 2nd encounter following SAL or CNO injection. **Bottom:** Heat maps representing investigation time across all animals during Nov or Fam investigation following SAL or CNO. Each line represents one rat. **c.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter. SAL treated animals showed a clear preference for Nov over Fam stimuli, whereas the same animals showed no clear preference for the Nov or Fam stimuli after CNO injection (two-way RM ANOVA, social preference (Fam v Nov) x treatment (Saline v CNO) ($F_{1,26}$ = 10.51,***P*=0.0032, n=14), effect of social preference ($F_{1,26}$ =12.34, ***P*=0.0016), effect of treatment ($F_{1,26}$ = 0.0005, *P*=0.98), post-hoc, Sidak multiple comparison test, Sal (Fam v Nov) *****P*<0.0001, CNO (Fam v Nov, *P*=0.973*, ns*). **d.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter for short bouts of interaction

(≤6sec). No significant differences in the preference for Nov over Fam stimuli following SAL or CNO injection (effect of treatment, $F_{1, 26} = 0.002$, $P=0.957$), effect of social preference (Fam v Nov, $F_{1, 26} = 0.01$, $P=0.920$, treatment x social preference, $F_{1, 26} = 2.396$, $P=0.133$). **e.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter for long bouts of interaction (≥6sec). There was a significant difference in the preference for Nov over Fam stimuli following SAL, whereas as the same animals with treated with CNO showed no clear preference for Nov or Fam stimuli (treatment x social preference, $F_{1,24} = 5.244$, $^{*}P=0.03$) effect of treatment, ($F_{1,24} = 0.032$, $P=0.857$), effect of social preference, $F_{1,24} = 11.10$, $^{**}P=0.0028$) (post-hoc Sidak multiple comparison test, SAL (Fam v Nov, $^{***}P=0.0005$), CNO (Fam v Nov, $P=0.681$, *ns*).

Figure 4

OXT fibers in the SuM originate from the PVH and not the SON. a. Immunohistochemical staining for OXT in the PVH and SuM**. Left:** A representative PVH section stained with specific anti OXT antibodies and developed using diaminobenzidiene (DAB) based enzymatic staining. **Middle:** A representative SuM section from the same animal as above was stained for cresyl violet to highlight anatomical structures within the SuM. **Right:** An immediately adjacent SuM section (to the middle image) stained with anti OXT antibodies to highlight OXT fibers in the SuMm and the SuMl (10x). Inset shows a higher magnification (40x) image of SuMm (highlighted by red box). Black arrows show the presence of axonal varicosities. **b.** Immunohistochemical staining for Venus in SuM from PVH injected with AAV1/2-OXTp-Venus. **Left:** a representative image of the PVH injected unilaterally with the AAV1/2-OXTp-Venus. Venus was identified using anti GFP antibodies and developed enzymatically using DAB based staining. **Middle:** Cresyl violet staining of the SuM to highlight the SuMl and SUMm. **Right:** An immediately adjacent SuM section (to the middle image) shows Venus positive fiber distribution across the SuMm and SuMl (4x). Inset shows a higher magnification (40x) image of the SuMm (highlighted by red box), confirming the presence of axonal varicosities within the SuMm. **c.** Immunohistochemical staining for Venus in

SuM from SON injected with AAV1/2-OXTp-Venus. **Left:** AAV1/2-OXTp-Venus injected into the SON identified using GFP antibody. **Middle:** Cresyl violet staining of SON injected group to highlight anatomical structures in the SuM. **Right:** An immediately adjacent SuM section (to the middle image) shows absence of Venus positive fibers across the SuMm and SuMl (4x). **d.** Immunofluorescent labeling for OXT in the PVH and SuM**. Left:** PVH tissue stained with anti-OXT antibodies. **Middle:** SuM tissue co-stained with anti OXT and anti-calretinin (a marker for the SuM) antibodies. **Right:** SuM tissue co-stained with anti-OXT and anti-parvalbumin (a marker for the MNu) antibodies. **e**. Immunofluorescent staining for Venus in the PVH and SuM from PVH injected with AAV1/2-OXTp-Venus**. Left**: Venus was identified using anti GFP antibodies and developed using fluorescently labeled antibodies. AAV1/2-OXTp-Venus is localized to the injected hemisphere. **Middle:** SuM section co-stained with anti-GFP and anti-calretinin antibodies. **Right:** SuM section co-stained with anti-GFP and parvalbumin antibodies. Scale bar 100um, 3V, 3rd ventricle, PVH, paraventricular nucleus of the hypothalamus. SuMm, medial supramammillary nucleus, SuMl, lateral supramammillary nucleus, MNu, Mammillary nucleus, pm, principal mammillary tract.

Figure 5

OXTR are differentially distributed in SuM neurons

a. RNAscope was performed on SuM tissue using probes for *Oxtr*, *vgat1* (marker for GABAergic neurons) and *vglut2* (marker for glutamatergic neurons). **Left:** Lower magnification (10x) shows *Oxtr* along with *vglut2* and *vgat1* to highlight the SuM. **Right:** Higher magnification (40x) of SuM tissue shows distribution of *Oxtr* across *vglut2*, *vgat1* positive neurons (*vlgut2*$^+$ and *vgat1*$^+$, respectively). **b.** Higher magnification (63x) of SuM tissue showing *Oxtr* localized to *vlgut2*$^+$ neurons (**Left**), *vgat1*$^+$ neurons (**Middle**), or *vlgut2*$^+$:*vgat1*$^+$ neurons (**Right**), highlighted by white arrows. **c.** Quantification of *vglut2*$^+$ (83.1±0.7, # of *vglut2*$^+$ neurons, 344±2.3, total number of neurons, 413.6±2.72), *vgat1*$^+$ (6.04±0.8%, # of *vgat1*$^+$ neurons, 25±3.4), and *vglut2*$^+$*:vgat1*$^+$

(10.8±0.7%, # of *vglut2*$^{+}$*:vgat1*$^{+}$ neurons, 44.6±3.17) neurons in the SuM. **d.** Quantification of *Oxtr* distribution across *vlgut2*$^{+}$ (61.3±2.0%, # of *vglut2*$^{+}$/*oxtr*$^{+}$, 211±25.1) **Left),** *vgat1*$^{+}$ (13.3±0.8, # of *vgat1*$^{+}$/*oxtr*$^{+}$ neurons, 3.3±0.8, **Middle**) and *vglut2*$^{+}$*:vgat1*$^{+}$ (48.6±0.1%, # of *vglut2*$^{+}$*:vgat1*$^{+}$*/oxtr*$^{+}$ neurons, 21.6±1.2, **Right**) neurons. Scale bar 100um, SuM, Supramammillary nucleus. MNu, Mammillary nucleus, pm, principal mammillary tract. Data presented as Mean±SEM.

Figure 6

OXTR antagonism in the SuM affects short-term SRM. a. A schematic showing experimental study design for the SRM experiments. Saline and OXTR antagonist treatment were counterbalanced between test days and short and long-term SRM was counterbalanced between cohorts. **b.** A schematic of the behavioral paradigm. Saline or OXTR antagonist was infused 10min prior to 1st encounter. **c. Top:** A representative trace from one animal per treatment during the 2nd encounter following SAL or OXTR anta infusion. **Bottom:** Heat maps representing investigation time of all animals during Nov or Fam investigation following Sal or OXTR anta infusion. **d.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter. SAL infused animals showed a clear preference for Nov over Fam stimuli, whereas the same animals showed no clear preference for Nov or Fam stimuli after OXTR anta (two-way RM ANOVA, social preference (Fam v Nov) x treatment (Sal v OXTR anta) ($F_{1,18}$=3.829, *P*=0.06, n=10), effect of social preference ($F_{1,18}$=0.007, *P*=0.93), effect of treatment ($F_{1,18}$ =13.3, **P*=0.0019), post-hoc, Sidak multiple comparison test, SAL (Fam v Nov) ***P*=0.0036, OXTR anta (Fam v Nov, *P*=0.53*, ns*). **e.** Total investigation time of the Nov v Fam stimuli during the 2nd encounter for short bouts of interaction (≤6sec). There was no significant differences for preference for Nov over Fam following SAL or OXTR anta infusion (treatment x social preference ($F_{1,18}$ = 0.37, *P*=0.55, *ns*), effect of treatment ($F_{1,18}$ = 0.193, *P*=0.66, *ns*), effect of social preference (Nov v Fam, $F_{1,18}$ = 0.014, *P*=0.90, *ns*) in short bouts. **f.** Total investigation time for the Nov vs. Fam stimuli during the 2nd encounter for long bouts of interaction (≥6sec). There was a significant difference in the

preference for Nov over Fam stimuli following SAL, whereas the same animals no clear preference for Nov or Fam stimuli following OXTR anta infusion (treatment x social preference ($F_{1,18}$ = 4.257, *P*=0.053), effect of treatment ($F_{1,18}$ = 0.027, *P*=0.87, *ns*), effect of social preference (Nov v Fam, $F_{1,18}$ = 5.797, **P*=0.02). post-hoc, Sidak multiple comparison test, SAL (Fam v Nov, ***P*=0.007), OXTR anta (Fam v Nov, *P*=0.54*, ns*). **f.** SAL, Saline, OXTRanta, OXTR antagonist. Fam, Familiar, Nov, Novel.

Figure 7

OXTR antagonism in the SuM impairs long-term SRM

a. A schematic of the behavioral paradigm. Saline or OXTR antagonist was infused 10min prior to 1st encounter. **b. Top:** A representative trace from one rat per treatment during 2nd encounter following SAL or OXTR anta. **Bottom:** Heat maps representing investigation time across all animals during Nov or Fam investigation following SAL or OXTR anta infusion. **c.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter. SAL infused rats showed a clear preference for Nov over Fam stimuli, whereas the same animals showed no clear preference for the Nov or the Fam stimuli after OXTR anta infusion (Two-way RM ANOVA, social preference x treatment (SAL v OXTRanta), $F_{1,22}$ = 15.17, ****P*=0.0008, n=12), effect of social preference ($F_{1,22}$ = 18.23, ****P*=0.0003) and effect of treatment ($F_{1,22}$ = 3.496, *P*=0.07, *ns*). post-hoc, Sidak multiple comparison test, SAL (Fam v Nov) *****P*<0.0001, OXTR anta (Fam v Nov, *P*=0.73*, ns*). **d**. Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter for the short bouts of interaction (≤6sec). No significant differences for preference for Nov over Fam following SAL or OXTR anta infusion (treatment x social preference ($F_{1,22}$ = 1.17, *P*=0.29, *ns*), effect of treatment ($F_{1,22}$ = 3.06, *P*=0.09, *ns*), effect of social preference (Nov v Fam, $F_{1,22}$ = 0.0003, *P*=0.98, *ns*) in short bouts. **e.** Total investigation time of the Nov vs. Fam stimuli during the 2nd encounter for the long bouts of interaction (≥6sec). There was a significant preference for Nov over Fam following SAL infusion on long bouts, however the same animals after OXTR anta

infusion did not show a clear preference for the Nov or the Fam stimuli (treatment x social preference ($F_{1,22}$ = 15.53, **P*=0.0007), effect of treatment ($F_{1,22}$ = 2.83, *P*=0.1, *ns*), effect of social preference (Nov v Fam, $F_{1,22}$ = 21.66, ***P*=0.0001). post-hoc, Sidak multiple comparison test, SAL (Fam v Nov) *****P*<0.0001, OXTRanta (Fam v Nov, *P*=0.72*, ns*). **e.**. SAL, Saline, OXTRanta, OXTR antagonist. Fam, Familiar, Nov, Novel.

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