

Oxytocin receptor-expressing neurons in the medial preoptic area affect social behaviour

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1. Abstract

Oxytocin (OT) is known to affect emotional behaviour via the oxytocin receptor (OTR) located in several forebrain regions. The medial preoptic area (MPOA), located in the anterior part of the hypothalamus, is well known for its role in maternal behaviour. However, recent evidence suggests that it is also involved in the control of affiliative social behaviour. Since OTR is abundant in the MPOA, we hypothesized that OTR-expressing neurons in the MPOA regulate social behaviour in rodents. The aim of this study was to investigate the functional role of OTR neurons in interfemale social behaviour by fiber photometry and chemogenetic manipulation. To this end, I used OTR-Cre female rats, which expressed Cre recombinase in OTR-expressing cells, allowing the use of Cre-dependent viral vectors to express calcium sensor proteins and excitatory receptors for chemogenetics. The control group received a Cre-dependent viral vector lacking excitatory receptors. I also used fiber photometry to investigate the neuronal activity of OTR⁺ cells in the MPOA during direct social interaction. With anterogradely spreading viral vector, I revealed the projections of MPOA OTR-expressing neurons.

Stimulation of OTR-expressing neurons led to a significant increase in the duration of chasing and subsequent direct contact behaviours, such as mounting and allogrooming without altering the animals' sociability, social preference, depression and anxiety-like behaviours. Using fiber photometry, I found that OTR⁺ neurons in the MPOA are activated during anogenital sniffing, a behavioural element, which I showed to be highly significantly followed by chasing. Neuronal tracing revealed that OTR neurons project to brain regions, such as the ventral subdivision of the lateral septum, the ventrolateral periaqueductal grey and the ventral tegmental area. These are all brain regions that constitute the social brain network, which is responsible for the control of social behaviours.

Here I present the first evidence of a function of MPOA OTR⁺ neurons by demonstrating their role in the control of affiliative social interactions. The activation of OTR⁺ neurons during anogenital sniffing is conveyed by their projections to the lateral septum, the periaqueductal grey matter, and the ventral tegmental area for distribution in the limbic system, motor control, and reward processing, respectively, which may facilitate a chain of behavioural elements to occur leading to direct contact between the animals. These findings suggest that MPOA OTR-expressing neurons may play a central role in regulating social interactions between adult female conspecifics.

2. List of abbreviations

3rd: third ventricle

ac: anterior commissure

AAV: adeno-associated virus

AHA: anterior hypothalamic area, anterior part

AHC: anterior hypothalamic area, central part

AVPV: anteroventral periventricular nucleus of the hypothalamus

Aq: aqueduct

AUC: area under curve

CNO: clozapine-N-oxide

CLi: caudal linear nucleus of the raphe

DAB: 3,3'-diaminobenzidine

DMH: dorsomedial hypothalamus

dMPA: medial preoptic area, dorsal part

DMSO: dimethyl sulfoxide

DRD: dorsal raphe nucleus, dorsal part

DREADD: Designer Receptors Exclusively Activated by Designer Drugs

EPM: elevated plus maze

ESO: episupraoptic nucleus

FST: forced swim test

GFP: green fluorescent protein

hM3D: human M3 muscarinic receptor

IF: interfascicular nucleus

IP: interpeduncular nucleus

LDT: laterodorsal tegmental nucleus

LPO: lateral preoptic area

MnR: median raphe nucleus

MePD: medial amygdaloid nucleus, posterodorsal part

MPOA: medial preoptic area

MRt: mesencephalic reticular formation

MS: medial septum

Mtu: medial tuberal nucleus

och: optic chiasm

OT: oxytocin

OTR: oxytocin receptor
PaAP: paraventricular hypothalamic nucleus, anterior parvicellular part
PAG: periaqueductal grey
PBP: parabrachial pigmented nucleus of the VTA
Pe: periventricular nucleus
PeF: perifornical nucleus
PH: posterior hypothalamic nucleus
PIL: posterior intralaminar complex of the thalamus
PMV: premamillary nucleus, ventral part
PP: peripeduncular nucleus
PS: parastrial nucleus
PSTh: parasubthalamic nucleus
PV: paraventricular thalamic nucleus
PVN: paraventricular nucleus
PTH2: parathyroid hormone 2
PTH2R: parathyroid hormone 2 receptor
RN: red nucleus
RRF: retrorubral field
RtTg: reticulotegmental nucleus of the pons
SHy: septohypothalamic nucleus
SON: supraoptic nucleus
STMA: bed nucleus of the stria terminalis, medial division, anterior part
SuB: subbrachial nucleus
SuM: supramammillary nucleus
TH: tyrosine hydroxylase
TuLH: tuberal part of the lateral hypothalamus
VLPO: ventrolateral preoptic nucleus
vIPAG: ventrolateral subdivision of the periaqueductal grey
VMH: ventromedial hypothalamic nucleus
VMPO: ventromedial preoptic nucleus
VOLT: vascular organ of the lamina terminalis
VTA: ventral tegmental area
vBNST: bed nucleus of the stria terminalis, ventral part
LSv: ventral lateral septum
VTM: ventral tuberomammillary nucleus

3. Introduction

Social behaviour, defined as the set of interactions between conspecifics, is usually divided into three main groups: reproductive, prosocial and antisocial [1]. Prosocial behaviour encompasses affiliative behaviours such as maternal behaviour, social preference, social reward, social approach or social recognition and memory [2], [3]. Affiliative social behaviour helps to strengthen social bonds between conspecifics. Allogrooming, or brushing another individual's coat, is a type of affiliative social touch that is reassuring to other conspecifics [4].

One of the most important regulatory centre for social behaviour is the hypothalamus, whose anterior region, the medial preoptic area (MPOA) (Fig. 1A) has been extensively studied for its role in maternal, and male sexual behaviour. In rodents, it has been shown that preoptic GABAergic neurons promote maternal behaviour and are activated after pup exposure [5], [6]. In addition, neurons in the MPOA are activated during pup-directed actions, such as pup grooming during which the MPOA-periaqueductal grey (PAG) projection is activated [7].

Despite the paucity of research on the role of the MPOA in adult affiliative behaviour, emerging evidence suggests a crucial function. Recent findings indicate that distinct neuron populations within the MPOA help maintain the "social homeostasis" underlying social interactions [8]. MPOA also controls the contact-seeking behaviour [9], probably driven by social touch information transmitted here by the posterior intralaminar complex of the thalamus (PIL)-MPOA pathway [10]. MPOA is further involved in the regulation of allogrooming through the medial amygdala-MPOA pathway [4]. In addition, the MPOA may also be involved in the regulation of juvenile social play [11].

Various neuronal populations regulate the wide range of behavioural and homeostatic functions in the MPOA. Among these, clusters of neurons express the oxytocin receptor (OTR), known for its role in the regulation of social behaviour (Fig. 1.) [12].

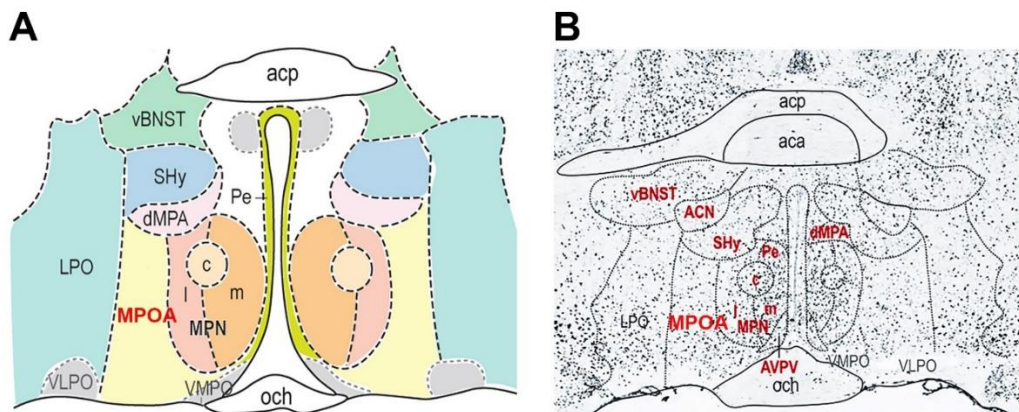


Figure 1. Oxytocin receptor expression in the preoptic region. (A) Schematic diagram of the preoptic region. (B) The distribution of oxytocin receptors in the preoptic region [13], [14].

Abbreviations: aca: anterior commissure, anterior part, acp: anterior commissure, posterior part, ACN: anterior commissural nuclei, AVPV: anteroventral periventricular nucleus of the hypothalamus, dMPA: dorsomedial preoptic area, LPO: lateral preoptic area, MPN: medial preoptic nucleus, (m) medial, (c) central and (l) lateral regions, MPOA: medial preoptic area, och: optic chiasm, Pe: periventricular nucleus, SHy: septohypothalamic nucleus, vBNST: bed nucleus of the stria terminalis, ventral part, VLPO: ventrolateral preoptic area.

OTR is a G-coupled receptor that mediates the effects of oxytocin (OT), a neuropeptide synthesized in the paraventricular (PVN) and the supraoptic nuclei (SON) of the hypothalamus. The MPOA neurons receiving OT inputs from the PVN [15] play a role in maternal behaviour [16]. OT-OTR binding in modulating affiliative social behaviour among female conspecifics has been demonstrated – for instance, in social reward processing within the nucleus accumbens [17] or social touch with the involvement of PVN [18]. However, the role of OT-OTR system in the MPOA in affiliative social behaviour remains unexamined. Therefore, this study aims to investigate the role of MPOA OTR⁺ neuronal contribution to affiliative social behaviours.

4. Objectives

1. To investigate whether the chemogenetic manipulation of MPOA OTR neurons affects affiliative social interactions among adult female rats
 - a. To investigate the excitatory effect of MPOA OTR neurons on social behaviour among freely interacting animals
 - b. To investigate the excitatory effect of MPOA OTR neurons on social motivation, memory and recognition
 - c. To test whether locomotor alteration has an effect on social behaviour due to chemogenetic manipulation
2. To determine the influence of chemogenetic stimulation of MPOA OTR neurons on anxiety- and depression-like behaviours
3. To measure the activity of MPOA OTR neurons during direct social interactions
4. To examine the efferent projections of MPOA OTR neurons
 - a. To perform whole brain mapping based on tract tracing in order to determine target sites of MPOA OTR neurons
 - b. To characterize MPOA OTR target neurons

5. Materials and methods

5.1. Animals

The Workplace Animal Welfare Committee of the National Scientific Ethical Committee on Animal Experimentation at Eötvös Loránd University, Budapest, approved this study (PE/EA/00403-4/2023). All procedures on rats were carried out according to the experimental protocols approved by the Ministry of Agriculture, Hungary (40/2013), which is in accordance with EU Directive 2010/63/EU.

This study used 22 female Sprague Dawley rats (8–12 weeks old, 180–240 g): 10 for chemogenetic stimulation, 8 for control, 2 for fiber photometry, and 1 for neuronal tract tracing. The rats expressed Cre recombinase in OTR⁺ cells, allowing the use of Cre-dependent viral vectors to express designer receptors exclusively activated by designer drugs (DREADDs) [19]. They were housed on a 12:12 light/dark cycle (lights on at 6:00 am) with free access to food and water. We maintain a heterozygous OTR-Cre strain, so each animal's genotype was confirmed by PCR from a tail DNA sample.

5.2. Viral vectors and stereotaxic injection

All the used viral vectors were Cre-recombinase-dependent adeno-associated virus (AAV) and contained mCherry reporter gene, which was used to determine the injection sites. Viral vectors encode DREADDs as double-floxed genes, which can only be expressed due to Cre-recombinase [20]. In excitatory group, I injected pAAV-hSyn-hM3D(Gq)-mCherry (RRID: Addgene_50474) while the control group (n=8) received pAAV-hSyn-DIO-mCherry (RRID: Addgene_50459) that lacks excitatory DREADDs. Animals were deeply anaesthetized with an intraperitoneal injection (i.p.) of a mix containing 75 mg/kg body weight ketamine and 10 mg/kg body weight xylazine. The virus (50 nl) was injected into the MPOA both sides of the brain with a nanoinjector (Nanoliter2010 by WPI) (Fig. 2A). The stereotaxic coordinates used for the MPOA were the following: AP= -0.48 mm from bregma, LM= +/-0.45 mm from the midline, DV= -7.85 from the brain surface (Fig. 2B) determined using the Paxinos and Watson rat brain atlas [21]. The ligand to act on hM3Dq was the clozapine N-oxide (CNO) [20].

Animals used for fiber photometry (n=2) received a virus injection (75 nl) of the Cre-dependent pAAV.Syn.Flex.GCaMP6f.WPRE.SV40 (RRID: Addgene_100833) into the MPOA. Additionally, a cannula implant was placed above the MPOA. For neuronal tract-tracing, I injected pAAV-hSyn-DIO-mCherry (100 nl) in the left MPOA.

5.3. Social behavioural tests

5.3.1. Chemogenetic manipulation of OTR+ neurons

After stereotaxic surgery, animals recovered for 2 weeks, reunited for 1 week, and were separated again 24 hours before testing. Behaviour was measured on 3 days. On day 1 (control), animals received an i.p. injection of vehicle (5% dimethyl sulfoxide (DMSO) in distilled water). On the treatment day, CNO (0.3 mg/kg in 5% DMSO) was injected i.p. 45 minutes before testing. Two days later, DMSO was administered again as a second control (Fig. 2C).

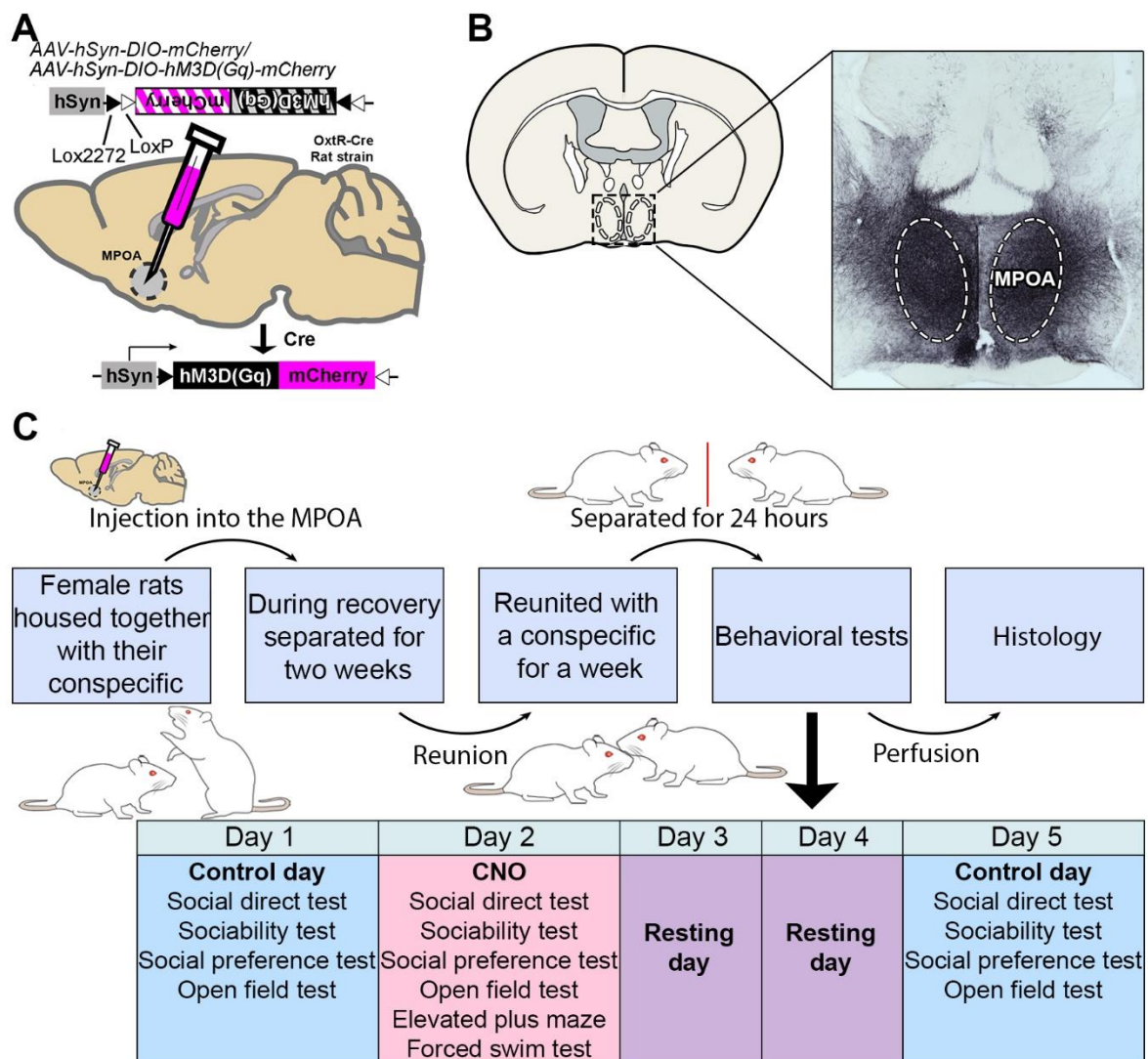


Figure 2. The experimental protocol of the chemogenetic manipulation of MPOA OTR+ neurons. (A) Schematic illustration of the virus construct and its injection site. (B) The validation of the injection site within the MPOA is demonstrated. The dashed lines marking the boundaries of MPOA. (C) Summary of the experimental protocol of the study. Abbreviations: CNO: clozapine N-oxide, MPOA: medial preoptic area,

5.3.2. Direct social interaction test

Subject animals were placed with their cagemate in an open field for 10 minutes, allowing free interaction while being video recorded. Behaviours were coded using Solomon Coder software, which provided total duration and frequency data for statistical analysis (Fig. 3).

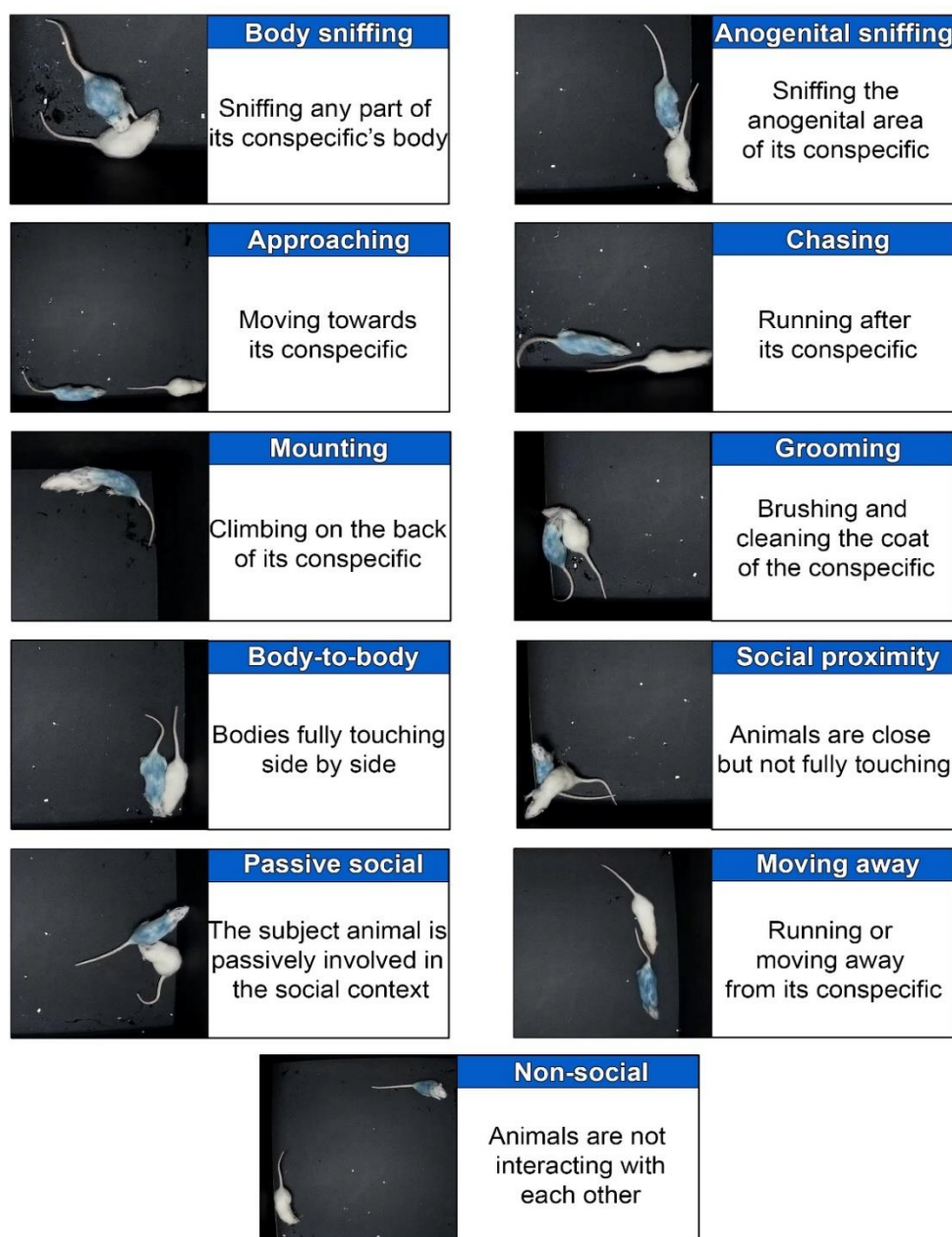


Figure 3. The summary of the distinguished behavioural elements in direct social interaction test. The figure describes the behavioural elements that were distinguished in the case of direct social interaction test. The fur of the subject animals was dyed blue for discrimination of the animals during the video coding.

5.3.3. Sociability and social novelty tests

The experiment used a three-chamber apparatus. After i.p. injections of DMSO or CNO, subjects were placed in the centre chamber to assess sociability, allowing them to choose between their cagemate or an empty chamber. In the social novelty test, they could choose between a familiar cagemate or an unfamiliar conspecific. Each 10-minute session was recorded, and behaviour was analysed with SMART Video Tracking Software v3.0 (Panlab, Barcelona, Spain).

5.3.4. Anxiety- and depression-like behaviour tests

I used the open field arena to measure the anxiety level and locomotor activity of the subject animals. Their behaviour was recorded for 20 min. To measure the anxiety level of the subject animals, they were placed in an elevated plus maze (EPM). I placed the subject animal in the middle of the EPM and recorded a 10-minute video. I distinguished the following elements: time spent in the open and closed arms and in the middle of the apparatus. Depression-like behaviour was assessed by forced swim test (FST), which I recorded for 6-min video and distinguished floating, swimming, and climbing behaviours.

5.4. Fiber photometry

Fiber photometry was used to monitor neuronal activity of OTR⁺ cells in the MPOA during social interaction. After virus injection and cannula implantation, animals recovered, spent one week with a conspecific, and were separated 24 hours before testing. Recordings were taken using the R811 Dual Color Multichannel Fiber Photometry System at 40 μ W and 60 fps. The implanted cannula was connected to an optical fiber to capture GCaMP fluorescence in freely moving animals. I recorded 60 seconds video before the test when the subject animal was alone in the apparatus (F0), as it was used to correct and smooth the recorded fluorescent signal with a smoothing coefficient of 15 and PLS baseline Fit coefficient of 8. We did every analysis on this normalized $\Delta F/F_0$ signal. Social interaction tests were recorded for 5 minutes on two days. Behaviours analysed included snout-to-snout, head-to-head, chasing, grooming, mounting, and various sniffing interactions. Peri-event analysis used a 2-second baseline before each event, analysing 5 seconds after.

5.5 Brain tissue processing

Rats were deeply anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH = 7.2). The brains were removed

and postfixed overnight and transferred to PB containing 20% sucrose. Coronal sections were cut at 50 μm on cryostat (Leica).

5.6 Immunohistochemistry

5.6.1. Determining the injection sites using peroxidase-based immunohistochemistry

To validate injection sites, peroxidase-based immunohistochemistry was used (Figure 2B). Tissues were treated with 0.1% H_2O_2 in PB to block endogenous peroxidase, then incubated in a solution with BSA, FBS, and TritonX-100 to reduce non-specific labelling. Primary antibody mCherry (1:3000) was applied overnight, followed by a biotinylated secondary antibody (1:1000) for 1 hour, and ABC (1:500) for visualization with 3,3'-diaminobenzidine (DAB) and H_2O_2 .

5.6.2. Fluorescent immunolabeling

Following blocking endogenous peroxidase activity and the blocking buffer as described above, I applied the following 1st antibodies overnight: anti-c-Fos (1:10000, guinea pig, Synaptic Systems, #225004), anti-parathyroid hormone 2 (1:3000, AB_1235466, rabbit), anti-tyrosine hydroxylase (TH) (1:2000, mouse, Sigma), anti-mCherry (1:3000, chicken, EnCor Biotechnology, #CPCA-mCherry), anti-GFP (1:6000, guinea pig, Synaptic Systems, #132005) and anti-oxytocin (1:1000, mouse, #MAB5296) followed by another 1st antibody for double labelling. Then, I used the following 2nd antibodies (1:500) for 2 h: donkey anti-guinea pig Alexa488, donkey anti-chicken Alexa594 and donkey anti-mouse Alexa488 (Jackson ImmunoResearch).

5.7. Microscopy and image acquiring

I have taken images using a Nikon Eclipse light microscope and a Zeiss LSM 800 laser scanning confocal microscope using 10x (Plan-Apochromat 10x/0.45) and 20x (Plan-Apochromat 20x/0.8) objectives. Images were adjusted using the ZEN software (Zeiss).

5.8. Analysis of neuronal tracing

Two weeks after the viral injection, the animals were sacrificed and then immunolabeled as described above. Whole-brain mapping was applied to identify target sites of MPOA OTR+ neurons. Quantification of area density of mCherry+ nerve fibers was conducted using QuPath's machine learning based pixel classification function [22].

5.9 Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.0.2 software. The frequency and duration of behavioural elements in the direct social test, the results of the three-chambers test, open field test, EPM and FST were analysed using two-way ANOVA followed by post-hoc Tukey test. The order of behavioural elements during social interaction in rats were analysed with binomial test. The results of fiber photometry experiments were analysed with paired t-test.

6. Results

6.1. The effect of stimulating MPOA OTR+ neurons on direct social interaction

First, I injected AAV-P_{hSYN}-hM3D(Gq)-mCherry into the MPOA of the subject animals (Fig. 4A-B) where oxytocinergic fibers are localized (Fig. 4C) to examine the effect of the activation of MPOA OTR+ neurons on social behaviour. I revealed numerous changes in the duration and number of various behavioural elements (Fig. 4).

As a result, the duration and number of grooming, mounting, chasing and body-to-body contact, social proximity and body sniffing were significantly increased after the CNO administration compared to the control days when the subject animals received vehicle. In turn, the duration of passive social interactions, moving away and non-social behaviour were significantly decreased following CNO injection compared to the vehicle days. The CNO administration did not alter the duration of anogenital sniffing and approaching. Moreover, the frequency of passive social interaction and moving away were significantly decreased compared to the control days. The CNO administration did not alter the frequency of anogenital sniffing. In the control group, the animals lacked excitatory DREADDs. Following CNO administration, none of the behavioural elements were affected. Their behaviour was similar to the control days of the excitatory group (Table 1).

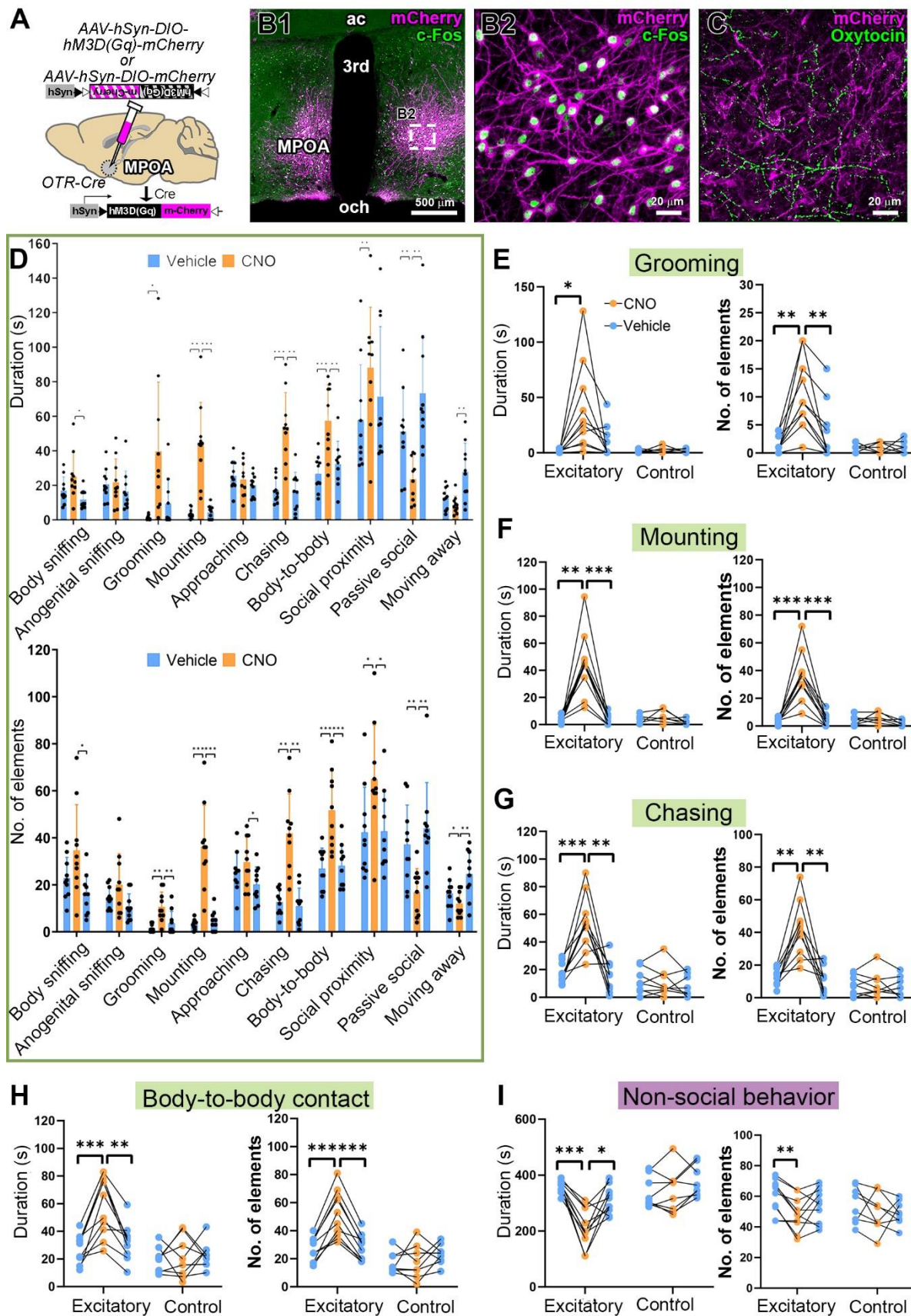


Figure 4. The effect of chemogenetic stimulation of the OTR+ neurons in the MPOA during direct social interaction. (A) Schematic figure of the injection of viral vector expressing DREADD. (B1-2) Neurons infected by the viral vector express the fluorescent

reporter protein (mCherry) are activated due to CNO injection indicated by c-Fos⁺ nuclei. (C) Oxytocinergic fibers are located in the same area. (D) The effect of chemogenetic stimulation resulted in changes in the frequency and duration of various behavioural elements. (E-I) The CNO activation of DREADDs resulted in a significant increase in the duration and frequency of grooming. (E), mounting (F), chasing. (G), body contact (H) without any changes when control viral vector without DREADDs was injected. The activation of DREADDs resulted in a significant decrease in the duration and frequency of non-social behaviour (I). N=10 for excitatory viral injection, N=8 for control viral injection, two-way ANOVA followed by post hoc Tukey tests (Excitatory group frequencies $F(1.702, 168.5) = 40.01$ and duration $F(1.510, 149.4) = 0.002908$; control group frequencies $F(1.197, 152.0) = 6.404$ and duration $F(1.742, 123.2) = 1.861 \times 10^{-29}$). The horizontal lines above bars indicate that p value is significant (*: $0.01 < p < 0.05$, **: $0.001 < p < 0.01$, ***: $p < 0.001$). Abbreviations: 3rd: third ventricle, ac: anterior commissure, CNO: clozapine N-oxide, MPOA: medial preoptic area, och: optic chiasm.

Duration of the behavior elements during the 10-minute direct social interaction test		
	p-value	
Behavior element	1 st control day vs CNO	CNO vs 2 nd control day
grooming	0.0344	n.s.
mounting	0.0013	0.0006
chasing	0.0007	0.0045
body-to-body contact	0.0008	0.0019
social proximity	0.0085	n.s.
body sniffing	0.0126	n.s.
passive social interaction.s.	0.0059	0.0051
moving away	n.s.	0.0031
non-social behavior	<0.0001	0.0122
anogenital sniffing	n.s.	n.s.
approaching	n.s.	n.s.
Number of the behavior elements during the 10-minute direct social interaction test		
	p-value	
Behavior element	1 st control day vs CNO	CNO vs 2 nd control day
grooming	0.0027	0.0063
mounting	0.0009	0.0005
chasing	0.0010	0.0030
body-to-body contact	0.0002	<0.0001
social proximity	0.0150	0.0448
body sniffing	n.s.	0.145
approaching	n.s.	0.316
passive social interaction.s.	0.0026	0.0014
moving away	0.0313	0.0025
non-social behavior	0.0033	n.s.

Table 1. Significance values of behaviour elements of direct social interactions.

6.2. The effect of chemogenetic stimulation on sociability and social preference of the animals

I tested the animals' sociability and social novelty. In the sociability test, subjects chose between their cagemate and an empty cage, spending more time with the cagemate as expected [23]. CNO administration did not affect this behaviour (Fig. 5A-B). In the social novelty test, animals chose between familiar and unfamiliar conspecifics, spending more time with the unfamiliar one; CNO did not alter this preference (Fig. 5C-D).

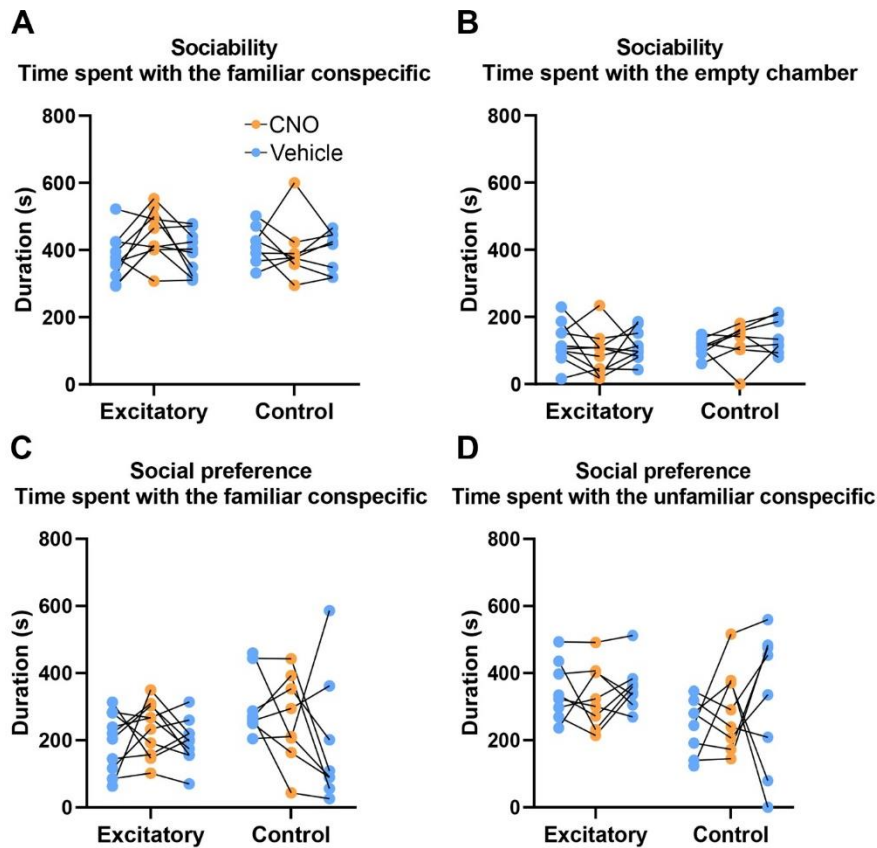


Figure 5. Sociability and social preference of the subject animals. (A-B) The administration of CNO did not cause any changes in the sociability of the subject animals ($F(2, 32) = 1.453$ and $F(2, 32) = 1.061$). CNO administration did not alter the social preference of the animals ($F(2, 32) = 1.797$ and $F(2, 32) = 0.8252$).

6.3. Anxiety-like and depression-like behaviours

I assessed anxiety- and depression-like behaviours to explore emotional influences on social interactions. CNO administration caused no significant changes in time spent across different areas of the open field arena (Fig. 6A-B) or in the total distance travelled in the excitatory group (Fig. 6C; excitatory group: $F(1.586, 33.31) = 2.507 \times 10^{-9}$, control group: $F(1.968, 35.43) = 2.446 \times 10^{-9}$). Both excitatory and control groups preferred the closed arm over the centre and open arm, with no changes in anxiety levels due to DREADD activation (Fig. 6D; $F(2, 45) =$

25.96). In the forced swim test, both groups spent more time swimming than floating, with no effect from DREADD activation (Fig. 6E; $F(2, 28) = 32.38$).

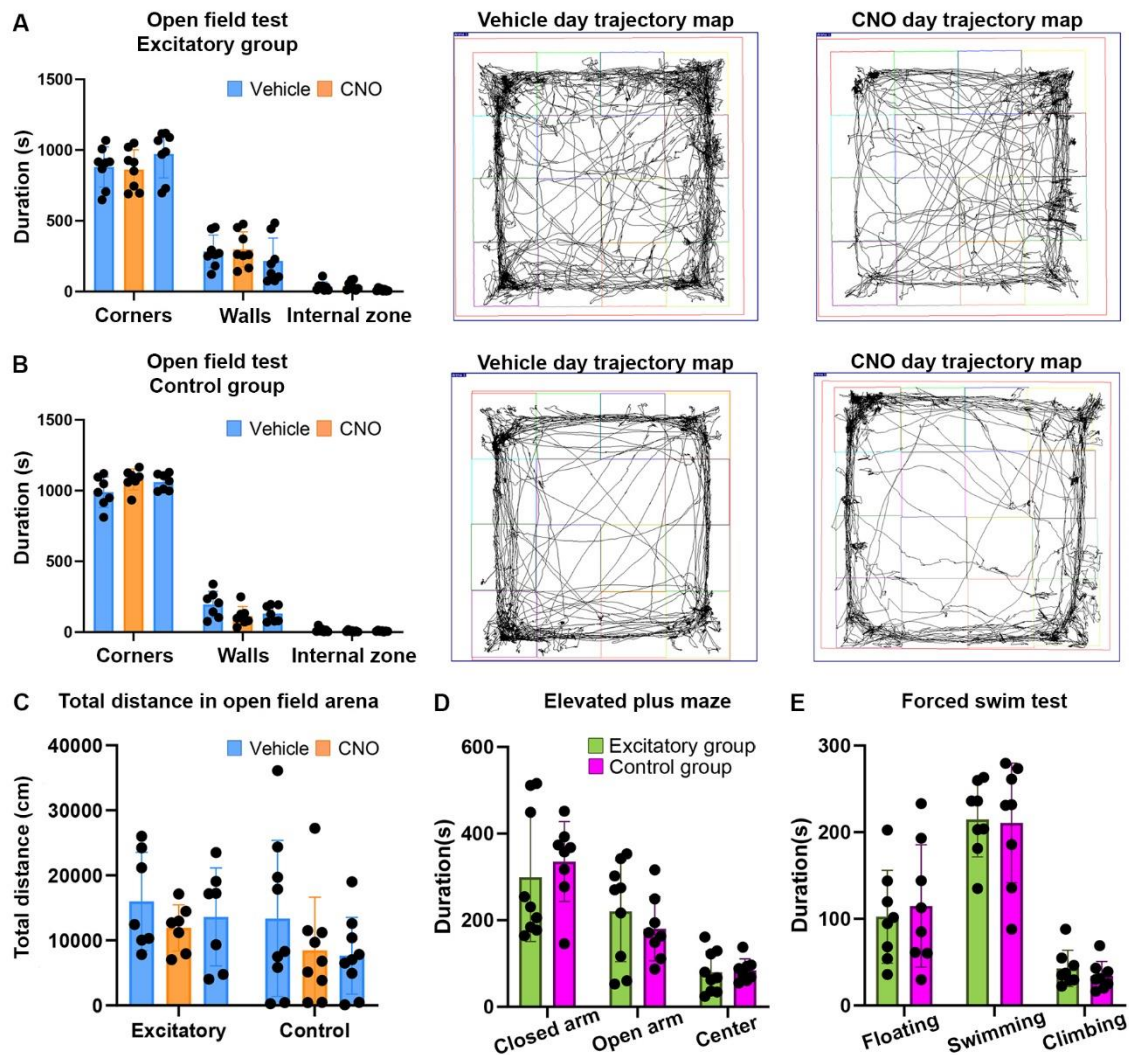


Figure 6. The effect of chemogenetic stimulation on anxiety- and depression-like behaviour. (A-B) The activation of MPOA OTR⁺ neurons did not alter the anxiety level of the animals compared to the control days or the control group. (C) The two groups also showed similar locomotor activity (Two-way ANOVA followed by post-hoc Tukey test, $F(1.566, 21.92) = 4.170$). (D) The activation of DREADDs did not result in significant changes in the elevated plus maze compared to the control group. (E) The activation did not cause any significant changes in the forced swim test compared to the control group.

6.4. Activity of MPOA OTR⁺ neurons during direct social interaction

I analysed changes in the normalized $\Delta F/F_0$ signal in MPOA OTR⁺ neurons using fiber photometry and examined fluorescence intensity, with the aim of analysing fluorescence intensity in correlation with social behavioural elements (Fig. 7A). The baseline was compared to the area under curve (AUC), which was determined between 0-1.5 seconds after the start of

the particular behaviour (Fig. 7B, C). For the analysis, baseline was calculated by averaging the fluorescent intensity values 2 seconds before the initiation of the behaviour. In the case of anogenital sniffing, the AUC was significantly larger than baseline ($p < 0.01$), using a two-tailed paired t-test ($N = 27$, Mean of the baseline: 0.5255, Mean of the AUC: 148.8, Difference between means (AUC - Baseline) $\pm$ SEM: $148,3 \pm 47,14$). However, no increase in intensity signal was observed during other behavioural elements.

6.5. The order of behavioural elements during social interaction in rats

To determine if anogenital sniffing, during which an increased activity of MPOA OTR+ neurons was found is specifically followed by particular behavioural elements, I analysed the alternations of behavioural elements following chemogenetic stimulation of MPOA OTR+ neurons. The total number of behavioural elements was 3670, which I used to determine the occurrence of a behavioural element from the total elements. I found that anogenital sniffing serves as the initial phase of a behavioural sequence. There were cases where anogenital sniffing (Total No.: 200) was followed by these behavioural elements: body-to-body contact (alternations: 32, Total No.: 517), mounting (alternations: 10, Total No.: 362), moving away (alternations: 3, Total No.: 120), body sniffing (alternations: 8, Total No.: 346), social proximity (alternations: 54, Total No.: 650), non-social behaviour (alternations: 24, Total No.: 485), chasing (alternations: 60, Total No.: 419). The expected occurrence of chasing after anogenital sniffing is 22, while for social proximity, it is 35. However, the observed occurrences were 60 for chasing and 54 for social proximity. Based on the results of the binomial test ($p < 0.0001$), I could confidently reject the possibility that these deviations are due to random chance. I analysed further the chasing behaviour and found that it is followed by mounting (alternations: 85, Total No.: 362), anogenital sniffing (alternations: 65, Total No.: 200), body sniffing (alternations: 99, Total No.: 346) or body-to-body contact (alternations: 72, Total No.: 517). The expected occurrence of mounting after chasing was 41, while for anogenital sniffing was 22, for body sniffing was 39 and for body-to-body contact was 59. In contrast we observed 85 chasing $\rightarrow$ mounting alternations ($p < 0.0001$), 65 chasing $\rightarrow$ anogenital sniffing alternations ($p < 0.0001$) and 99 chasing $\rightarrow$ body sniffing alternations ($p < 0.0001$), the body-to-body contact was not significant. So the chasing is followed by mounting or body sniffing, or alternatively, the animal returning to anogenital sniffing. As the following step I analysed the events after mounting behaviour. After mounting, the animals preferred to engage in the following behaviours: chasing (alternations: 79, Total No.: 419), grooming (alternations: 59, Total No.: 106), body-to-body contact (alternations: 81, Total No.: 517), social proximity

(alternations: 88, Total No.: 650). The results of the binomial test are chasing significantly follows mounting ($p < 0.0001$), its expected occurrence was 41, but I observed 79 events; grooming also significantly follows mounting ($p < 0.0001$), as the expected occurrence of grooming was 10, but we observed a remarkably higher number, 59. Body-to-body contact followed mounting 81 times, but the expected number of this event was 51, these two behavioural elements significantly ($p < 0.0001$) alternate. Finally, social proximity was also frequent after mounting, we observed 88 social proximity events, however the expected number of this behavioural element was 64, it still significantly ($p = 0.0018$) follows mounting behaviour. Thus, the final phase of the behavioural sequence is grooming or body-to-body contact/social proximity interaction, which succeeds in mounting behaviour (Fig. 7D).

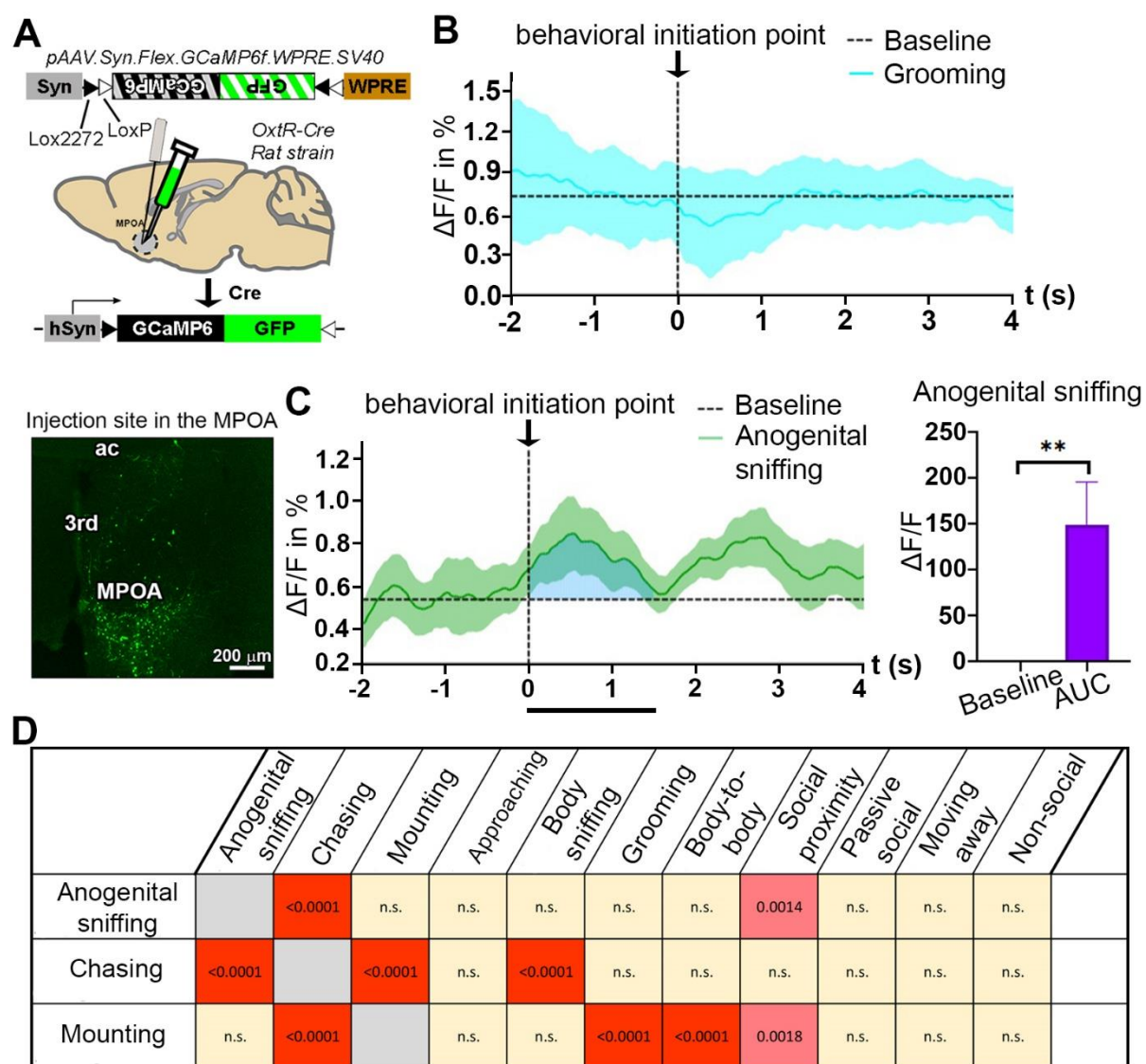


Figure 7. The changes of fluorescence intensity indicating neuronal activation of OTR+ neurons during social interaction, and the determination of subsequent behaviours. A:

Schematic illustration of the behaviour test paradigm and a representative image of the GCaMP injection into the MPOA. (B-C): Illustration of the analysis of the $\Delta F/F$ signal in case of grooming (B) and anogenital sniffing (C). The horizontal line indicates the mean time spent with the particular behaviour. The diagram shows the results of the statistical analysis of the comparison of baseline and AUC value (**: $0.001 < p < 0.01$, two-tailed paired t-test) between 0 to 1.5 s after the onset of anogenital sniffing. I also analysed the second fluorescence intensity peak between 1.5 to 3.4 s after the onset of anogenital sniffing, but the comparison of AUC and baseline was not significant albeit a tendency for increase is present ($p = 0.058$). The results indicate that OTR+ neurons are activated during anogenital sniffing, which typically last for 1.5 s. In contrast, there was no elevated activity during grooming (and other behavioural elements not shown in the figure). (D): Anogenital sniffing is significantly ($p < 0.0001$ with Binomial test) followed by chasing, and also significantly by mounting and social proximity. It was also found that chasing is highly significantly followed by anogenital sniffing, mounting and body sniffing. The end point of the behavioural chain is the grooming behaviour or side-to-side (body-to-body) contact or remaining in social proximity as these behavioural elements take place after mounting. Abbreviations: 3rd: third ventricle, ac: anterior commissure, AUC: area under curve, MPOA: medial preoptic area.

6.6. Oxytocin receptor-expressing neurons in the MPOA send projections to broad regions of the rat brain

MPOA OTR+ neurons project to several brain regions involved in maintaining homeostasis. They also project to brain regions involved in social behaviour, such as the ventrolateral subdivision of the PAG (vlPAG), ventral lateral septum (LSv) or ventral tegmental area (VTA). In addition, these cells also send projections to the PVN (Fig. 8).

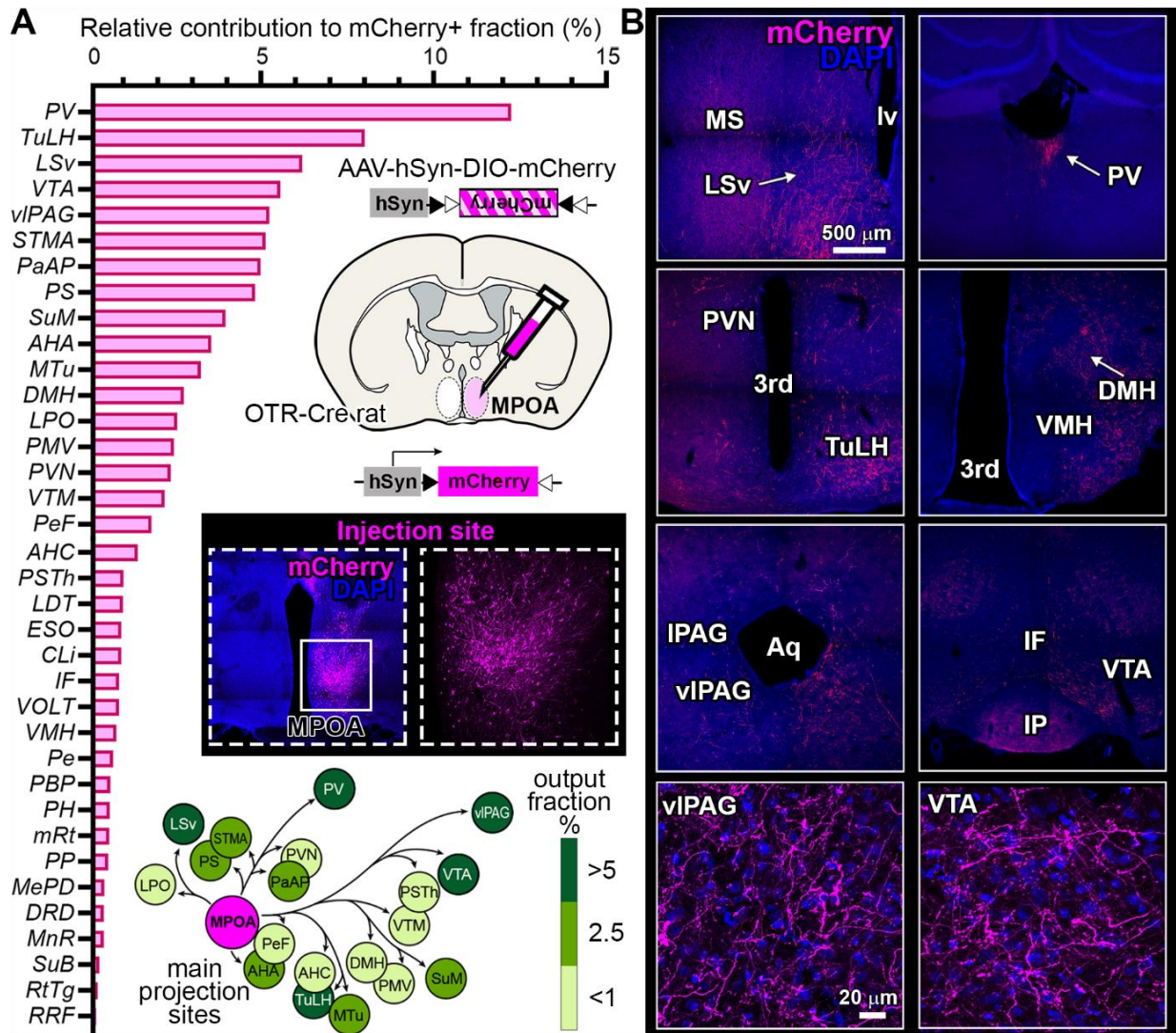


Figure 8. The target regions of the OTR+ medial preoptic neurons. (A) Relative fiber density of MPOA OTR+ neuronal projection targets. The confocal microscopic images show the injection site of the viral vector. (B) Some of the target areas are demonstrated. High density of mCherry+ fibers are located in the LSv, PVN, MTu, DMH, VTA and vIPAG. Abbreviations: 3rd: third ventricle, Aq: aqueduct, AHA-AHC: anterior hypothalamic area, anterior-central part; CLi: caudal linear nucleus of the raphe, DMH: dorsomedial hypothalamus, IF: interfascicular nucleus, DRD: dorsal raphe nucleus, dorsal part, ESO: episupraoptic nucleus, LDT: laterodorsal tegmental nucleus, LPO: lateral preoptic area, MnR: median raphe nucleus, MePD: medial amygdaloid nucleus, posterodorsal part, MPOA: medial preoptic area, mRt: mesencephalic reticular formation, MS: medial septum, MTu: medial tuberal nucleus, PBP: parabrachial pigmented nucleus of the ventral tegmental area, PaAP: paraventricular hypothalamic nucleus, anterior parvicellular part, Pe: periventricular nucleus, PeF: perifornical nucleus, PH: posterior hypothalamic nucleus, PMV: premammillary nucleus, ventral part, PP: peripeduncular nucleus, PS: parastrial nucleus, PSTh: parasubthalamic nucleus, PV: paraventricular thalamic nucleus, PVN: paraventricular nucleus, RRF: retrorubral field, RtTg: reticulotegmental nucleus of the pons, STMA: bed nucleus of the stria terminalis, medial division, anterior part, SuB. subbrachial nucleus, SuM: supramammillary nucleus, TuLH: tuberal part of the lateral hypothalamus, vIPAG: ventrolateral subdivision of the periaqueductal grey, VMH: ventromedial hypothalamic nucleus, VOLT: vascular organ of the lamina terminalis, VTA: ventral tegmental area, LSv: ventral lateral septum, VTM: ventral tuberomammillary nucleus.

Next, we examined 3 target areas involved in social behaviour by the OTR system [3]. In the VTA, we revealed that some TH positive dopaminergic neurons (Fig. 9A1-A2) and calbindin neurons (Fig. 9B) are surrounded by fibers projected from the MPOA. We further described similar localization patterns of mCherry+ fibers in the vIPAG (Fig. 9C1-C3) and LSv (Fig. 9D1-3) in association of calbindin neurons.

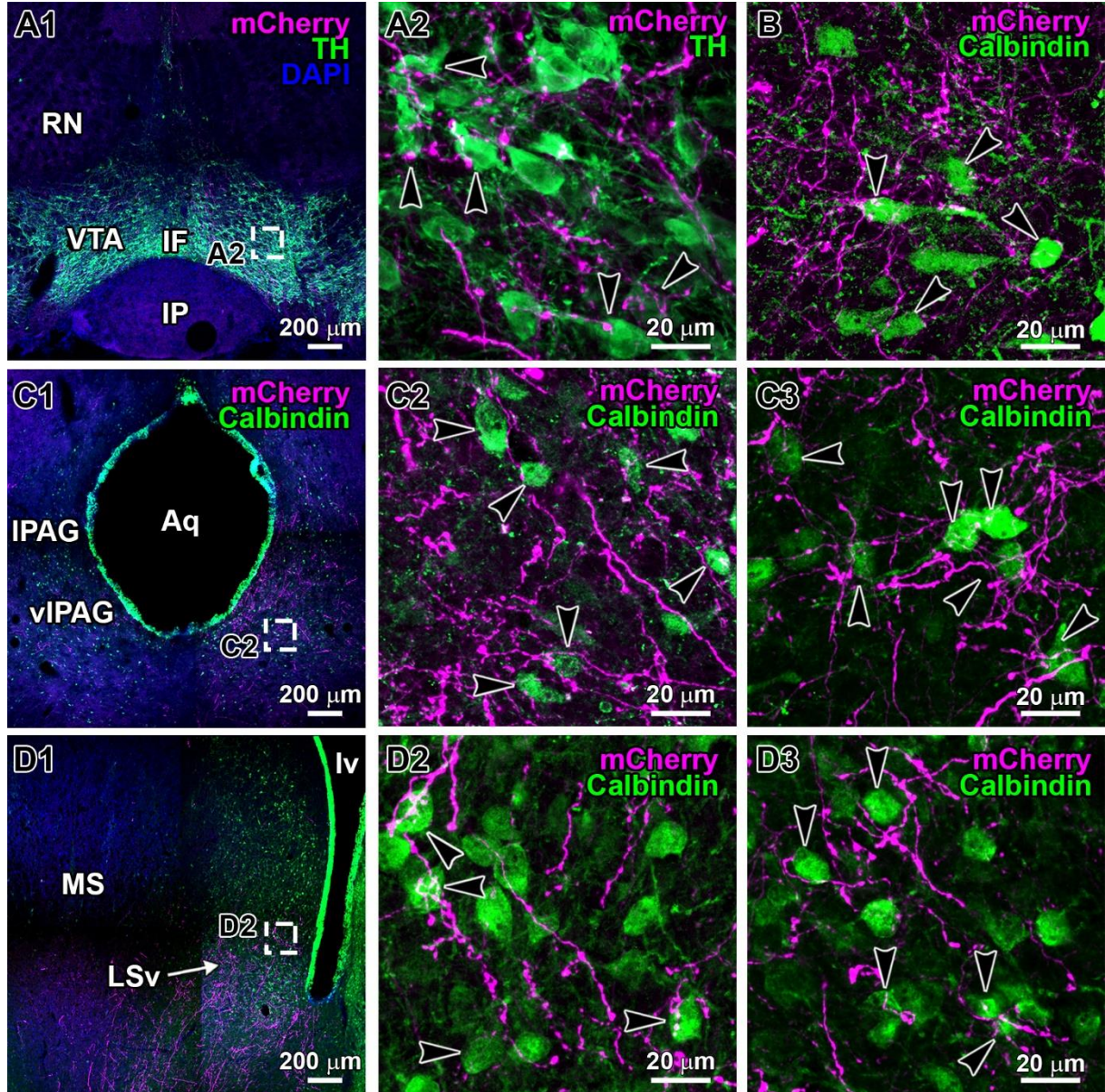


Figure 9. Projection targets of OTR+ preoptic neurons surrounded in the VTA and vIPAG. (A-B) VTA-projecting MPOA mCherry+ fibers located close to TH+ (A1-2) and calbindin (B) cells. (C-D) Calbindin neurons are closely surrounded by mCherry+ fibers in the vIPAG (C) and LSv (D), although calbindin neurons are not densely located in the area. Abbreviations: Aq: aqueduct, IF: interfascicular nucleus, IP: interpeduncular nucleus, LSv: lateral septum, ventral subdivision, IPAG: lateral periaqueductal grey, MS: medial septum, RN: red nucleus, TH: tyrosine-hydroxylase, vIPAG: ventrolateral periaqueductal grey, VTA: ventral tegmental area.

Since MPOA neurons receive touch-related input from PTH2+ neurons in the PIL, I examined whether OTR neurons are modulated by this system. PTH2 immunolabeling of brain slices showed a high number of PTH2+ fibers near mCherry+ cell bodies and processes (Fig. 10A-B).

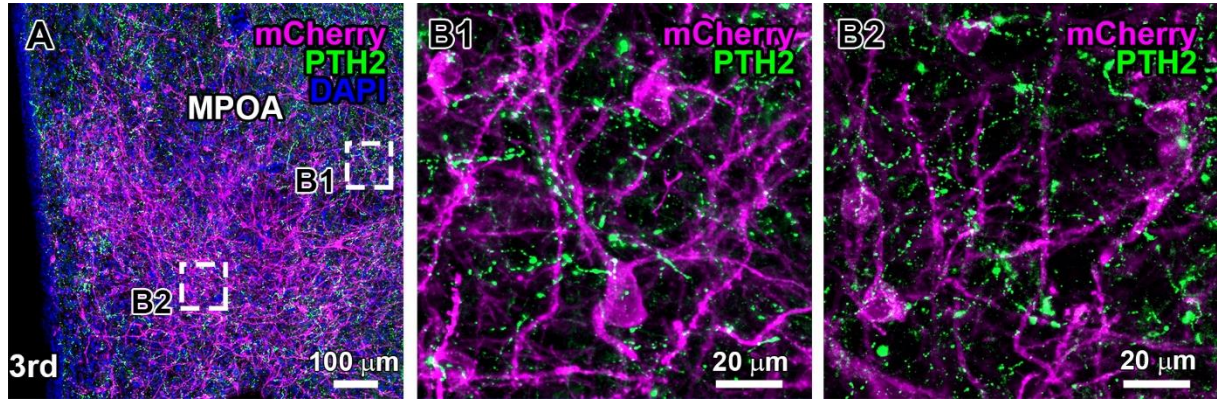


Figure 10. PTH2+ terminals around oxytocin receptor neurons in the MPOA for potential delivery of social input. (A) Low magnification image shows the injection site of the viral tracer in the MPOA to show OTR+ neurons. (B1, B2) High magnification images demonstrate prominent PTH2+ network in the area with OTR+ neurons being closely opposed by PTH2+ terminals. Abbreviations: PTH2: parathyroid hormone-2, MPOA: medial preoptic area, 3rd: third ventricle.

7. Discussion

7.1. Histological verification of chemogenetics

Histological analysis of viral injection sites confirmed the presence of OTR⁺ neurons in the MPOA, consistent with previous rat studies using in situ hybridization histochemistry [24], [25]. This also supports the hypothesis that OTR distribution in rats mirrors that in mice, where genetic methods have shown OTR expression in the MPOA [26], [27]. Injection sites were verified for each animal to avoid mistargeting outside the MPOA.

The efficacy of DREADDs was assessed using c-Fos immunohistochemistry [28], [29] with most DREADD-expressing MPOA neurons showing c-Fos positivity after CNO injection, indicating effective activation. This also verifies the CNO dose was sufficient for action at the target site.

7.2. Behavioural effect of chemogenetic stimulation of oxytocin receptor neurons

The specificity of the chemogenetic results was ensured by including two different controls in the behavioural experiments: first, vehicle was injected, followed by testing; the next day, CNO was administered, and tests were repeated. This allowed for within-animal comparison, controlling for individual differences. Tests were repeated two days later with vehicle to confirm CNO-induced changes returned to baseline, indicating no long-term effects or spontaneous shifts in social behaviour. Additional controls used rats with a virus expressing only the fluorescent protein (no DREADD), showing no changes with CNO, confirming that the observed effects were specific to DREADD activation.

The rats predominantly engaged in social behaviours throughout the test, consistent with previous studies [30], [31], confirming normal behaviour in our experiments. Since the experimental animals were familiar females, no aggression was observed, as expected. Chemogenetic activation of MPOA OTR⁺ neurons increased affiliative behaviours like body contact, grooming, and mounting, suggesting that these neurons promote positive social interactions [32]. This is the first evidence of a role for MPOA OTR⁺ neurons in rats and mice. Importantly, control experiments showed no changes in sociability, anxiety, or depression-like behaviour following CNO treatment, indicating that MPOA OTR⁺ neuron activity specifically enhanced direct social contact without affecting overall social behaviour or emotional states of the animals.

7.3. Inputs to MPOA OTR+ neurons and their potential functions

Our findings show OT fibers in the MPOA alongside OTR+ neurons, suggesting OT release in this region to activate OTR+ neurons. Since OTRs are excitatory G-protein coupled receptors, OT is likely to activate these neurons [18], [33], [34]. These results suggest that MPOA OTR+ neurons may contribute to the affiliative behaviour increasing effect of OT. Another potential source of input affecting OTR+ neurons in the MPOA is the PTH2-containing fiber terminals ascending from the posterior intralaminar thalamic nucleus [35], [36]. PTH2 terminals were observed to be in close apposition to MPOA OTR+ neurons, and the PTH2 receptor (PTH2R) is known to be expressed in the MPOA [37], thereby providing the possibility that PTH2 can affect OTR neurons via the PTH2R, which is also a stimulatory G-protein coupled receptor [38]. Furthermore, PTH2+ neurons contain glutamate [39] indicating that the PIL to MPOA pathway may utilize the excitatory amino acid neurotransmitter, potentially on OTR+ neurons, too. The PIL-MPOA pathway, which facilitates allogrooming behaviour [10], may transmit somatosensory input related to social touch [40]. Our data provide new insights into the PTH2+ pathway's role in the MPOA by identifying OTR+ neurons as one of their preoptic targets.

7.4. Activity of MPOA oxytocin receptor neurons during social behaviours

The c-Fos technique has shown MPOA neuron activation during affiliative social interactions in female rats [10], but its limited temporal resolution and inability to label OTR+ neurons hindered behavioural correlations. To overcome this, we used fiber photometry to correlate OTR+ neuronal activity with different behavioural elements. OTR+ neuronal activity was increased only during anogenital sniffing, linking this behaviour to MPOA OTR+ neuronal activation. The elevated activity could be sustained beyond the end of anogenital sniffing, as a trend of increased OTR+ activation persisted until 3.4 s after the onset of anogenital sniffing, a period exceeds the typical duration of anogenital sniffing. This study is the first to show a correlation between anogenital sniffing and OTR+ neuronal activation. Previous research using fiber photometry or microendoscopy has only linked social behaviours with the activity of glutamatergic or GABAergic neurons during social isolation or rebound [8]. We also determined the order of social behavioural elements. We found that anogenital sniffing is most frequently followed by chasing, which is in turn followed by mounting and then allogrooming, a behavioural transition which has been previously described [41], [42]. These findings are consistent with our results of increased chasing, mounting and grooming behaviours in response to chemogenetic stimulation of MPOA OTR+ neurons. Taken together, these the two

approaches suggest that a physiological function of MPOA OTR+ neurons may be to initiate a behavioural cascade of chasing, mounting and grooming following anogenital sniffing.

7.5. Projections of MPOA oxytocin receptor neurons and their target cells' potential functions

The anterograde spread of the DREADD-mCherry fusion protein enables the identification of the projection sites of specific cell types [43], [44], [45]. In the present study, MPOA OTR+ neurons were observed to project to a diverse range of brain regions, with the highest density of anterogradely labelled fibers detected in the LSv, the vIPAG, and the VTA. These are all brain regions that constitute the social brain network, which is responsible for the control of social behaviours [46]. The LSv is a limbic region that may be involved in the emotional processing of social interactions [47], [48]. The vIPAG is an executive member of the social brain network that possesses motor output towards brainstem and spinal cord motoneurons as part of the descending emotional motor system [49]. The VTA represents the initial point of the brain reward system, exhibiting dopaminergic projections to the accumbens nucleus and the medial prefrontal cortex for reward processing [50] and has been shown to play a role in social behavioural modulation [51], [52]. It has been shown that VTA-projecting OT neurons of the paraventricular hypothalamic nucleus promote affiliative social interaction [53] MPOA OTR+ neurons also project to the VTA and are activated during social play in juvenile rodents [11]. Given that we have demonstrated the close apposition of VTA dopaminergic neurons by terminals of MPOA OTR+ neuronal projections, it is probable that this projection also conveys the rewarding value of social interactions to further promote social interactions (Fig. 11).

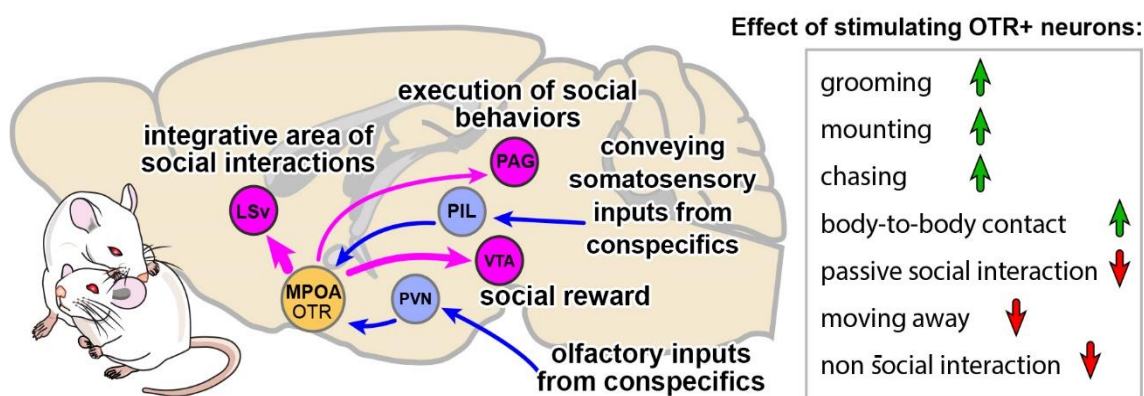


Figure 11. The MPOA receives somatosensory and olfactory inputs from the PIL and PVN, modulating social behaviours. MPOA OTR+ neurons project to the PAG, LSv, and VTA, key areas for affiliative behaviour and the social reward system. Our results highlight the MPOA's role in social behaviour, as stimulation of OTR+ neurons increased the animals' interest in conspecifics, spending more time in social contexts.

8. Conclusion

In conclusion, we identified OTR neurons and their inputs in the preoptic area of female rats, implicating them in the regulation of affiliative social interactions. Anogenital sniffing activated MPOA OTR⁺ neurons, which, when stimulated, increased chasing, and direct contact behaviours, such as mounting and allogrooming. This suggests a potential function of MPOA OTR⁺ neurons in facilitating these direct contact behaviours following anogenital sniffing [40], [41]. Therefore, the presented work contributes to the understanding of social interaction regulation.

9. Author contribution

Hereby, I, Máté Egyed, declare that:

- I performed the stereotaxic surgeries for the chemogenetic, neuronal tract tracing and fiber photometry experiments and carried out the behavioural tests under proper supervision.
- I recorded videos during the tests and analysed the behaviour of the animals manually (Solomon Coder) or with automated software (Smart) and processed the results with GraphPad Prism.
- I carried out brain sample processing, including perfusion, sectioning brain samples with a cryostat, and performed immunohistological procedures, such as peroxidase-based and fluorescent immunolabeling.
- I acquired images using both light and confocal microscopes; for neuronal tract-tracing I also examined the confocal images using QuPath.

I hereby declare that I have not previously won a TDK conference. If I am awarded a place at another TDK conference before the current BTDK Conference, I will inform the organizers of the BTDK before the conference and will send the winning essay to them.

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