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Thalamo-preoptic projections controlling affiliative and aggressive social behaviours in rodents

PhD thesis

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List of abbreviations.

1. 3V – 3rd ventricle
2. 4V – 4th ventricle
3. ac – Anterior commissure
4. aca – anterior part of the anterior comissure
5. APT – Anterior pretectal area
6. ASt – Amygdalostriatal transition area
7. BLA – Basolateral amygdala
8. BMA – Basomedial amygdala
9. ca – Cerebral aqueduct
10. CeA – Central amygdala
11. Cx – Cerebral cortex
12. DMH – Dorsomedial hypothalamic nucleus
13. DLPAG – Dorsolateral periaqueductal grey
14. DMPAG – Dorsomedial periaqueductal grey
15. DR – Dorsal raphe nucleus
16. DTg – Dorsal tegmental nucleus
17. ECIC – External cortex of the inferior colliculus
18. f – Fornix
19. fmi – Forceps minor of the corpus callosum
20. ILC – Infralimbic cortex
21. LaDA – Lateral amygdaloid nucleus, dorsal anterior part
22. LPAG – Lateral periaqueductal grey
23. LPB – Lateral parabrachial nucleus
24. LS – Lateral septal nucleus
25. LT – Laterla thalamus
26. MeA – Medial amygdala
27. MG – Medial geniculate body
28. ml – medial lemniscus
29. MPA – Medial preoptic region
30. MPN – Medial preoptic nucleus
31. MPOA – Medial preoptic area
32. MS – Medial septal nucleus
33. Och – Optic chiasm
34. PAG – Periaqueductal central grey

35. PBL – Parabrachial nucleus
36. PH – Posterior hypothalamic nucleus
37. PIL – Posterior intralaminar thalamic nucleus
38. PnO – Pontine reticular nucleus, oral part
39. PP – Peripeduncular area
40. PTH2 – Parathyroid hormon 2
41. PVN – Paraventricular nucleus
42. scp – Superior cerebellar peduncle
43. SG – Supragenicula te nuclues
44. SN – Substantia nigra
45. TIP39 – Tuberoinfundibular peptide of 39 residues
46. TuLH – Tuberal region of lateral hypothalamus
47. vBNST – Ventral bed nucleus of stria terminalis
48. VLPAG – Ventrolateral periaqueductal grey
49. VMH – Ventromedial hypothalamic nucleus

1. INTRODUCTION

1.1 Social behaviours

Social behaviour is defined as the interaction between an animal and one or more conspecifics. In the context of interaction between members of the same species, there is a demonstrable alteration in the animal's behaviour, with the manifestation of distinctly delineated behavioural patterns. In contrast to humans, rodents' social behaviours are predominantly innate and governed by subcortical regions. However, certain components of social behaviours, such as internal state recognition and empathy-like behaviours associated with cortical functions, have been observed in rodents in select cases (1).

In rodents, a variety of innate social behaviours can be observed when two conspecifics encounter each other. The following are examples of affiliative (or friendly) social behaviours exhibited by adults in a familiar context; (2) aggressive behaviours between males; (3) sexual behaviour between opposite-sex adults; (4) social play between adolescent animals; and parental behaviour between an adult female and pups (5). To elaborate, a wide array of behaviours may transpire between two animals. The aforementioned examples represent merely the most commonplace cases. For instance, while affiliative behaviour manifests predominantly among female rats, familiar male rats have been observed to exhibit affiliative behavioural elements as well. The behaviour of the mother is the primary contributor to the overall care of the offspring. However, in certain species, the father may also assume a role in nurturing the pups (2, 3). A review of the extant literature reveals that rodents of both sexes may exhibit aggression (4), which is, however, more prevalent between males. In addition, mothers also display aggression in the case of a potential threat, such as an unfamiliar male conspecific (5).

The role of sensory inputs in regulating social behaviour in animals has been well-documented. However, the precise mechanisms through which these sensory signals interact with each other to influence behaviour remain to be fully elucidated. As demonstrated in the extant literature, olfactory cues, such as pheromones (6), and somatosensory inputs are of great importance in rodents (7). Vocalization as an auditory input also plays a significant role in regulating rodent innate behaviours, such as the influence of pup vocalization during parental care and ultrasonic vocalization (USV) during mating and aggression (8).

Despite the numerous novel discoveries that have emerged in recent years, the neuronal regulation of innate behaviours remains to be established.

1.1.1 Affiliative social behaviours

Affiliative social behaviour is a type of prosocial behaviour that is commonly observed among familiar conspecifics. Affiliative behaviour is defined as friendly interaction between animals, which typically includes allogrooming, side-to-side contacts, body sniffing and anogenital sniffing (9). While the major brain regions controlling affiliative behaviours have not been established, a recent paper suggested the central role of the MPOA in its regulation (10). The phenomenon of affiliative behaviour has been demonstrated to be rewarding to the animals (10).

1.1.2 Aggressive behaviour

Aggressive behaviour is defined as a form of antisocial behaviour typically exhibited by unfamiliar adult male animals. Aggressive behaviour is a typical manifestation of the animal's endeavour to establish resources such as territory, sustenance, hierarchical structure, and females for the purpose of procreation (11). The behavioural elements that are most frequently displayed are those that fall under the category of "aggression." These elements include fighting, in which the animals typically engage in acts of boxing, kicking, and brawling with each other. Furthermore, mounting and biting behaviours are also frequently exhibited during instances of aggression (9). Male laboratory rats typically do not display aggressive behaviour towards their peers. However, aggression can be induced by social isolation. Rodents exhibit elevated levels of aggression when isolated from their conspecifics for extended periods, typically spanning over two weeks. However, this temporal element is subject to variation between different species and strains (12). Aggressive behaviour is generally contained, as conspecifics do not inflict severe harm upon each other (13).

The phenomenon of aggression is governed by a multitude of cerebral regions including the primary regulator ventromedial hypothalamic nucleus (VMH), but also the medial (MeA) and central amygdaloid nuclei (CeA) (14), and the periaqueductal gray (PAG), which are facilitated by the aforementioned VMH through the transmission of afferent signals (15).

1.2 Brain structures that are involved in the control of social behaviours

The regulation of social behaviours is orchestrated by various cerebral regions, collectively termed the social brain network (16). Despite the multifaceted nature of the regulatory mechanisms governing social behaviour, which encompasses numerous brain regions (17), the ensuing discussion will focus exclusively on those brain regions that have been the subject of investigation in the studies underpinning this thesis. Thus, the posterior intralaminar thalamic nucleus (PIL), the medial preoptic area (MPOA) and the ventromedial hypothalamic nucleus (VMH) are discussed.

1.2.1 Posterior intralaminar thalamic nucleus (PIL)

The PIL is a thalamic nucleus located at the posterior part of the thalamus, ventromedial to the medial geniculate body (MG), ventrolateral to the anterior pretectal nucleus (APT), and dorsal to the substantia nigra (SN).

The PIL is the recipient of multimodal sensory inputs, including somatosensory information from the spinal cord (thoracic and lumbar regions), as well as from the cuneate and gracile nuclei. Additionally, it receives auditory inputs from the external cortex of the inferior colliculus (ECIC) and hypothalamic inputs from the VMH (18).

PIL neurons have been found to express calbindin, a protein that can be used to distinguish these neurons from their surrounding area (19). In rats, the medial part of the PIL has been observed to contain neurons that express a neuropeptide known as parathyroid hormone 2 (PTH2) (Figure 1). This neuropeptide was previously designated tuberoinfundibular peptide 39 (TIP39) (20). It has been demonstrated that these neurons may play a regulatory role in lactation in mother rats (21). PIL neurons were shown to convey auditory inputs from the inferior colliculus to the paraventricular hypothalamic nucleus in mother mice. These neurons promote lactation in response to pup vocalization (22).

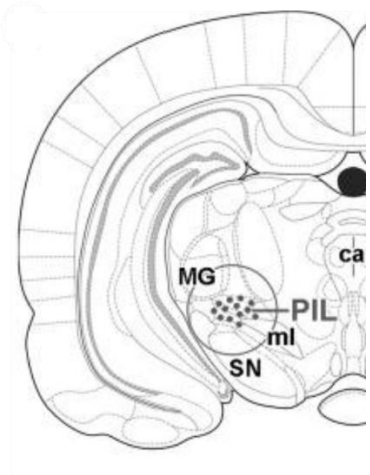

Figure 1. Localization of PTH2 neurons in the PIL

PIL neurons have been found to express PTH2 neuropeptide. In contrast, the surrounding brain areas have not been observed to express this neuropeptide. Gray dots in the figure represent the PTH2-expressing neurons in the PIL. Source:(23)

1.2.2 Medial preoptic area (MPOA)

The MPOA is a hypothalamic region located in the rostral part of the hypothalamus, between the anterior commissure and the optic chiasm. The structure under consideration is adjacent to the third ventricle on the medial aspect and to the lateral preoptic region on the lateral aspect.

The chemoarchitecture of the medial preoptic area consists of both GABAergic and glutamatergic neurons. It has been determined that the GABAergic cell population of the MPOA can promote maternal care in mice, and the glutamatergic MPOA neurons can suppress this behaviour (24). Additionally, research has indicated that a population of GABAergic neurons in the MPOA that express oestrogen receptor 1 (ESR1) projects to the ventromedial hypothalamus, thereby modulating aggression via this pathway (25).

The MPOA has been demonstrated to play a crucial role in the regulation of body homeostasis and controlling social behaviours such as parental, male sexual, affiliative and aggressive behaviours (8, 10, 24-26) (Figure 2).

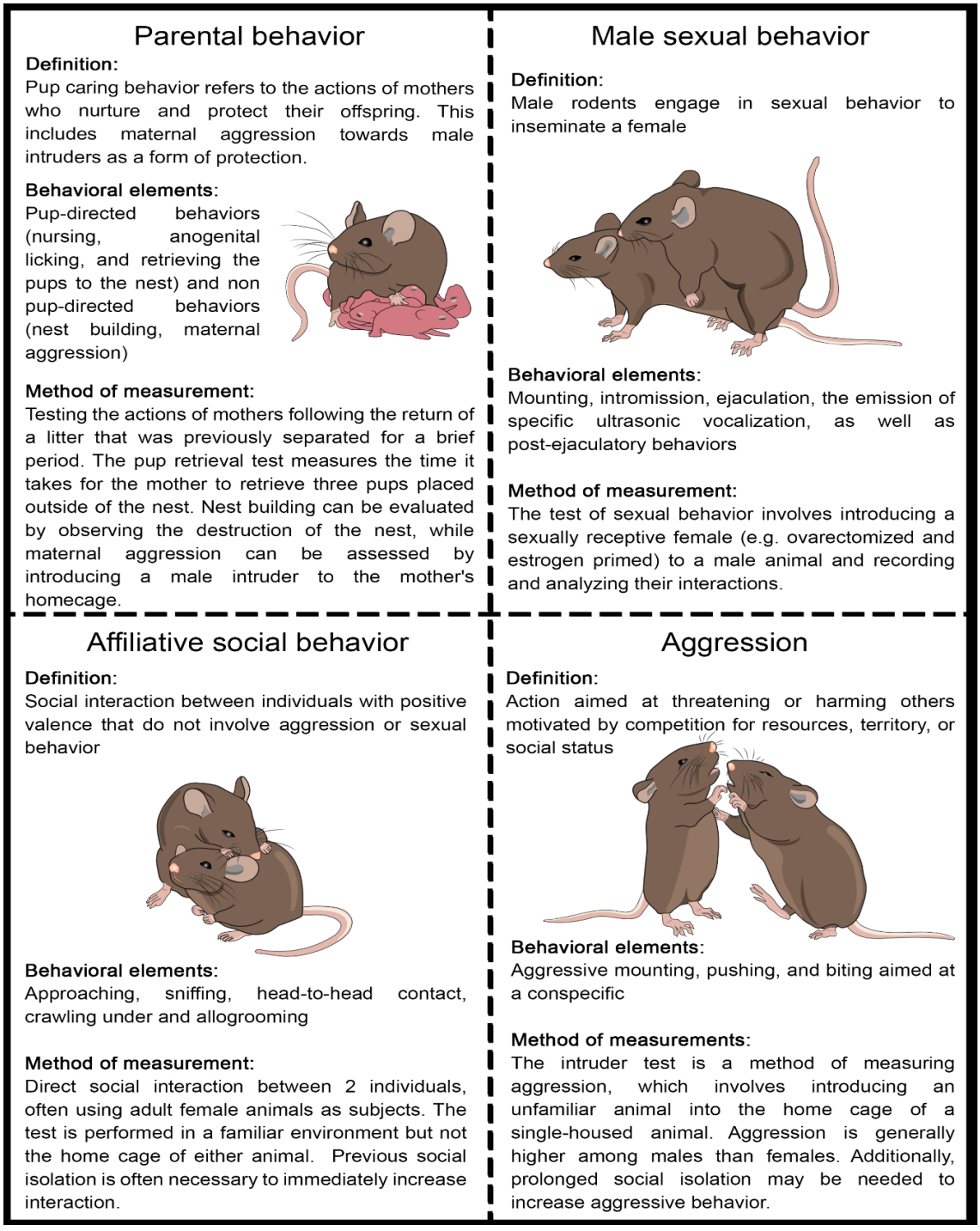

Figure 2. Summary of different types of innate social behaviour governed by the medial preoptic area (MPOA). The figure illustrates four primary categories of social behaviour influenced by the MPOA: parental behaviour, male sexual behaviour, affiliative behaviour, and aggressive behaviour. The figure incorporates the most prevalent behavioural elements, and the methodologies employed to measure each type of social behaviour. Source:(27)

1.2.3 Ventromedial hypothalamic nucleus (VMH)

The VMH has been identified as a critical region in the promotion of aggressive behaviour. The VMH can be subdivided into three regions: the dorsomedial, central, and ventrolateral parts. The ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) has been demonstrated to be implicated in the modulation of aggression. As demonstrated in the extant literature, optogenetic stimulation of this area has been shown to increase aggression, while inhibition of the VMHvl has been demonstrated to reduce aggression in mice (28).

The VMH is comprised predominantly of glutamatergic neurons; however, GABAergic projection neurons have also been documented in this region. The present study hypothesizes that glutamatergic neurons projecting to the PAG are a causative factor in aggressive behaviour (15). A neuronal population that express estrogen receptor alpha (ERα) (29) has been identified as playing a crucial role in regulating aggression (30).

The VMH has been shown to receive olfactory inputs, which is a potential catalyst for aggressive behaviour (31). Nevertheless, further research is necessary to determine the extent to which other sensory modalities, such as somatosensory or visual cues, contribute to aggressive behaviour.

2. OBJECTIVES

Characterization of the anatomical properties of PIL neurons and their projections to the MPOA

- Mapping the brain projections of neurons originating from the PIL.
- Characterisation of MPOA neurons closely apposed by PIL neurons.

Functional investigation of the PIL and the PIL to MPOA pathway during affiliative behaviour

- Investigation of the role of PIL neurons in regulating affiliative behaviours in male rats.
- Characterization of the functional contribution of the PIL to MPOA pathway to affiliative behaviour.
- Fiber photometric measurement of the activity of PIL neurons projecting to the MPOA during social encounters.

Exploration of the involvement of PIL neurons and the PIL to MPOA pathway in aggressive behaviours

- Assessment of the impact of chronic social isolation on the social behaviour in male rats.
- Manipulation of PIL neurons that are activated during aggressive interactions using an activity-dependent tagging technique.
- Investigation of the c-Fos activation of the PIL and its target areas in response to direct experimental manipulation of PIL activity.
- Determination of the role of the PIL–MPOA pathway in the regulation of aggressive behaviour.

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3. METHODS

3.1 Animals

Research on affiliative social behaviour has utilized adult female and male Wistar rats. Conversely, the evaluation of aggression was conducted using adult male Wistar rats. To investigate GABAergic neurons in the MPOA, the VGAT-ZsGreen transgenic mouse line was utilized. The line under discussion was created by crossing VGAT-Cre mice with a ZsGreen reporter line. This process resulted in offspring that expressed the ZsGreen fluorescent protein in GABAergic cells. A total of 143 animals were utilized in the course of the studies, including 18 female Wistar rats, 30 female VGAT-ZsGreen mice, and 95 male Wistar rats. The Workplace Animal Welfare Committee of the National Scientific Ethical Committee on Animal Experimentation at Semmelweis and Eötvös Loránd Universities, Budapest, specifically approved this study (PE/EA/926-7/2021 and PE/EA/568-7/2020, respectively). Thus, the procedures involving rats and mice were carried out according to experimental protocols that meet the guidelines of the Animal Hygiene and Food Control Department, Ministry of Agriculture, Hungary (40/2013), which is in accordance with EU Directive 2010/63/EU for animal experiments.

3.2 Tracer injections

The posterior intralaminar thalamic nucleus (PIL) was targeted and injected with the anterograde neuronal tracer biotinylated dextran amine (BDA). The stereotactic coordinates for the PIL were determined relative to the bregma point: anteroposterior (AP) = -5.2 mm, mediolateral (ML) = +2.6 mm, and dorsoventral (DV) = -6.8 mm, the latter measured from the dura mater. To gain access to the PIL, a hole was drilled at these coordinates, and a micropipette containing a 10% solution of BDA in phosphate buffer (PB) was administered via iontophoresis. The iontophoretic current was set at +6 mA, with a pulse duration of 7 seconds on and 7 seconds off, applied for a period of 15 minutes. Subsequent to this, the pipette was maintained in its position for a further 10 minutes without current. Thereafter, it was withdrawn under negative current. Injections that were misplaced served as controls.

3.3 Immunohistochemistry

Subsequent to transcardial perfusion, the brains were sectioned into 40 µm thick coronal slices. A subsample of each fifth section was selected for immunohistochemical analysis. Free-floating sections were subjected to a treatment comprising 3% hydrogen peroxide for a duration of 15 minutes. Subsequently, the sections were treated with PB containing 0.5% Triton X-100 and 3% bovine serum albumin for a duration of 1 hour. Subsequently, primary antibodies were applied to the sections for a duration of 12 hours. Thereafter, an incubation in phosphate-buffered saline (PBS) containing secondary antibodies was conducted for a period of 60 minutes. The following procedure was carried out: the sample was left for one hour in a solution of avidin-biotin complex (ABC, 1:500). The verification of viral injection sites was achieved through Ni-DAB visualization using chicken anti-mCherry antibodies at a dilution of 1:1000. To characterize MPOA neurons that are approached by PIL neurons, PTH2 immunolabeling (rabbit anti-PTH2 antibodies at a dilution of 1:3,000) was performed on sections containing MPOA in vGAT-ZsGreen mice. The visualization of PTH2 terminals was performed with Alexa594 labeled anti-rabbit IgG secondary antibody. Subsequently, the sections were treated with DAPI (1:10,000) for a period of five minutes, with the objective of visualizing DNA in the nuclei. The antibodies and histological reagents utilized in the study are listed in Table S3.

3.4 Chemogenetic studies

For specific chemogenetic manipulation of PIL neurons, AAV5-hSyn-hM3D(Gq)-mCherry, AAV5-hSyn-hM4D(Gi)-mCherry, or AAV5-hSyn-mCherry was injected into the PIL. The viral vectors were injected bilaterally into the PIL of head-fixed animals. The coordinates were measured from the bregma as follows: anteroposterior (AP) = -5.2 mm, mediolateral (ML) = ±2.6 mm, and dorsoventral (DV) = -6.8 mm from the dura mater. Subsequent to the surgical procedure, the animals received an injection of enrofloxacin (5 mg/kg bw) to prevent any potential infection.

To achieve activity-dependent manipulation of PIL neurons, a recently developed viral system was employed, known as vGATE (Virus-delivered Genetic Activity-induced Tagging of Cell Ensembles). This system was used for the activity-dependent tagging of PIL neurons. A 300-nanoliter viral cocktail containing rAAV-(tetO)7-Pfos-rtTA; rAAV-Ptetbi-Cre/YC3.60; and rAAV-PhSYN-DIO-hM3D(Gq)-mCherry or rAAV-PhSYN-DIO-hM4D(Gi)-mCherry was injected into the PIL. Three weeks later, following the recovery period for the animals, doxycycline (5 mg/kg bw) was injected intraperitoneally to open a time window during which the Cre recombinase is induced in neurons, which express c-Fos by neuronal activation. On the subsequent day, the subjects were exposed to a male intruder test, wherein they were presented with a social stimulus. The social stimulus prompted the expression of c-Fos in PIL neurons, and the infected neurons concurrently expressed reverse tetracycline transactivator (rtTA). In the presence of doxycycline, rtTA was bound to the promoter region of the second virus (Ptetbi, a bidirectional tetracycline-responsive promoter), thereby enabling CRE-recombinase to be expressed. The third virus was an AAV Cre-dependently expressing inhibitory or stimulatory DREADD. In the course of the study, neurons that demonstrated activity during social interaction were tagged with DREADDs, which were then subjected to subsequent chemogenetic manipulations.

A series of steps were taken to achieve selective manipulation of the PIL to MPOA pathway. The following procedure was undertaken in order to accomplish this. Initially, a retrograde virus (AAVrg-Ef1a-mCherry-IRES-Cre) was injected into the MPOA bilaterally (AP = -0.5 mm, ML = ±0.6 mm, and DV = -6.7 mm and -7.7 mm from the dura mater). A total of 100 nanoliters of virus was injected at both levels of the MPOA. Subsequent to a recovery period of three weeks, a second virus (pAAV-hSyn-DIO-HA-hM3D(Gq)-IRES-mCitrine or pAAV-hSyn-DIO-HA-hM4D(Gi)-IRES-mCitrine) was injected bilaterally into the PIL.

In order to achieve selective manipulation of the PIL neurons' axon terminals in the MPOA, stimulatory or inhibitory DREADD-encoding viral vectors (AAV5-hSyn-hM3D(Gq)-mCherry, AAV5-hSyn-hM4D(Gi)-mCherry) were bilaterally injected into the PIL (AP = -5.2 mm, ML = ±2.6 mm, measured from the bregma, and DV = -6.8 mm from the dura mater). Subsequently, intracerebral cannulas were implanted bilaterally

above the MPOA (AP = -0.5 mm, ML = ±3 mm, DV = -7.2 mm) at a 16.5-degree angle. The cannulas were affixed to the skull with Duracryl, a self-curing dental resin.

3.5 Fiber photometric analysis of PIL neurons that project to the MPOA

Male Wistar rats were anesthetized, and the MPOA (AP = -0.5 mm, ML = -0.6 mm, and DV = -6.7 mm and -7.7 mm) was targeted on one side with 100 nL of a retrogradely spreading virus (AAV-EF1a-jGCaMP8m-WPRE) at both levels. The subsequent step involved the implantation of a fiber optic cannula (RWD fiber optic cannula with a 400 μm core) above the PIL (AP = -5.2 mm, ML = -2.6 mm, DV = -6.8 mm). This procedure was performed on the same side as the virus injection in the MPOA. The cannulas were affixed to the skull with Duracryl.

Three weeks after the surgical procedure, the animals recovered and were subjected to behavioural testing. The activity of PIL neurons was measured using an R810 Dual Colour Multichannel Fiber Photometry System. Subsequent to the establishment of a connection between the fiber cannula and the photometry system via an optical fiber, the subject animal was positioned within an arena for a duration of one minute. Subsequently, an unfamiliar male Wistar rat was introduced into the arena. The animals were permitted to interact with each other for a period of five minutes, during which the activity of the PIL neurons was recorded. Video documentation of the animals' behaviour was also taken. The 410-nm light was utilized as isosbestic light, with the objective of recording noise and artefacts caused by movement. The excitation of GCaMP was achieved through the utilization of 470-nm light, and the subsequent collection of data pertaining to the activation of PIL neurons was facilitated.

The analysis was conducted using RWD software. Initially, the data underwent a process of smoothing and motion correction, with the isosbestic data being extracted from the recording to ensure the removal of motion artefacts. The activity measured in the PIL was then correlated with the behavioural elements displayed by the animals. The peri-event analyses were initiated two seconds prior to the presentation of the behavioural element, subsequently followed by a six-second recording of the activity of PIL neurons. The physical contact between the animals (i.e., when the subjects were in proximity to each other) and the subsequent separation of the subjects (i.e., when the subject was

moving away from their partner animal) were analyzed. A total of 47 events involving physical contact and 28 events involving movement were recorded. Z-scores were calculated by the software for each event. The area under the curve (AUC) was calculated for the Z-scores during the 2-second period following the presentation of the behavioural elements and the 2 seconds prior to this.

3.6 c-Fos studies

The utilization of c-Fos proved to be instrumental in identifying the target areas of PIL projections, which are activated by inputs from the PIL. The animals, which had previously been injected with an AAV encoding stimulatory DREADDs and expressed with vGATE (n = 8), were divided into two groups: half of the animals were administered CNO, while the other half received vehicle. Subsequently, the animals were isolated for a period of two hours, after which they underwent a transcardial perfusion.

The animals that expressed inhibitory DREADDs in their PIL neurons (n = 8) were also divided into two groups: half received CNO and the other half received vehicle intraperitoneally. Subsequent to the administration of CNO or vehicle, a male intruder test was performed on the animals for a duration of 30 minutes. Subsequently, the partner animals were removed, and the subjects were left undisturbed for a further 30 minutes prior to the beginning of the perfusion.

The brains were collected and sectioned coronally into 40 µm slices. A total of 20% of the slices were collected and processed for c-Fos immunolabeling. The immunolabeling of c-Fos was accomplished with a rabbit anti-c-Fos antibody at a dilution of 1:3000, followed by the visualization of the labeling through an immunoperoxidase reaction.

Microscopic images at low magnification were collected of the PIL and all its target areas on both sides. The number of c-Fos-positive cells was determined by means of cell counting with ImageJ. The predefined regions of the brain were utilized to enumerate the number of c-Fos immunoreactive (c-Fos-ir) neurons. For the purpose of statistical analysis, the two sides of each brain region were averaged. Statistical significance was assessed using a two-way analysis of variance (ANOVA), followed by the Šidák post hoc test, after verifying normal distribution with the Shapiro-Wilk test.

3.7 Behavioural protocols

Isolation of animals

In order to examine the impact of social isolation, male animals were either pair-housed or isolated for a period of 2 weeks. Subsequently, behavioural tests were conducted on the animals. The experimental animals were subjected to social interaction in an open arena and also a resident intruder test in their home cages to measure aggression.

Clozapine-N-oxide treatment

Clozapine-N-oxide (CNO) was utilized as a ligand to manipulate neurons expressing DREADDs. CNO was administered intraperitoneally to the animals (0.3 mg/kg bw CNO, dissolved in 5% DMSO in distilled water, 1 ml/kg bw). For the vehicle, 5% dimethyl sulfoxide (DMSO) dissolved in distilled water (1 ml/kg bw) was utilized. A local CNO injection was performed via the intracerebral cannula using a 333x dilution of the previously mentioned solution, with 100 nl of that being injected.

Testing of affiliative behaviours

The testing of affiliative behaviours was conducted through the utilization of a pair-housing method, wherein the subjects were placed in a shared environment with a conspecific. Twenty-four hours prior to the commencement of the behavioural test, the animals were isolated with a view to inducing social interactions upon their subsequent reunification during the behavioural tests. On the day prior to the experiment, the subject animals were habituated to the empty open field arena (40 x 80 cm). The experiment was conducted over a period of three days. On the first day of the experiment, the animals received an injection of a vehicle control solution. On the second day, CNO was administered to manipulate the neurons, followed by a two-day resting period. Over the course of two days, the subjects were reintroduced to the open field arena, and the vehicle injection was repeated on the following day. Within the designated arena, the animals were permitted to engage with each other without restraint. Ten minutes of footage of their behaviours were recorded and subsequently analysed.

The behaviour of the animals was evaluated manually using the SOLOMON coder (32). The behavioural elements analyzed included anogenital sniffing, wherein the animal sniffs its partner in the anogenital region; non-anogenital sniffing, where the sniffing occurs on a different body part; social grooming, where the subject gently touches the partner with its snout and licks it; chasing, where the subject follows the partner animal; and mounting, where the subject touches the partner with its front legs. Passive social behaviour is characterised by non-responsiveness to external stimuli, as well as the absence of any distinctive behavioural manifestations.

Testing of aggression

The measurement of aggression was conducted using the resident-intruder test. Subsequent to a one-month period of social isolation, the subject animals were subjected to resident-intruder tests within the confines of their home cage. As with the direct social interaction tests for affiliative behaviour, the experiment had a duration of three days. On the first day of the experiment, a vehicle was administered to the animals, and an unfamiliar male rat of similar size was introduced into the subject's cage, resulting in an aggressive response. On the subsequent day, CNO was injected to manipulate neuronal activity, and the same test was repeated. Subsequent to a 48-hour period of rest, the control injection was repeated. Thirty minutes of video footage were recorded and subsequently analyzed.

The behavioural patterns exhibited by the subjects were meticulously assessed through a manual evaluation process employing the Solomon coder. The assessment encompassed various social behavioural elements, including anogenital sniffing, non-anogenital body sniffing, positive-valence direct contacts (e.g., grooming, friendly mounting, and side-to-side contacts), and aggressive behaviour. Aggressive behavioural elements were further analyzed, including dominant mounting (where the subject pins down the partner), fighting (where the animals tumbled over, kicked, punched, and boxed each other) and biting.

3.8 Microscopy and image processing

The sections were examined using an Olympus BX60 light microscope equipped with fluorescent epi-illumination. The images were captured at 2048X2048 pixel resolution using a SPOTXplorer digital CCD camera (Diagnostic Instruments, Sterling Heights, MI) with a 4-40 X objective. Confocal images were acquired with a Zeiss LSM780 confocal microscope system, utilizing 40-63X objectives at an optical thickness of 1 mm for the enumeration of varicosities and 3 mm for the enumeration of labeled cell bodies. To illustrate this phenomenon, adjustments were implemented to the contrast and sharpness of the images by employing the "Levels" and "Sharpness" commands within the Adobe Photoshop CS9.0 software. It is noteworthy that throughout the process, the images maintained their full resolution until the final versions were adjusted to a resolution of 300 dots per inch (dpi).

3.9 Statistical analyses

The statistical calculations were performed using GraphPad Prism. Initially, the Shapiro-Wilk test was conducted on the entire dataset to ascertain whether the data followed a Gaussian distribution. For normally distributed data, two-tailed paired t-tests (AUC of moving away) or unpaired t-tests (effect of social isolation on social behaviour) were used. In instances where the data did not conform to a normal distribution, the Wilcoxon test (AUC of physical touch) or the Friedman test followed by the Dunn's post hoc test were employed. In instances where a greater number of variables were compared, a 2-way ANOVA (quantitative analysis of c-Fos positive cells) or a repeated measures 2-way ANOVA (testing aggressive behaviours) was employed. Significant changes are indicated as $0.010 < *p < 0.050$, $0.001 < **p < 0.010$, and $***p < 0.001$.

4. RESULTS

4.1 Projection pattern of PIL neurons in the brain

To analyze the projection of PIL, the anterograde tracer BDA was injected into the PIL (see Figures 3A, B, C). BDA was taken up by the PIL neurons and transported through their axon terminals, marking them with a signal. Subsequent to this, the BDA was detected using a peroxidase method. Subsequently, an analysis of the fibers in the target areas was conducted (Figure 3D). The highest density of fibers originating from the PIL was found in the medial preoptic area (MOPA). The distribution of projections from the PIL and SN utilized as controls is further detailed in Table S1.

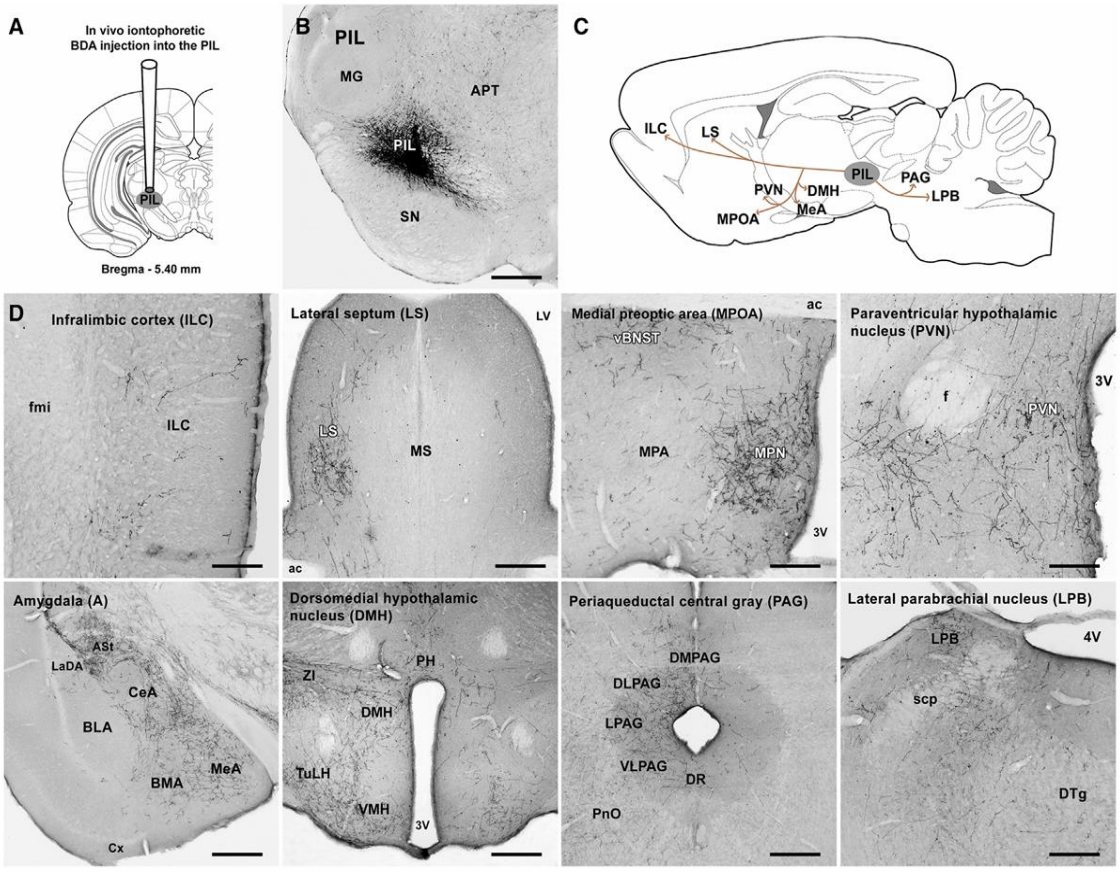

Figure 3. Projection of the posterior intralaminar (PIL) neurons.

A, B: The process of localizing the injection site of the virus in the PIL. **C**. Schematic representation of the projection of PIL neurons to their target area. **D**: Illustrative coronal

sections of the brain regions in which PIL neurons project. The presence of fiber terminals was observed in the infralimbic cortex (ILC), the lateral septum (LS), medial preoptic area (MPOA), the hypothalamic paraventricular nucleus (PVN), and the medial amygdala (MeA), the dorsomedial hypothalamus (DMH), the ventrolateral part of the periaqueductal grey (VLPAG), and the parabrachial nucleus (PBL). Scale bar: The PIL in panel B is 750 μm, while the ILC and PVN are 300 μm, the MPOA is 375 μm, the LPB is 450 μm, the LS is 600 μm, and the MeA, DMH, and PAG are 750 μm. Source: (33)

4.2 The role of PIL neurons in affiliative behaviour

To determine the effect of PIL neurons on affiliative behaviour in male rats, we injected a stimulatory or inhibitory DREADD-expressing adeno-associated virus (AAV), or a control virus (pAAV-hSyn-hM3D(Gq)-mCherry, pAAV-hSyn-hM4D(Gi)-mCherry, or pAAV-hSyn-mCherry), into the PIL. After a two-week recovery period, the animals were reunited with a familiar male partner for one week to eliminate potential aggression during the behavioural tests (Figure 4A). During the behavioural tests, ten minutes of behaviour were recorded, and distinct social behavioural elements were analyzed. Chemogenetic stimulation of PIL neurons increased the duration of social grooming compared to the two control days when the vehicle was injected (Figure 4B), while chemogenetic inhibition of PIL neurons decreased the duration of social grooming significantly compared to the control days (Figure 4D). We also investigated social interactions in which no direct contact was permitted. The partner animal and the subject were separated by a barrier through which they could see, hear, and smell each other but could not touch each other. Time spent around the partner animal was unaffected by chemogenetic manipulation of PIL neurons (Figures 4C, E, and F). We also injected a virus that did not express any DREADD to control for the CNO injection. Injection of CNO without DREADD expression had no effect on the behaviour of the animals (Figures 4F and 4G).

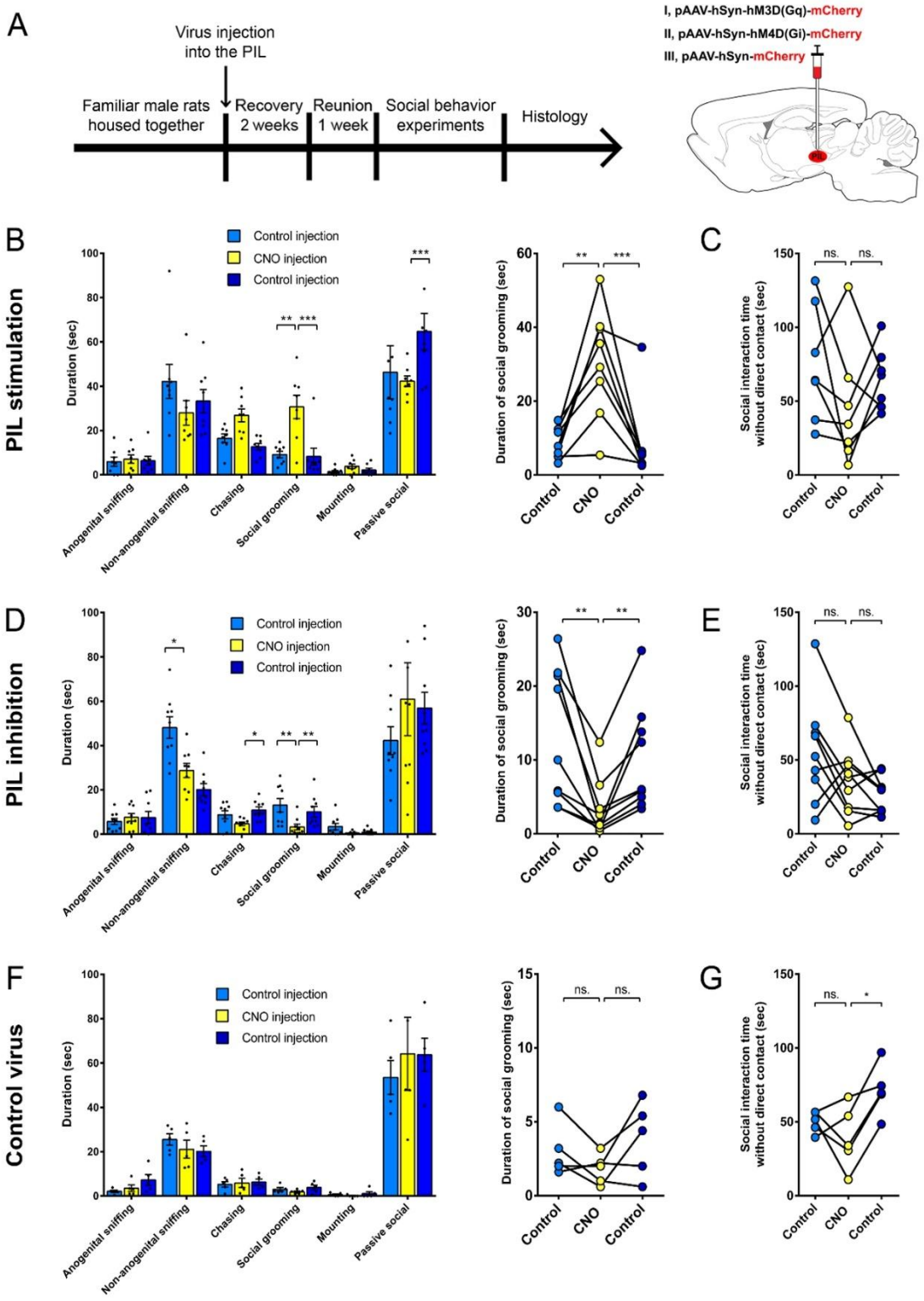

Figure 4. The effect of chemogenetic manipulation of PIL neurons in male Wistar rats on affiliative behaviour.

A. The behavioural protocol of the study and an image showing the location of the viruses used. **B.** The effect of chemogenetic stimulation of PIL neurons in freely moving male rats (n = 8). Statistical analysis was performed using a repeated-measures two-way ANOVA, followed by a Šidák post hoc test. Statistical significance was found in social grooming: $p=0.0013$ between the first control and CNO, and $p<0.001$ between the second control and CNO. **C**. Chemogenetic stimulation of PIL neurons without physical contact (n = 7). Statistics were tested using a one-way ANOVA, followed by a Šidák post hoc test. No significance was found ($p = 0.44$ between the first control and CNO, and $p = 0.61$ between the second control and CNO). **D**. The effect of chemogenetic inhibition of PIL neurons in freely moving male rats (n = 9). For social grooming, statistical analysis was performed using the Friedman test, followed by the Dunn's post hoc test. The results showed statistical significance at $p=0.0044$ for both the first and second controls compared to CNO. **E**. Chemogenetic inhibition of PIL neurons without physical contact (n=9). Statistics were tested using a one-way ANOVA followed by a Šidák post hoc test. No significance was found ($p = 0.28$ between the first control and CNO, and $p = 0.47$). **F.** Lack of effect of CNO administration on animals injected with the control virus (n = 5). Statistical analysis of social grooming was conducted using the Friedman test, followed by Dunn's post hoc test. No significant effect was found ($p > 0.99$ between the first control and CNO, and $p > 0.99$ between the second control and CNO). **G.** CNO administration to animals injected with a control virus in the absence of physical contact (n = 5). Statistics were tested using a one-way ANOVA followed by a Šidák post hoc test ($p = 0.73$ between the first control and CNO, and $p = 0.02$ between the second control and CNO). Source:(33)

4.3 The function of the PIL to MPOA pathway in affiliative behaviour

To test the PIL-to-MPOA pathway, we first injected the MPOA with a viral construct that spreads retrogradely and expresses Cre recombinase. A second viral injection into the PIL enabled us to mark PIL neurons with DREADDs that project to the MPOA (see Figures 5A and 5B). Next, we examined the effect of manipulating the tagged PIL neurons projecting to the MPOA using chemogenetics. Chemogenetic stimulation had no effect on social grooming (Figure 5C), but inhibiting these neurons significantly decreased social grooming duration compared to the two groups (Figure 5D).

Using another approach, we injected an anterogradely spreading, stimulatory, DREADD-expressing virus into the PIL and implanted an intracerebral cannula above the MPOA. This allowed us to selectively stimulate a fiber terminal originating from the PIL and reaching medial preoptic neurons (Figure 5E). Stimulating the PIL-to-MPOA pathway markedly increased the duration of social grooming (Figure 5F).

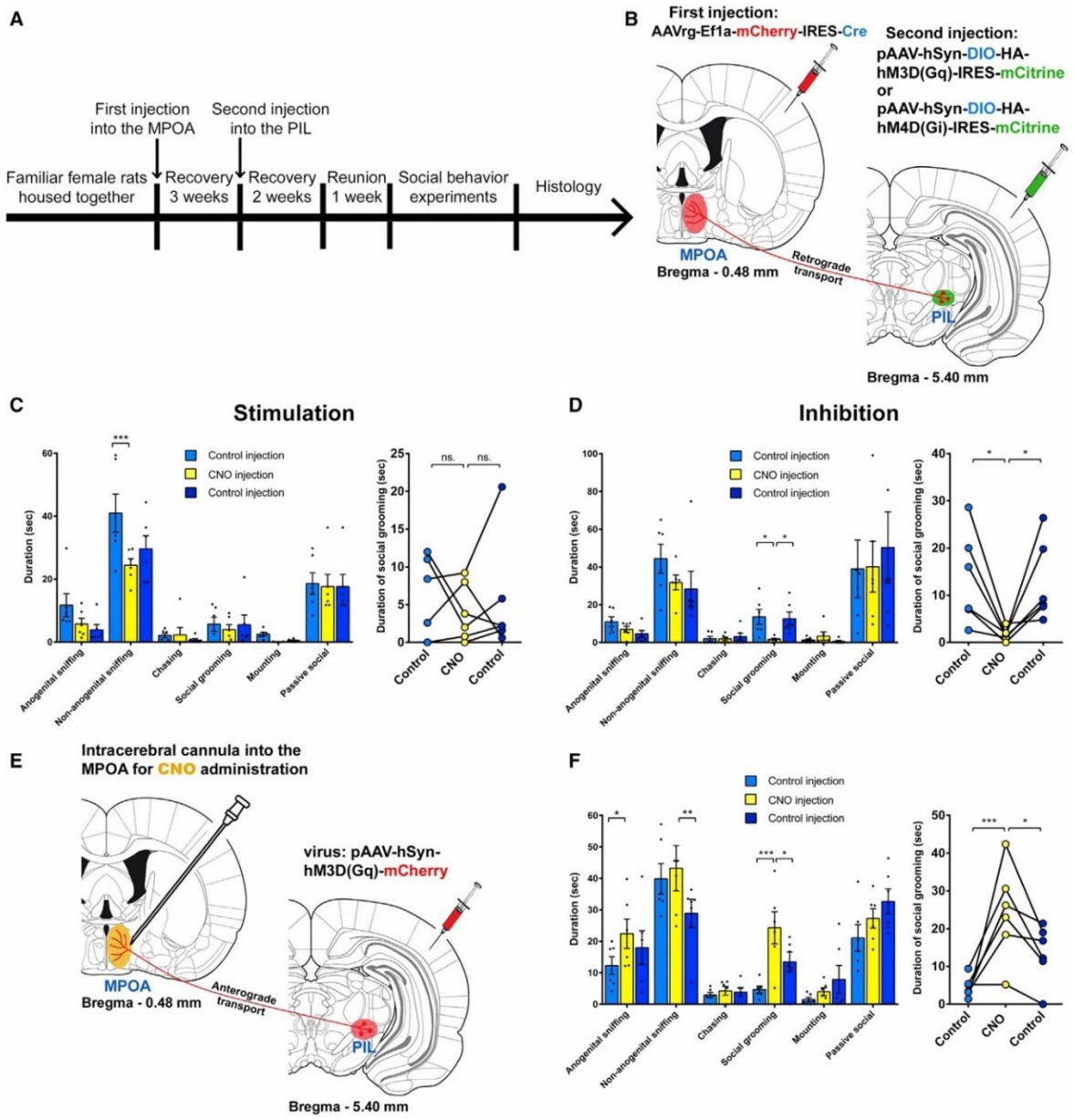

Figure 5. The effect of chemogenetic manipulation of the PIL to MPOA pathway on affiliative behaviour

A. Experimental design for testing affiliative behaviour. B. Overview of the viruses and injection sites used in the study**. C.** Effect of chemogenetic stimulation of PIL neurons projecting to the MPOA on affiliative social behaviour (n = 6). **D.** The effect of chemogenetic inhibition of PIL neurons projecting to the MPOA on affiliative social behaviour (n = 6). The statistical significance of social grooming was analysed using a Friedman test, followed by a Dunn's post hoc test: p = 0.028 (first control vs. CNO) and p = 0.028 (CNO vs. second control). **E.** Overview image of the experimental design involving local CNO injection into the MPOA. **F.** The effect of chemogenetic stimulation

of fiber terminals originating from PIL neurons reaching MPOA neurons on affiliative social behaviour. Statistical significance was tested using a repeated-measures ANOVA followed by Šidák's post hoc test for social grooming: $p < 0.001$ (first control vs. CNO) and $p = 0.029$ (CNO vs. second control). Source:(33)

4.4 PTH2-containing terminals from the PIL closely appose GABAergic MPOA projection neurons

In order to investigate GABAergic neurons in the MPOA, a transgenic mouse line was utilized that expresses the fluorescent protein ZsGreen in GABAergic, vesicular GABA transporter (VGAT)-positive cells. Additionally, a connection to the PIL was successfully established. A substantial presence of PTH2-positive fiber terminals was identified within the MPOA (see Figure 6A). Additionally, the close apposition of PTH2-ir terminals to VGAT-positive GABAergic neurons (see Figure 6B) was also described. The investigation aimed to ascertain whether socially active MPOA neurons receive inputs from PTH2-positive PIL neurons. The findings revealed that MPOA neurons demonstrating responsiveness to social interactions also express the PTH2 receptor (PTH2R) (Figure 6C). This finding indicates the presence of functional connections between PTH2 neurons and GABAergic MPOA neurons. Furthermore, the number of c-Fos-positive and PTH2R-positive cells during periods of social isolation and social interaction was quantified. MPOA neurons demonstrated a substantially higher degree of overlap compared to the isolated control group. GABAergic cells in the medial preoptic area (MPOA) project to multiple socially relevant brain regions, including the PVN, VMH, CeA and PAG. In contrast, glutamatergic MPOA neurons primarily project to the lateral hypothalamic area (LH) and the supramamillary nucleus (SuM) (Figure 7).

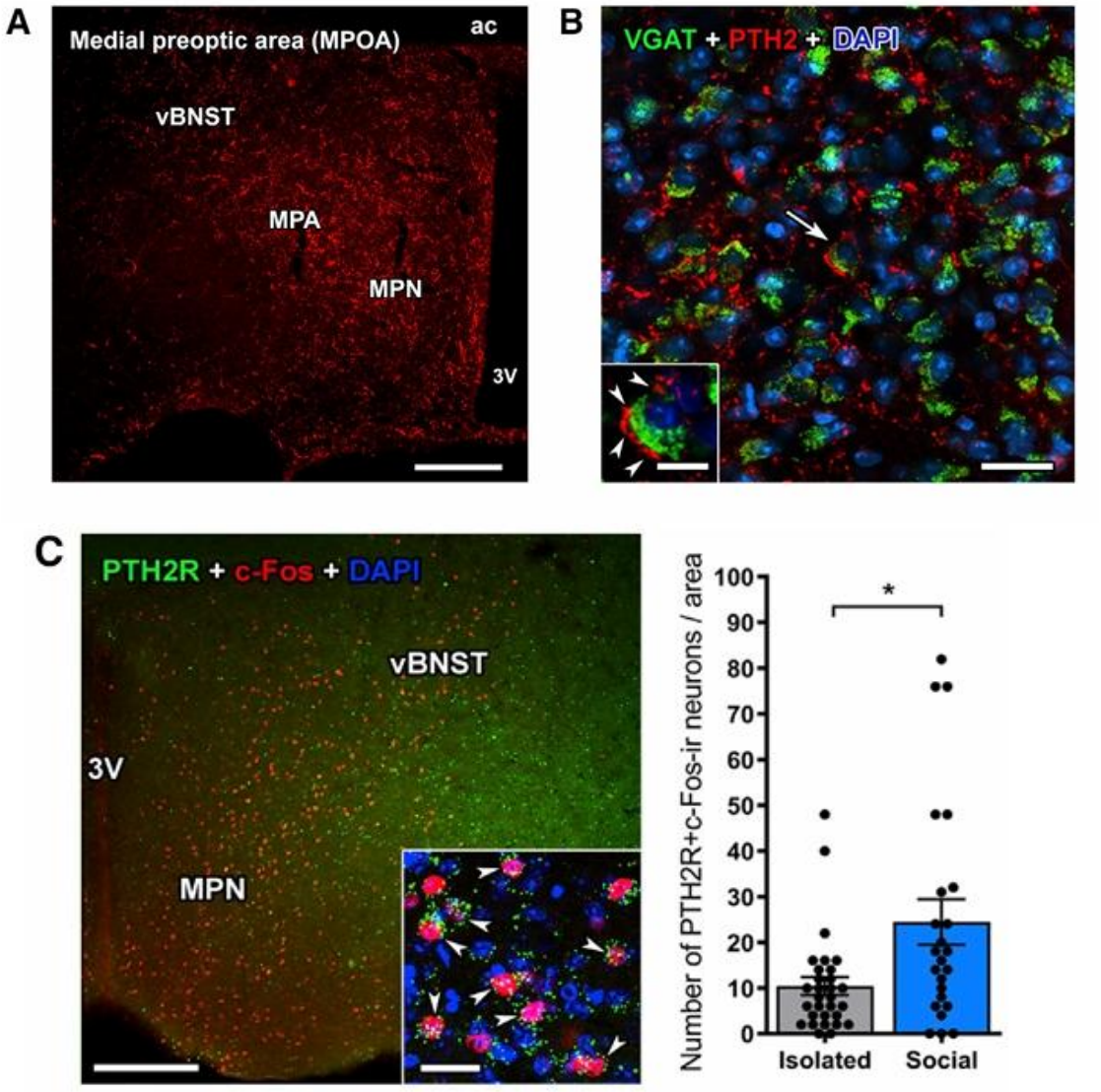

Figure 6. GABAergic nature of MPOA neurons that receives input from the PIL

A. PTH2-containing fiber terminals are present in significant numbers in the MPOA in VGAT-ZsGreen mice. **B.** PTH2-positive fiber terminals (red) approach GABAergic VGAT-positive cells (green cells) in the MPOA. The nuclei are visualised using DAPI in the blue channel. **C**. Socially activated MPOA neurons expressing c-Fos (red) also express the PTH2 receptor (green). Cell nuclei are visualised on the blue channel. The study investigated the number of double-labeled neurons expressing PTH2R and c-Fos in the MPOA. A significant increase in overlap among neurons was observed in animals engaged in social interactions (n = 30) compared to the group that did not participate in social interactions (n = 24). The statistical analysis employed a two-tailed unpaired t-test, with a significance level of p = 0.013. Scale bar: The scale bar in the main panels is 300 µm, while in the insets,50 µm. Abbreviations: 3V – third ventricle, ac – anterior

commissure, MPA – medial preoptic region, MPN – medial preoptic nucleus, vBNST – ventral subdivision of the bed nucleus of the stria terminalis. Source:(33)

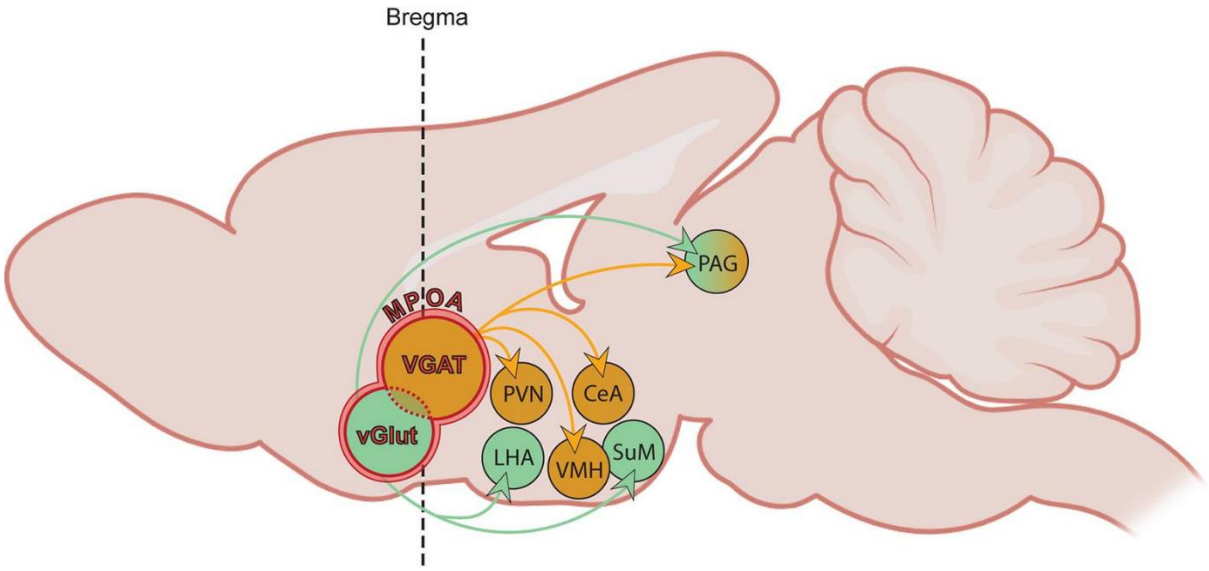

Figure 7. GABAergic and glutamatergic projections of MPOA neurons

VGAT-expressing GABAergic neurons (yellow) project to the paraventricular hypothalamic nucleus (PVN), the ventromedial hypothalamic nucleus (VMH), and the central amygdaloid nucleus (CeA). VGlut-expressing glutamatergic neurons (green) project to the lateral hypothalamic area (LHA) and the supramamillary nucleus (SUM). Notably, both glutamatergic and GABAergic MPOA neurons project to the periaqueductal gray (PAG). Source: (27)

4.5 The effect of social isolation on the behaviour of male rats

In order to investigate the effects of social isolation on male rats, adult male rats were isolated for a day. Subsequent to this period, a direct social interaction test was conducted with a familiar male rat introduced as a partner in an arena to measure affiliative behaviour. The test animals were habituated to the arena the previous day.

The results demonstrate that social isolation increases the duration of affiliative behaviours (Figure 8A). Affiliative behaviours included anogenital and body sniffing, mounting, and grooming (Figure 8B). Aggressive behaviour was assessed in the home cage environment through interactions with an unfamiliar male rodent after 2 weeks of social isolation by individual housing. Social isolation was demonstrated to induce

aggression (Figure 8C). A significant increase in dominant mounting was observed within the context of aggressive behaviour (Figure 8D).

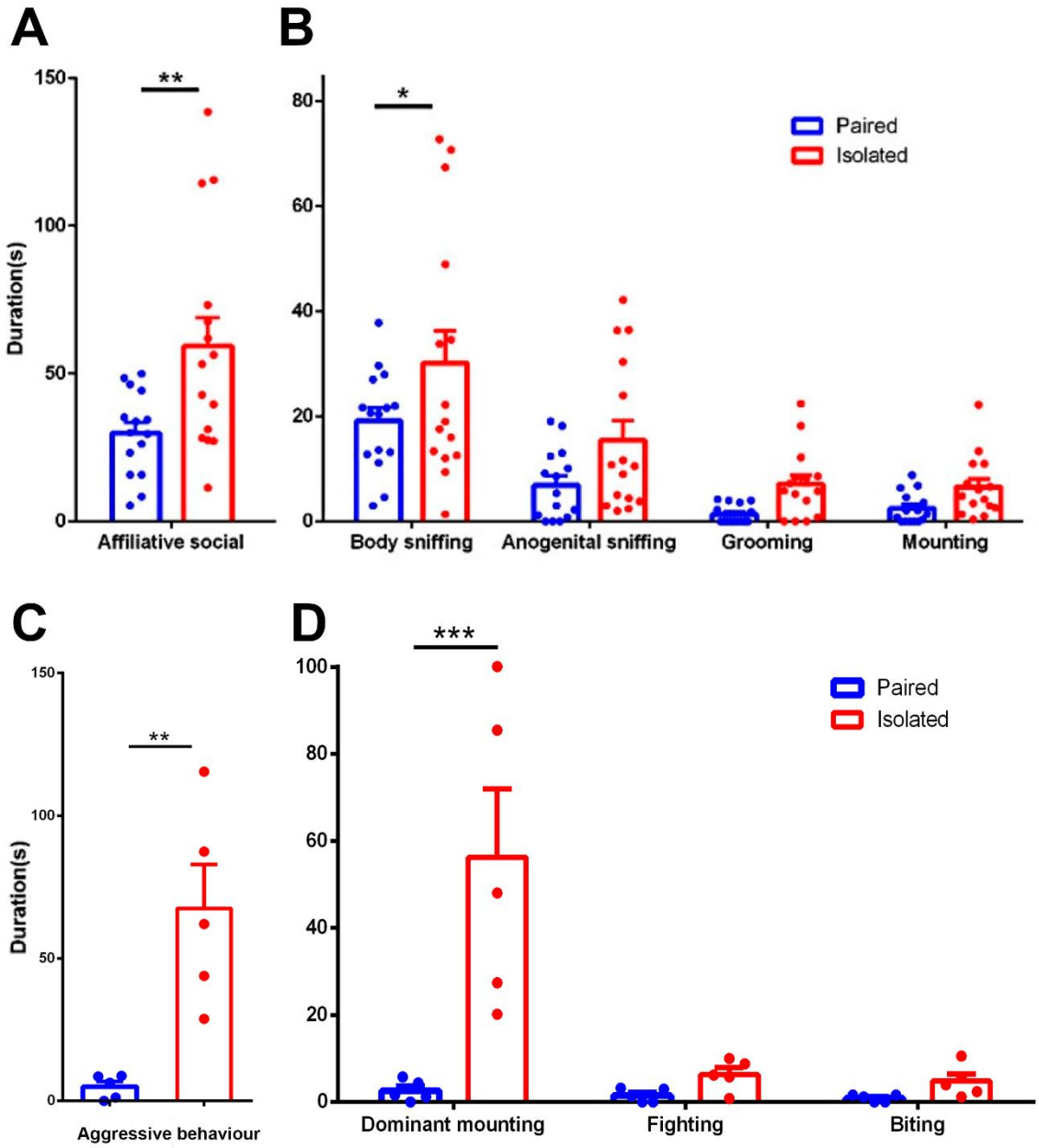

Figure 8. The effect of social isolation on affiliative behaviour and aggression in male rats.

A. The impact of social isolation on affiliative behaviour in male rats. Affiliative social behaviours included body and anogenital sniffing, mounting, and grooming. The statistical analysis was conducted using an unpaired t-test. A substantial difference was identified between the isolated and control groups ($p < 0.01$; $n = 15$ for the isolated group

and n = 15 for the paired group). **B.** When affiliative behavioural elements were assessed, a significant increase was observed in the duration of body sniffing compared to the control group, as determined by a two-way ANOVA followed by Šidák's post hoc test ($p < 0.05$). **C.** The effect of social isolation on aggression. The statistical significance of these results was determined by conducting an unpaired t-test, which yielded a p-value of 0.0019 (n = 5 for the isolated group and n = 5 for the paired group). **D.** Within the context of aggressive behaviour, dominant mounting exhibited a significant increase in the isolated group. Statistical analysis was conducted using a two-way analysis of variance (ANOVA) followed by Šidák's multiple comparison test to assess the differences between groups ($p < 0.0001$). Source: (33)

4.6 The role of social activity-tagged PIL neurons during aggression

Initially, it was determined that the PIL neurons, exhibit c-Fos expression in response to the interaction of male rats with an unfamiliar male conspecific, leading to the manifestation of aggressive behaviour. (Figures 9A and 9B). In order to investigate the role of PIL neurons in aggression, it was required to selectively target and tag only those PIL neurons that respond to direct social interactions. This objective was accomplished by integrating the vGATE system into the PIL. This facilitated the tagging of PIL neurons that expressed c-Fos during the resident intruder test using stimulatory or inhibitory DREADDs (see Figures 9C, D).

Inhibiting the socially tagged neurons in the PIL significantly increased the duration of aggression compared to the two control days and decreased the duration of positive-valence direct contacts (Figure 9E, G). Within the context of aggression, a subsequent analysis was conducted to identify the specific type of aggressive behaviour that underwent change. Dominant mounting and fighting behaviours exhibited a significant increase following chemogenetic inhibition of PIL neurons (Figure 9F).

Aggressive behaviour was found to be significantly reduced in subjects given chemogenetic stimulation of socially tagged PIL neurons (see Figure 9H, J). Subsequent analysis of each aggressive behavioural element indicated that dominant mounting exhibited a significant decrease in duration when the socially tagged PIL neurons were activated (Figure 9I).

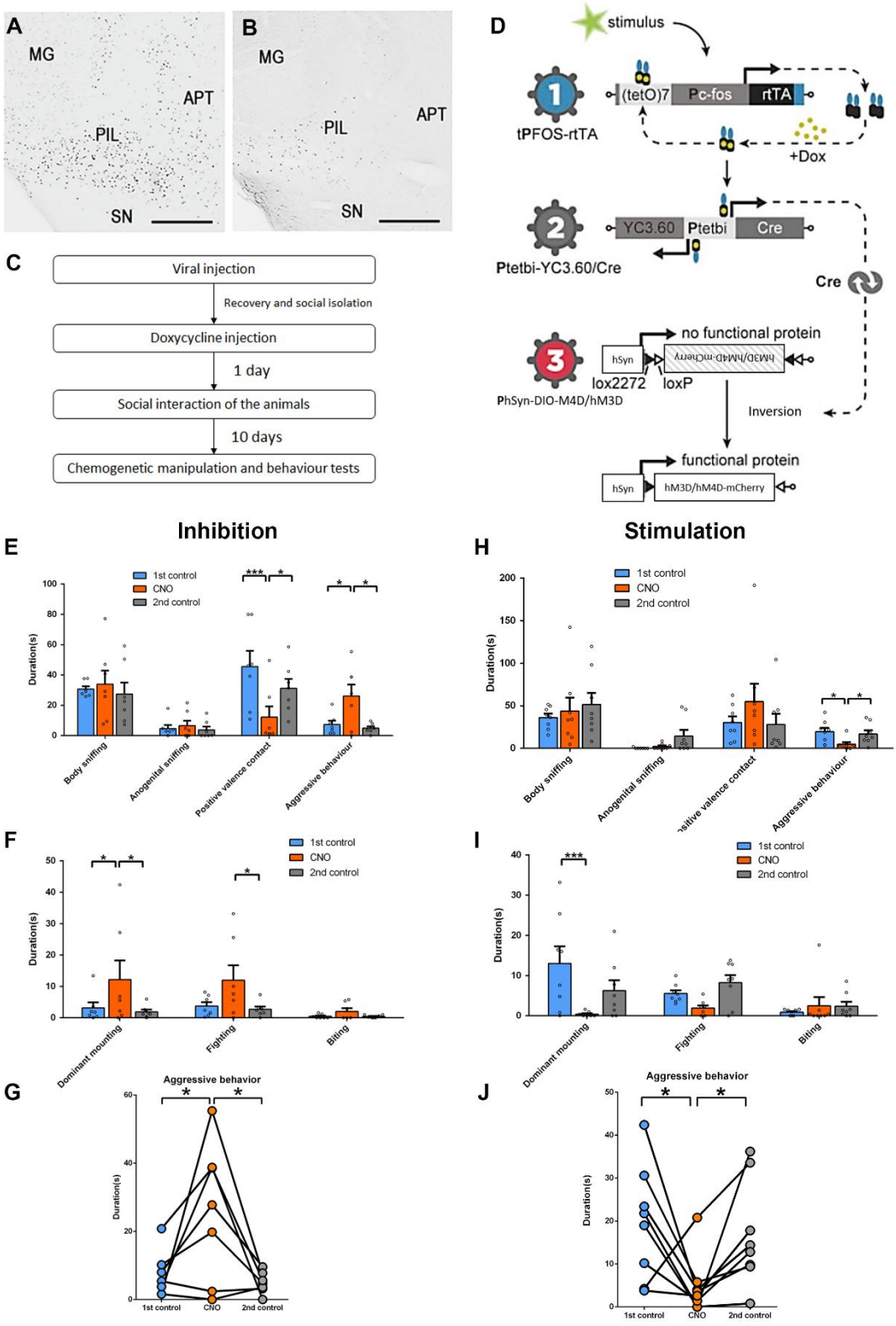

Figure 9. Chemogenetic manipulation of socially activated PIL neurons tagged with the vGATE system.

A. Social interaction between unfamiliar male rats, where aggression is present, was demonstrated to induce c-Fos expression in the PIL. **B.** In the control group, socially isolated animals were utilized, and c-Fos was not expressed in their PIL neurons. Scale bar: 500 μm for panels A and B. **C.** A synopsis of the protocol that was utilized to tag socially activated PIL neurons. Initially, the viral mixture was injected into the PIL. During the recovery period, the animals were subjected to a social isolation process, which was designed to elicit aggressive behaviours. The activation of the vGATE (Virus-delivered Genetic Activity-induced Tagging of Cell Ensembles) system was initiated through the administration of doxycycline to the animals, followed by the execution of the resident-intruder test the subsequent day. Subsequent to a 10-day period of social isolation, during which the DREADDs were expressed in adequate quantities, behavioural tests were conducted on the animals employing chemogenetic manipulation. **D.** A schematic representation of the vGATE system shows that a combination of three viruses (rAAV-(tetO)7-CAG-GC-hCFC-IRES-mCherry; rAAV-Ptet-Cre/YFP; and rAAV-Phsyn-DIO-hM3D(Gq)-mCherry or rAAV-Phsyn-DIO-hM4D(Gi)-mCherry) was injected into the PIL. **E.** The application of chemogenetic inhibition to socially tagged PIL neurons was demonstrated to result in a decline in positive valence contact duration and an escalation in aggression. Statistical significance was identified in both behavioural elements. Statistical analysis was performed using a repeated-measures two-way ANOVA, followed by a Tukey's multiple comparisons test ($p < 0.001$ for positive valence contacts between the first control and CNO, and $p = 0.0417$ for the second control and CNO). In aggression, a significant difference was identified between the first control and CNO ($p = 0.0444$), as well as between the second control and CNO ($p = 0.0205$). **F.** Within aggression, mounting and fighting increased significantly in response to the chemogenetic inhibition of PIL neurons. The statistical analysis was conducted using a repeated-measures two-way ANOVA, followed by a Tukey's post hoc test. The results demonstrated a significant difference between the first control and CNO for dominant mounting ($p = 0.0438$), and between the second control and CNO ($p = 0.0187$). In the case of fighting, the p-value was 0.0716 for the comparison between the first control and CNO, and 0.0372 for the comparison between the second control and CNO. **G.** Individual data concerning the animals' aggressive behaviours during chemogenetic inhibition of PIL neurons. **H**. The chemogenetic stimulation of socially tagged PIL neurons led to a

significant decrease in the time spent engaging in aggressive behaviour. The statistical analysis of aggressive behaviour was conducted using a Friedman test, followed by Dunn's post hoc test. This analysis revealed a significant difference between the first control and CNO groups ($p = 0.0374$) and between the second control and CNO groups ($p = 0.0179$). **I.** The present study demonstrated a significant decrease in dominant mounting behaviour following stimulation of PIL neurons. Statistical analysis was performed using a two-way analysis of variance (ANOVA) with a Tukey's multiple comparisons test, which revealed a statistically significant difference ($p < 0.001$) between the first control and CNO, and a statistically non-significant difference ($p = 0.109$) between the second control and CNO injection. **J.** Individual data concerning the animals' aggressive behaviours during chemogenetic stimulation of PIL neurons.
Abbreviations: APT - anterior pretectal nucleus, MG – medial geniculate body, PIL – posterior intralaminar thalamic nucleus, SN – substantia nigra. Source:(34)

The c-Fos technique was employed to assess neuronal activation following the chemogenetic manipulation of socially tagged PIL neurons. In the initial experiment, neuronal activation in response to CNO was measured in the absence of social interaction. This experiment was performed on animals that had previously been injected with a DREADD that expresses a stimulatory receptor. Subsequently, the animals were injected with CNO or a vehicle control, and perfused after a two-hour period of isolation (see Figure 10E). To this end, we performed c-Fos immunolabelling on brain sections to visualize neuronal activation in the PIL and target areas. As anticipated, the PIL demonstrated augmented c-Fos expression subsequent to chemogenetic stimulation of PIL neurons. This phenomenon is widely accepted as the direct consequence of chemogenetic stimulation, thereby validating the functionality of the stimulatory DREADDs. The activation of PIL neurons also induced the activation of one of their most significant target areas, the MPOA (see Figures 10A, B, C, D, F).

In a separate experiment, we investigated whether chemogenetic inhibition of PIL neurons could also inhibit c-Fos expression in target areas. CNO or vehicle was injected into the PIL of animals expressing inhibitory DREADDs in the PIL. Subsequently, the animals were subjected to a resident-intruder test, during which they exhibited aggressive behaviour towards the intruder (Figure 10K). Subsequent to perfusion, visualization of

the activated neurons expressing c-Fos was conducted in the PIL and its target areas. The results demonstrate that socially induced c-Fos activation can be suppressed by CNO administration, thereby demonstrating the effect of chemogenetic inhibition. In addition, the inhibition of PIL neurons led to a reduction in c-Fos expression in the MPOA, even in the absence of social interaction (see Figures 10G, H, I, J, L). This finding indicates that PIL neurons may serve as a predominant input to the MPOA during social interactions.

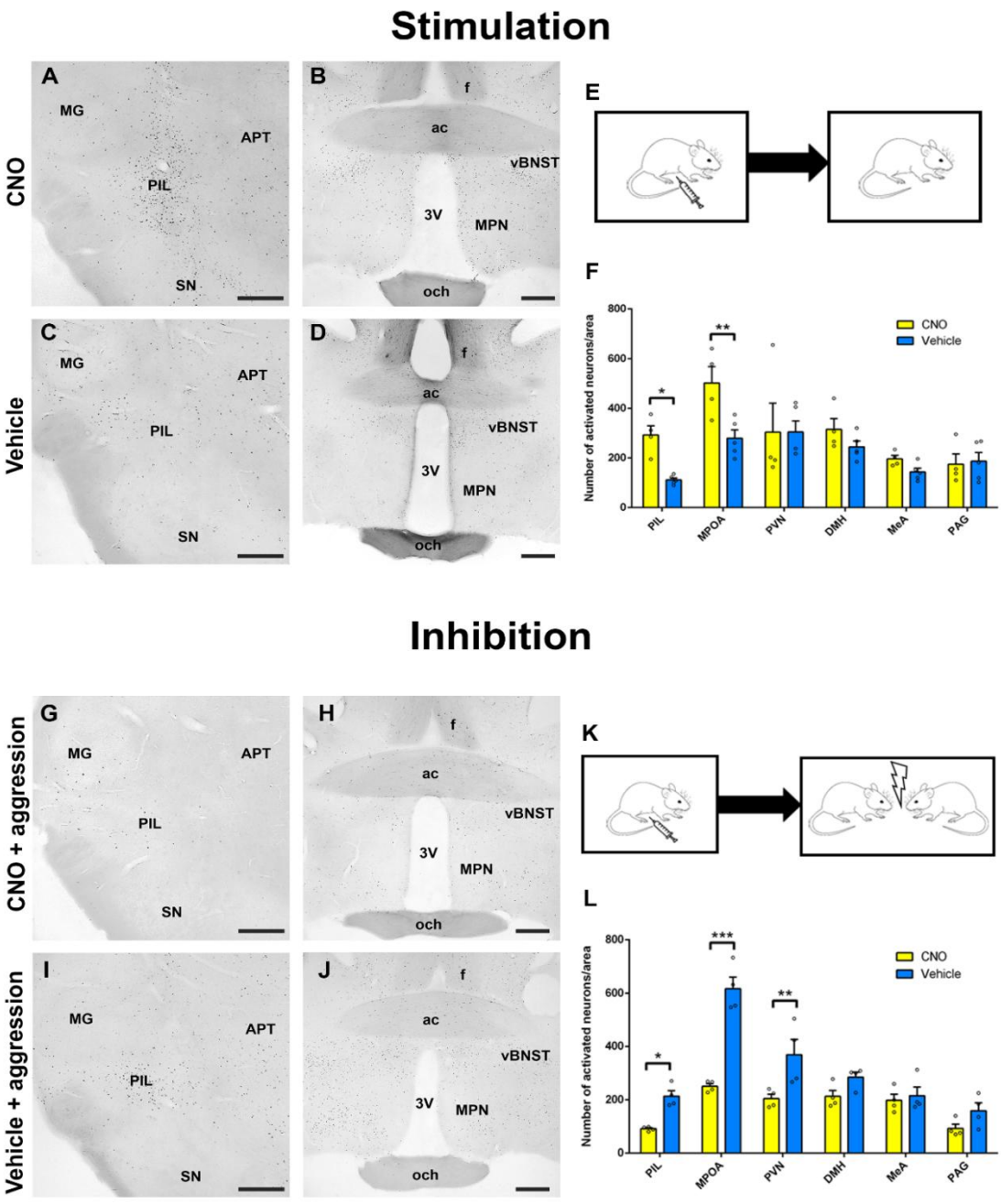

Figure 10. c-Fos expression in response to chemogenetically manipulating socially tagged PIL neurons

A. An abundance of c-Fos-expressing neurons has been identified in the PIL following chemogenetic stimulation of PIL neurons in the absence of social interaction. **B** Elevated c-Fos expression in the medial preoptic area (MPOA) has been observed in response to chemogenetic stimulation of PIL neurons in the absence of social interaction. **C.** Control c-Fos expression in PIL neurons following vehicle administration in the absence of social interaction. **D**. Control c-Fos expression in MPOA neurons following vehicle administration in the absence of social interaction. **E**. Schematic design of the experiment on animals expressing stimulatory DREADDs in the PIL. **F.** Quantitative analysis of c-Fos-immunoreactive neurons in the PIL and its target brain areas. Statistical significance was analyzed using a two-way analysis of variance (ANOVA) followed by a Šidák post hoc test. A statistically significant difference was identified in the PIL ($p = 0.0434$) and MPOA ($p = 0.0073$) between CNO and vehicle injections. **G.** The absence of c-Fos expression in PIL neurons following aggressive interaction and chemogenetic inhibition of the PIL via CNO administration. **H.** Reduced c-Fos expression in the MPOA neurons following aggressive interaction and chemogenetic inhibition of the PIL via CNO administration. **I.** Social interaction-induced c-Fos expression in the PIL utilized as control. **J.** Social interaction-induced c-Fos expression in the MPOA was utilized as a control. **K.** Schematic of the experiment on animals expressing inhibitory DREADDs in the PIL that underwent aggressive interactions prior to perfusion. **L**. Quantitative analysis of c-Fos-immunoreactive neurons in the PIL and its target areas. Statistical significance was analysed using a two-way ANOVA followed by Šidák's post hoc test. Significant differences were observed in the PIL ($p = 0.0248$), MPOA ($p < 0.0001$) and PVN ($p = 0.0013$) following CNO and vehicle injections. Scale bar = 500 μm in every panel.

Abbreviations: ac – anterior commissure, APT - anterior pretectal nucleus, ac – anterior commissure, f – fornix, MG – medial geniculate body, MPN – medial preoptic nucleus, PIL – posterior intralaminar thalamic nucleus, och – optic chiasm, SN – substantia nigra, vBNST – ventral subdivision of the bed nucleus of the stria terminalis, 3V- third ventricle. Source:(34)

4.7 The role of the PIL to MPOA pathway in aggression

We also tested the role of the PIL-to-MPOA pathway in aggressive behaviour during interactions between adult male rats. As in our previous experiment, the PIL was targeted using an AAV that expressed either stimulatory or inhibitory DREADDs (pAAV-hSyn-hM3D-mCherry or pAAV-hSyn-hM4D-mCherry). Intracerebral cannulas were implanted above the MPOA, enabling us to administer CNO locally and manipulate only the fiber terminals originating from the PIL that reach the MPOA (Figure 11A).

After chemogenetically manipulating the PIL-to-MPOA pathway, a resident-intruder test was performed on the animals. Chemogenetic inhibition of the pathway resulted in elevated aggressive behaviour and decreased positive valence contact duration (Figures 11B and 11D). Conversely, stimulation of the PIL-MPOA pathway produced the opposite effects: the duration of positive valence contacts increased, and aggression decreased significantly (Figure 11E, G).

The duration of dominant mounting changed in response to the chemogenetic manipulation of the pathway. Following chemogenetic inhibition, dominant mounting increased (Figure 11C). In contrast, stimulation resulted in a decrease in dominant mounting (Figure 11F).

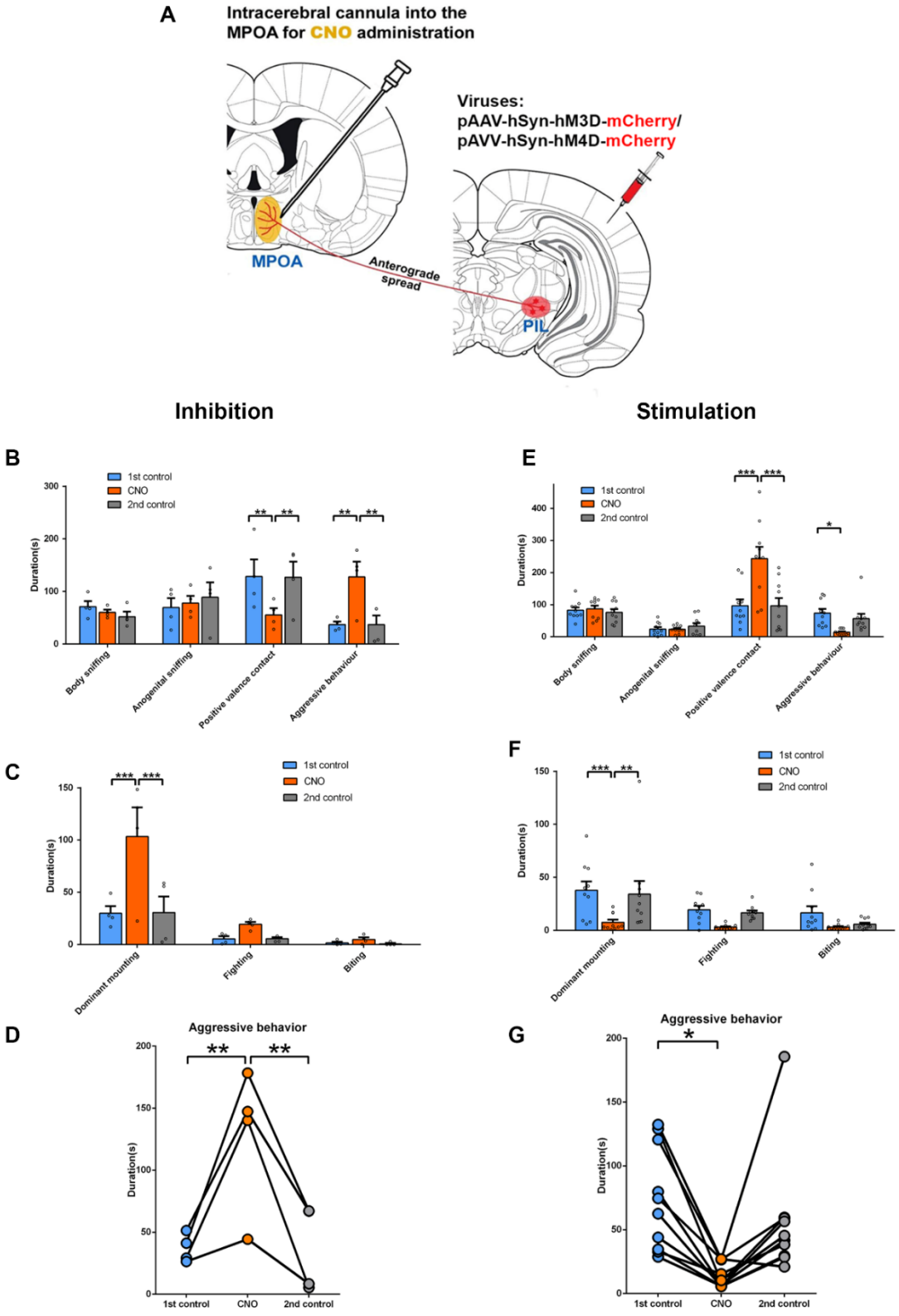

Figure 11. Chemogenetic manipulation of the PIL to MPOA pathway during aggressive behaviour of male rats.

A. The following schematic figure illustrates the experimental design for manipulating the PIL-to-MPOA pathway. **B.** The effect of chemogenetically inhibiting the PIL-to-MPOA pathway on behaviour in response to a male intruder. To ensure the robustness of the findings, a repeated measure 2-way analysis of variance (ANOVA) was employed for the statistical analyses. Statistical significance was identified in the duration of positive-valence contacts ($p = 0.0079$ between the first control and CNO; $p = 0.009$ between the second control and CNO injection) and in aggression ($p = 0.0011$ between the first control and CNO; $p = 0.0011$ between the second control and CNO injection). **C.** The effect of chemogenetic inhibition on elements of aggressive behaviour. To this end, a repeated measure 2-way ANOVA was employed for the purpose of conducting the requisite statistical analyses. This analysis yielded a significant increase in dominant mounting ($p < 0.0001$ between the first control and CNO, and between the second control and CNO injection). **D**. Changes in aggression exhibited by individual animals in response to chemogenetic inhibition of the pathway. **E**. The present study investigated the impact of chemogenetically stimulating the PIL-MPOA pathway on social interactions between male rats. The statistical analysis, conducted using a two-way repeated measures (RM) ANOVA, revealed a significant increase in positive valence contacts ($p < 0.0001$ between the first control and CNO; $p < 0.0001$ between the second control and CNO) and aggressive behaviour ($p = 0.0229$ between the first control and CNO; $p = 0.1399$ between the second control and CNO). **F.** The impact of chemogenetic stimulation on components of aggressive behaviour. To this end, a repeated measure 2-way ANOVA was employed for the purpose of statistical analysis. This analysis yielded a significant decrease in dominant mounting in comparison to the control days ($p = 0.0003$ between the first control and CNO injection, and $p = 0.0013$ between the second control and CNO injection). **G.** Changes in aggression exhibited by individual animals in response to chemogenetic stimulation of the PIL-MPOA pathway. Source:(34)

4.8 Activation of PIL neurons that reach the MPOA in response to social stimuli measured by fiber photometry

In order to enhance our comprehension of the activation of the PIL-MPOA pathway, an alternative approach was employed to achieve a significantly enhanced time

resolution of the activating neurons in the PIL during social interactions. Initially, a retrograde AAV was injected into the MPOA, where fibers withdrew the virus and expressed a calcium sensor (gCAMP8). Subsequently, a fiber cannula was implanted above the PIL neurons to detect activation of the PIL-to-MPOA pathway during social interactions. (Figure 12A, B).

The present study employed a direct social test to assess PIL activation, wherein the subject was permitted to move and interact with a conspecific for a duration of five minutes. PIL activity was continuously recorded during the behaviour test. A significant increase in PIL activation was observed when the animals touched each other, and a significant decrease in activation was observed when the animals moved away from each other (Figures 12C, D, E, and F). This finding indicates that PIL neurons that project to the MPOA are likely to receive somatosensory inputs from their cage mate.

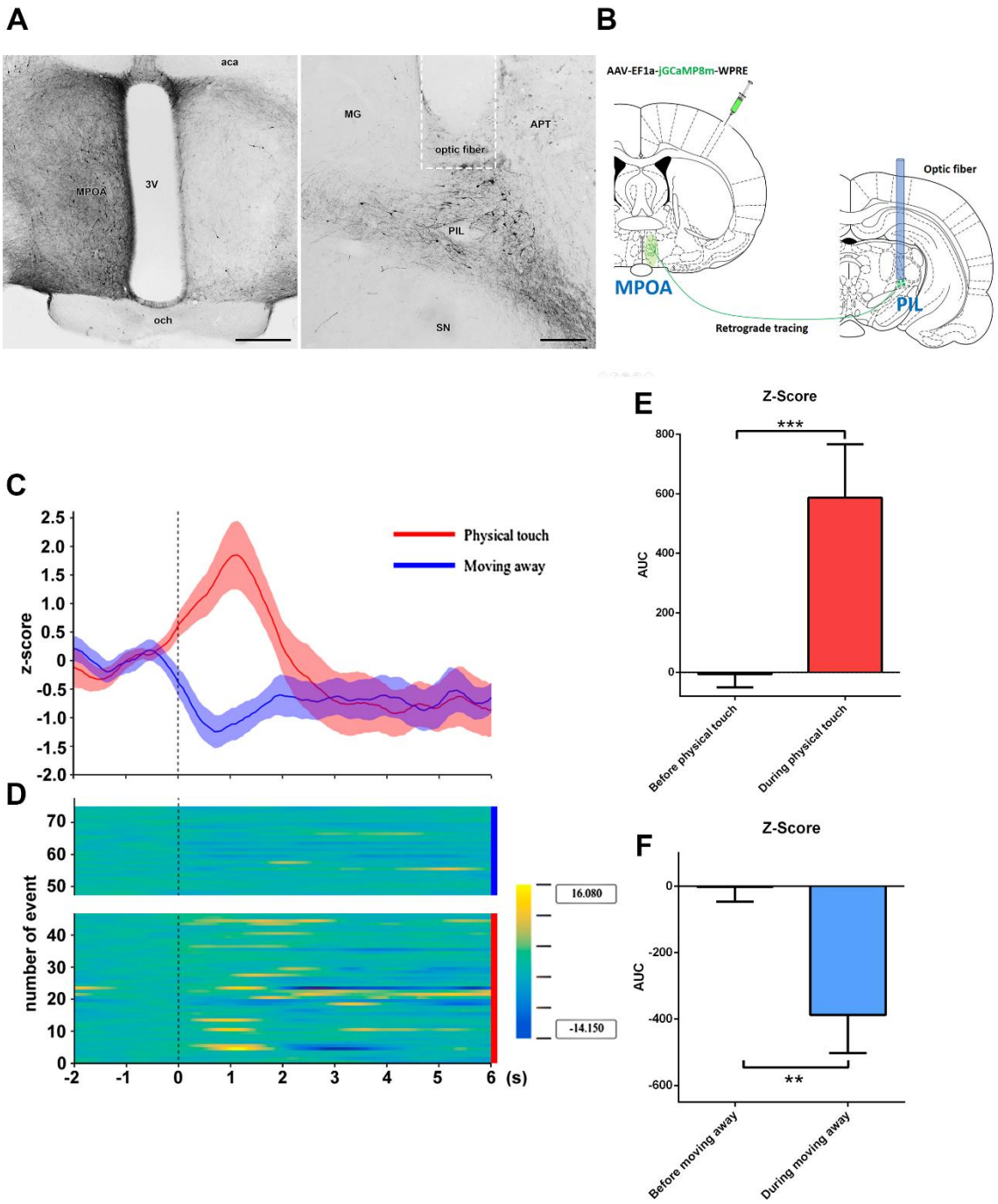

Figure 11. Fiber photometric analysis of PIL neurons projecting to the MPOA

A. Histological images of the injection site in the medial preoptic area are presented, demonstrating the precise location of the optic cannula above the PIL. The visualization of infected cell bodies expressing GCaMP is a viable method for analysis. Scale bar: 500 µm for the MPOA and 250 µm for the PIL. **B**. A schematic figure is provided below to illustrate the surgical protocol. The MPOA was targeted with a retrogradely spreading

virus expressing green fluorescent protein (GCaMP) (AAV-EF1a-jGCaMP8m-WPRE), and optic fibers were implanted above the PIL. **C**. The mean and standard error of the mean (SEM) of calcium activity Z-scores of PIL neurons projecting to the MPOA during physical contact (visualized in red) and when moving away (visualized in blue) (n = 3). The 0-time stamp on the X-axis demarcates the onset of the behavioural elements. Furthermore, calcium activity is observed for a duration of two seconds prior to the manifestation of the behaviour, and for a duration of six seconds following the initial onset of physical contact or movement away from the subject. Z-scores are displayed on the y-axis. **D**. Heat map of the Z-scores for each event. The heat map illustrates a total of 75 events, of which 47 are designated as physical touch (marked with a red line at the bottom) and 28 are marked as moving away (marked with a blue line at the top). The scale of calcium activity is observable on the right side of the image. **E.** Statistical analysis of physical touch: The two-second Z-score AUC was compared to the two-second Z-score AUC two seconds prior to physical touch. The Wilcoxon test indicated a statistically significant difference ($p = 0.0012$). **F.** Statistical analysis of the phenomenon of moving away: the 2-second Z-score area under the curve (AUC) was compared to the AUC of the Z-scores 2 seconds prior to the occurrence of movement away from the mean ($p = 0.0022$). Source:(34)

5. DISCUSSION

5.1 The posterior intralaminar thalamic nucleus

The posterior intralaminar thalamus (PIL) has been designated with a variety of names over the course of its historical classification (Table S2). The activation of the region has been established in social behaviour, primarily in the regulation of maternal behaviours. Recent studies also cite the PIL as a constituent of the lateral thalamus (LT). The lateral thalamus is regarded as a complex comprising the PIL and adjacent brain regions, such as the peripeduncular area (PP), the supragenicular nucleus (SG), and the medial division of the medial geniculate body (MG) (35).

The PIL can be subdivided into two parts, a lateral and a medial subdivision (36). While the two regions are chemoarchitectonically similar, with both containing neurons expressing calbindin and calretinin, their projections and functions differ. Recent research has demonstrated that neurons within the medial subdivision that express PTH2 are known to project to both the MPOA and the PVN. In contrast, neurons in the lateral subdivision primarily project to the amygdala and regulate fear conditioning (37). While the majority of studies do not differentiate between the two components of the PIL due to their proximity, the functional connection and distinction between the two subdivisions remains ambiguous.

The lateral thalamus (LT) is a recipient of multimodal sensory inputs, including auditory, somatosensory, and visual stimuli. In contrast, the PIL neurons receive predominantly auditory and somatosensory inputs. PIL neurons receive inputs from the cuneate and gracile nuclei, as well as from dorsal horn neurons in the spinal cord directly. These inputs carry social touch information to the PIL and pain information from the lateral parabrachial nucleus (PBL) (18, 38). The auditory inputs are derived from the external cortex of the inferior colliculus (ECIC). The PIL also receives inputs from the VMH (18). Given the established role of VMH neurons in the triggering of aggression, it is conceivable that the VMH-to-PIL pathway plays a significant role in the regulation of aggression.

The present findings demonstrate that the PIL neurons project to several socially relevant brain regions, including the MPOA, the lateral septal nucleus (LS), and the PVN. The following structures have been identified as relevant to the present study: the

infralimbic cortex (ILC), the dorsomedial hypothalamic nucleus (DMH), the PAG, and multiple regions of the amygdala, particularly the basomedial (BMA) and the medial amygdaloid nuclei (MeA). As the PIL receives multimodal sensory inputs and projects to regions governing social behaviours, it could potentially integrate and process social sensory information before conveying it to higher brain areas.

5.2 The function of PIL neurons in social behaviours

Social interactions can be interpreted on a continuous scale. On one side of the spectrum, prosocial behaviour is predominant, while on the other side, antisocial behaviour is prevalent. Prosocial behaviours encompass affiliative and parental behaviours, while antisocial behaviour is characterized by manifestations of aggression. PIL neurons have been demonstrated to exhibit a response to social interactions, as evidenced by c-Fos expression (39). It has been established that c-Fos expression is the result of friendly interactions between adult female rats. Furthermore, evidence has been presented demonstrating that social interactions between male rats result in the activation of PIL neurons. The results of chemogenetic studies have demonstrated that PIL neurons also play a role in regulating social behaviours. The results of this study demonstrate that chemogenetic manipulation of PIL neurons can exert a simultaneous influence on both aggressive and affiliative behaviours. Selective chemogenetic manipulation of socially activated PIL neurons corroborates these results. PIL neurons have been postulated to play a role in the regulation of social behaviours by promoting prosocial interactions.

One potential regulatory pathway that has the capacity to shift behaviour towards antisocial behaviour is the VMH-to-PIL pathway. While the PIL receives sensory information from various brain regions, inputs from the VMH are particularly salient. Given the established role of VMH neurons as the primary instigators of aggressive behaviour, it can be postulated that these neurons may also exert a regulatory influence over PIL neurons, thereby leading to a reduction in prosocial interactions and an escalation in aggressive tendencies.

Given that PIL neurons project to multiple brain regions that regulate social interaction, it is conceivable that they influence social behaviours through multiple pathways. Of these projections, one of the most salient is the one to the MPOA. The

results of this study demonstrate that chemogenetic stimulation of PIL neurons activates MPOA neurons, and that chemogenetic inhibition of PIL neurons can suppress social-induced c-Fos in the MPOA. This finding indicates that the transmission of social information received by the MPOA is facilitated by the PIL.

Another potential target area for PIL neurons is the PVN. A similar phenomenon has been observed in the PVN, which also exhibits a substantial presence of PTH2-containing fiber terminals derived from the PIL. It has been documented that auditory stimuli received by the pups can impact lactation through the PIL-to-PVN pathway. The hypothesis that the PIL-to-PVN pathway contributes to the regulation of social interactions between adult conspecifics by conveying auditory social information is a plausible one.

The lateral septal nucleus has also been the focus of recent research. The site has been described as significant for its role in regulating social cognition, maternal care, and maternal aggression (40). Recent findings have revealed that PTH2-expressing PIL neurons reach the LS and are active during maternal behaviour (41). Despite the absence of data regarding the PIL-to-LS pathway's role in regulating social interactions between adults, this potential avenue for research remains a viable prospect for future studies.

5.3 The importance of social touch

It has been widely acknowledged for some time that physical contact between individuals plays a significant role in the development and maintenance of healthy social interactions. This phenomenon was first demonstrated in Harlow's seminal experiments, in which infant monkeys exhibited a preference for proximity to warm, soft mother substitutes, despite the absence of milk (42).

Subsequent research has demonstrated that social and somatosensory isolation during early life can modify gene expression (43), consequently affecting animal behaviour (44, 45). Animals that are socially isolated generally exhibit an increase in antisocial behavioural patterns, including elevated levels of atypical aggression (46, 47). This finding suggests that somatosensory inputs are essential for optimal brain development (48).

In the present study, it was observed that animals subjected to 1isolation exhibited heightened levels of aggression toward unfamiliar conspecifics and increased friendly interactions upon reunion with familiar conspecifics. A recent study also established that social interactions and somatosensory contacts are homeostatically regulated, similarly to hunger and other homeostatic functions. A paucity of social interactions has been demonstrated to trigger social interaction-seeking behaviour. Once the animal has had enough interaction, social behaviours stop due to "social satiety." This process is subject to regulation by the MPOA, whereby neurons exhibit a response to social interactions, while others maintain an active state during periods of isolation (10).

The significance of tactile and other somatosensory interactions has been extensively researched in human subjects. A body of research has demonstrated that children under the age of eight who have exhibited behavioural problems and who received five to ten minutes of massage therapy seven times a week for a period of six months have demonstrated a significantly reduced tendency towards aggression. Furthermore, studies have demonstrated that massage therapy can reduce long-term aggression (49). Additionally, empirical evidence has demonstrated the efficacy of massage therapy in reducing aggressive behaviours in adolescents (50).

Numerous studies have demonstrated that social isolation and a paucity of social interaction can lead to aggressive behaviour. Conversely, sufficient somatosensory input has been shown to inhibit aggressive behaviour and promote friendly interaction. However, the precise sensory pathways involved in this process remain to be elucidated. One potential correlation could be identified in the PIL-to-MPOA pathway.

5.4 The role of the PIL in conveying social touch information to the MPOA to affect affiliative and aggressive innate behaviours

PIL neurons have an abundance of fiber terminals in the MPOA. The hypothesis posits that social information conveyed through the PIL reaches the MPOA, resulting in behavioural regulation. As previously discussed, social isolation and physical contact are critical factors in regulating social behaviour. Therefore, the PIL-to-MPOA pathway may serve as a regulatory neuronal pathway that fosters prosocial interaction. Utilizing fiber photometric analysis, we have demonstrated that PIL neurons that reach the MPOA respond to physical touch and become activated by direct social contact.

We have also collected functional evidence that the PIL-to-MPOA pathway regulates affiliative and aggressive behaviours. This finding suggests that social touch, transmitted through the PIL and reaching the MPOA, promotes increased prosocial behaviour and suppresses aggression (Figure 13).

Our findings demonstrate that PTH2-containing fiber terminals from the PIL approach and probably communicate with GABAergic cells in the MPOA via their receptors. A body of research has demonstrated that GABAergic cells within the medial prefrontal cortex (MPFC) have the capacity to encourage prosocial interaction (24, 51). However, the precise GABAergic cell population innervated by the PIL remains to be elucidated. The hypothesis that MPOA ERα-positive neurons could be a potential target for PIL neurons is predicated on the premise that these neurons have been described as participating in the regulation of parental behaviours (52) by projecting to the PVN and PAG (53), and to inhibit aggression through VMH (8, 25). The thyrotropin-releasing hormone receptor (TRHR) MPOA neurons, which are GABAergic, could also be a target for PIL neurons. These neurons become active when an animal reunites with its conspecific and respond to somatosensory inputs (10). Oxytocin-receptor-expressing GABAergic neurons in the MPOA (54) are also potential targets for PIL neurons, since oxytocin has been demonstrated to play a role in the regulation of social behaviours (55). Future research endeavours will ascertain the precise targets of PIL neurons within the MPOA, as well as the MPOA circuitry, which is subsequently responsible for promoting positive valence behaviours.

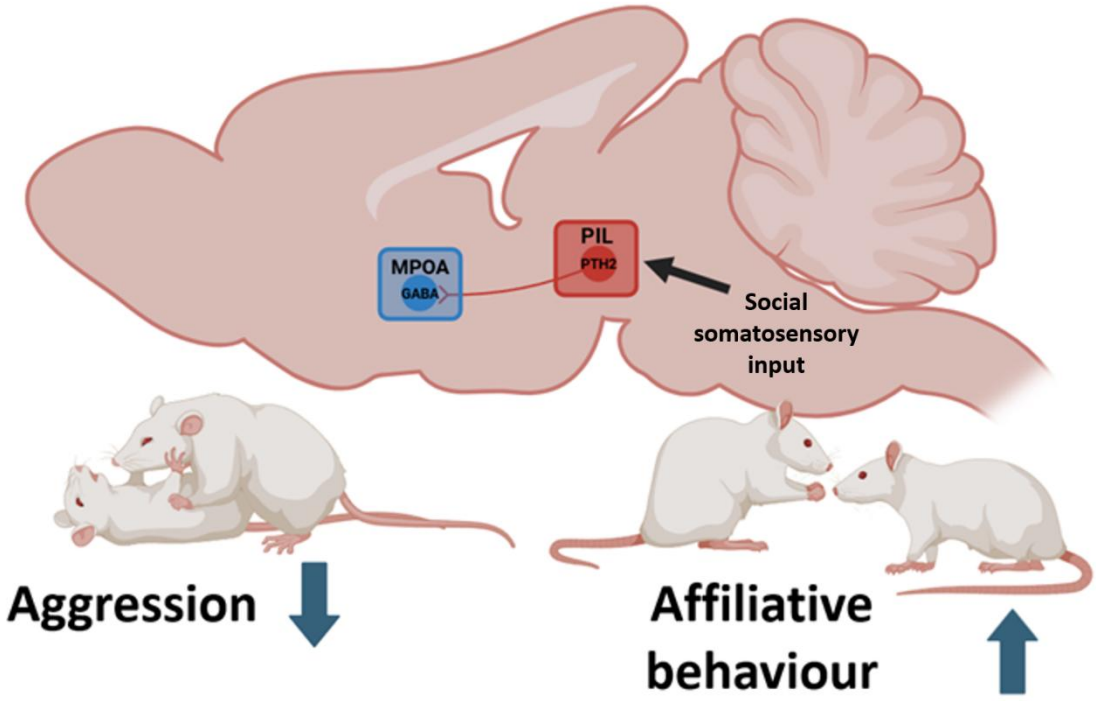

Figure 13. Summary of the neuronal pathway regulating aggression and affiliative behaviour

PIL neurons have been shown to be activated by somatosensory inputs, thereby promoting social grooming and reducing aggression through their projections to the MPOA. It is plausible that PTH2-positive PIL neurons are acting on GABA-ergic MPOA neurons.

6. CONCLUSIONS

1. PIL neurons project to several socially relevant brain regions, including the medial preoptic area, the medial amygdala and the paraventricular hypothalamic nucleus.

2. The chemogenetic stimulation of PIL neurons increases social grooming behaviour, while the inhibition of these neurons decreases its duration.

3. Selective stimulation of PIL neurons that project to the MPOA increases social grooming, while the opposite effect was observed with inhibition.

4. Selective chemogenetic stimulation of fiber terminals originating from the PIL in the MPOA increase grooming, while inhibition has the opposite effect.

5. PTH2-containing terminals were found in close proximity to GABAergic neurons in the MPOA.

6. Social isolation induces affiliative behaviour and aggression.

7. Chemogenetic inhibition of PIL neurons active during male social interaction increases aggression, while stimulation of these neurons decreases dominant mounting.

8. Selective chemogenetic stimulation of the PIL-to-MPOA pathway decreases, while its inhibition increases aggression.

9. Chemogenetic stimulation of socially tagged PIL neurons activates the MPOA, increasing c-Fos expression.

10. The chemogenetic inhibition of socially tagged PIL neurons suppress the activation of the PIL and MPOA during aggressive interactions.

11. Fiber photometric measurements showed that PIL neurons reaching the MPOA are activated by physical touch.

7. SUMMARY

We identified a novel neuronal pathway that conveys somatosensory social information from the posterior intralaminar thalamic nucleus (PIL) to the medial preoptic area (MPOA), regulating affiliative and aggressive behaviours. We mapped the efferent connections of the PIL and found an abundance of fibers in the MPOA. PTH2-containing PIL neurons were demonstrated to closely appose vGAT-positive GABAergic MPOA neurons. The application of chemogenetic stimulation to socially tagged PIL neurons resulted in the activation of neurons within the MPOA, suggesting that the PIL to MPOA projections are able to activate their MPOA targets. Functional experiments were also performed to examine whether the PIL-MPOA pathway exerts a regulatory influence on innate social behaviours. Stimulation of PIL neurons through chemogenetics resulted in an augmentation of affiliative grooming behaviour; conversely, inhibition of these neurons elicited a contrasting effect. Furthermore, we demonstrated that social isolation can induce aggressive behaviours by the absence of somatosensory social inputs and performed activity-dependent tagging of PIL neurons in aggressive male rats. Selective inhibition of these PIL neurons led to an augmentation in the duration of aggression. The regulatory effect of the PIL-MPOA pathway on aggression was also examined by chemogenetics. Stimulation of the pathway led to a decrease in aggression, while inhibition resulted in an increase. In addition, the inhibition of PIL neurons during aggressive behaviour led to a suppression of c-Fos expression in the MPOA. To gain further information on the activity of PIL neurons projecting to the MPOA under physiological conditions, fiber photometric measurements were performed, which revealed that PIL neurons reaching the MPOA are activated by direct contact between animals. The findings, when considered collectively, indicate that the PIL to MPOA pathway fosters positive valence interactions.

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9. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

Publications related to the dissertation:

Láng T, Dimén S, Oláh S, Puska G, Dobolyi A. (2024) Medial preoptic circuits governing instinctive social behaviours, *iScience* 27:110296. Doi: 10.1016/j.isci.2024.110296. (Q1, D1)

Csikós V†, Dóra F†, **Láng T†,** Darai L, Szendi V, Tóth A, Cservenák M, Dobolyi A. (2024) Social isolation induces changes in the monoaminergic signalling in the rat medial prefrontal cortex. *Cells* 13(12):1043. Doi: 10.3390/cells13121043. †: **equal contribution**. (Q1)

Keller D, **Lang T,** Cservenák M, Puska G, Barna J, Csillag V, Farkas I, Zelena D, Dóra F, Küppers S, Barteczko L, Usdin TB, Palkovits M, Hasan MT, Grinevich V, Dobolyi A. (2022) A thalamo-preoptic pathway promotes social grooming in rodents. *Current Biology* 32:4593-4606. Doi: 10.1016/j.cub.2022.08.062. (Q1, D1)

Submitted manuscript on the topic of the dissertation:

Tamás Láng, Botond B. Drahos, Fanni Dóra, Dávid Keller, Ingrid Csordás, Vivien Szendi, Gina Puska, Valery Grinevich, Árpád Dobolyi (2025) Social Touch Suppresses Aggression via Thalamic Mechanisms bioRxiv 2025.08.23.670446; doi: https://doi.org/10.1101/2025.08.23.670446

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11. SUPPLEMENTS

	Injection sites SN	PIL
Cerebral cortex		
Prelimbic cortex	0	+
Infralimbic cortex	0	+
Dorsal peduncular cortex	0	+
Perirhinal cortex	0	+
Frontal cortex	0	0
Somatomotor cortex	0	0
Somatosensory cortex (barrel)	0	0
Cingulate cortex	0	0
Piriform cortex	0	0
Entorhinal cortex	0	0
Ectorhinal cortex	0	+
Auditory cortex – primary	0	+
Auditory cortex – secondary	0	+
Olfactory bulb		
Anterior olfactory nucleus	0	+
Hippocampus		
CA1	0	0
CA2	0	0
CA3	0	0
Dentate gyrus	0	0
Tenia tecta	0	0
Septum		
Lateral septal nucleus		
Dorsal	0	+
Intermediate	0	++
Ventral	0	+++
Medial septal nucleus	0	+
Subfornical organ	0	0
Amygdala		
Central nucleus	0	++
Basolateral nucleus	0	+
Basomedial nucleus	0	++
Lateral nucleus		
Ventral	0	0
Dorsal	0	+
Medial nucleus	0	++
Cortical nucleus	0	0
Amygdalo-striatal transition area	0	++
Basal nuclei		
Caudate-putamen	+++	+
Globus pallidus	++	+
Claustrum	0	+
Nucleus accumbens		
Shell	0	++
Core	0	+
Ventral bed nucleus of the stria terminalis	0	+++
Ventral pallidum	0	++
Thalamus		
Anterodorsal nucleus	0	0
Anteroventral nucleus	0	0
Paraventricular nucleus	0	+
Reticular nucleus	0	0
Ventral anterior nucleus	0	0
Ventral lateral nucleus	0	0
Ventral posterolateral nucleus	0	0
Ventral posteromedial nucleus	0	0
Dorsomedial nuclei	0	0
Posterior nucleus	+	+
Periventricular grey area	0	+
Zona incerta	++	+++

	Injection sites SN	PIL
Hypothalamus		
Medial preoptic nucleus	0	+++
Medial preoptic area	0	++
Lateral preoptic area	0	+
Periventricular nucleus	0	++
Supraoptic nucleus	0	+
Suprachiasmatic nucleus	0	0
Paraventricular nucleus	0	++
Anterior hypothalamic nucleus	0	+
Arcuate nucleus	0	++
Median eminence	0	0
Ventromedial nucleus	0	++
Dorsomedial nucleus	0	+++
Dorsal hypothalamic area	0	++
Posterior hypothalamic nucleus	0	++
Tuberal part of lateral hypothalamus	0	+
Lateral hypothalamic area	0	+
Supraoptic decussations	0	++
Subthalamic nucleus	+	+
Midbrain		
Superior colliculus	0	+
Inferior colliculus		
Central nucleus	0	0
External cortex	0	+
Periaqueductal central grey		
Ventrolateral	0	++
Lateral	0	++
Dorsolateral	0	++
Dorsomedial	0	++
Dorsal raphe nucleus	0	+
Midline raphe nucleus	0	0
Ventral tegmental area	0	0
Interpeduncular nucleus	0	0
Red nucleus	0	0
Cuneiform nucleus	0	+++
Lateral parabrachial nucleus	0	++
Lateral lemniscal nuclei	0	++
Lateral lemniscus	0	+
Medial paralemniscal nucleus	0	+
Pons		
Locus coeruleus	0	+
Dorsal tegmental nucleus	0	+
Pontine nuclei	0	+
Superior olive	0	+
Pontine reticular formation	+	+
Sensory (principal) trigeminal nucleus	0	0
Motor trigeminal nucleus	0	0
Pontine raphe nucleus	0	+
A5 noradrenaline cell group	0	0
Vestibular nuclei	0	0
Cerebellum		
Cortex	0	0
Nuclei	0	0
Medulla oblongata		
Cochlear nuclei	0	0
Gigantocellular reticular nucleus	0	+
Parvocellular reticular nucleus	0	++
Spinal trigeminal nucleus		
Interpolar part	0	+
Caudal part	0	0
Medullary reticular formation	0	+
Inferior olive	0	0
Medullary raphe nuclei	0	0
Gracile nucleus	0	+
Cuneate nucleus	0	+

Table S1. Semi-quantitative analysis of labeled fibers in the rat brain following the injection of BDA into the PIL or SN (substantia nigra as a control injection site), compared to the distribution of PTH2-containing fibers.

The semi-quantitative analysis of the labeled mCherry-ir fibers represented as none to

low (0), moderate (+), high (++), and very high (+++).

Name of region	Description	Reference
Mid-brain area ventromedial to the medial geniculate body	Using microstimulation, the area ventromedial to the medial geniculate body, but not the caudally located lateral midbrain tegmentum, was determined to contain effective injection sites of the milk-ejection reflex.	Tindal and Knaggs (1975)
Lateral zona incerta	Retrograde tracer injected into the supraoptic nucleus labeled in the lateral zona incerta.	Tribollet *et al.* (1985)
Posterior intralaminar nucleus (PIN)	The posterior intralaminar nucleus is a triangular-shaped structure located ventromedial to the medial division of MG and it is retrogradely labeled following injection of axonal markers into the dorsal amygdala.	Ledoux *et al.* (1985) , Ledoux *et al.* 1987)
Peripeduncular nucleus (PPN)	PPN lesions reduced maternal aggression, partially inhibited lactation and caused disturbance in the neuroendocrine control of male and female copulatory behaviour.	Hansen and Köhler (1984) , López *et al.* (1985) , Factor *et al.* (1993)
Peripeduncular nucleus (PPN)	Stimuli applied to the PPN evoked potentials in the ventromedial hypothalamic nucleus, which involves synaptic activity in both the lateral amygdaloid nucleus and the bed nucleus of the stria terminalis.	López *et al.* (1985)
Parvicellular division of the subparafascicular and posterior intralaminar nuclei	These auditory-responsive posterior thalamic nuclei project to the medial parvocellular region of the hypothalamic paraventricular nucleus.	Campeau and Watson (2000)
Posterior intralaminar nucleus (PIN)	As part of the caudal paralaminar thalamic nuclei, PIN plays a role in processing sensory stimuli during emotional situations, to the amygdala.	Linke and Schwegler (2000)
Centromedian and parafascicular nuclei (Cm/Pf)	In macaque monkey studies, most Cm/Pf neurons show multimodal sensory activity, responding to auditory, visual and/or somatosensory stimuli. Cm/Pf neurons generally showed greater responses to unexpected sensory stimuli and responded to the sensory stimuli.	Matsumoto *et al.* (2000) , Saalmann (2014)
Intralaminar centromedian/ parafascicular thalamic complex (CM/PF)	The CM/PF has been related to motor control and pain, as well as attentional processing, and sexual behaviour in animal studies. High-resolution imaging at ultra-high field strength identified activation during attentional processes in the intralaminar CM/PF in humans.	Minamimoto and Kimura (2002) , Metzger *et al.* (2013)
Subparafascicular and posterior intralaminar nuclei	Subparafascicular and posterior intralaminar nuclei were labeled following the injection of retrograde tracer into the inferior colliculus.	Winer *et al.* (2002)

Parvocellular subparafascicular thalamic nucleus (SPFp)	The medial subdivision of SPFp contains a dense population of galanin-immunoreactive fibers, originating from galanin neurons in the lumbosacral spinal cord. In contrast, the lateral subdivision contains CGRP-positive fibers and neurons.	Coolen *et al.* (2003)
Posterior intralaminar complex of the thalamus (PIL)	A group of PTH2 neurons scattered throughout the posterior intralaminar complex of the thalamus (PIC), an area that includes the parvicellular subparafascicular nucleus, the posterior intralaminar thalamic nucleus, and cells in the most caudal and lateral part of the zona incerta. This area is situated in the rostral end of the lateral mesencephalic tegmentum, demonstrates retrogradely labeled neurons after mediobasal hypothalamic injections and Fos activation in lactating rats.	Palkovits *et al.* (2010), Cservenák *et al.* (2010), Cservenák *et al.* (2013)
Posterior intralaminar thalamic nucleus (PIL)	Using expression-based correlation maps and the manual mapping of mouse and human datasets, genes associated with GABAergic neurons (Gad1, Gad2, and Slc32a1), Calbindin 2, Parvalbumin and Insulin-like growth factor binding protein 4 were described in the PIL.	Nagalski *et al.* (2016)
Posterior intralaminar complex of the thalamus (PIL)	The PIL also corresponds to the posterior intralaminar complex, described as containing calbindin but not parvalbumin in the mouse. c-Fos study showed that the PIL is activated upon the social encounter of female rats. The PTH2-positve neurons of PIL innervate and activate the oxytocin cells of PVN.	Cservenák *et al. (*2017)
Posterior intralaminar thalamic nucleus (PIL)	Thalamic cells retrogradely labeled from the lateral amygdala with cholera toxin B subunit mainly located around the auditory thalamus in the PIL and the suprageniculate regions.	Barsy e*t al.* (2020)

Table S2. Previous anatomical and functional investigations of the PIL area.

The PIL was named in various ways by different research groups.. The functions of the area were also investigated, and its role in the regulation of maternal behaviour, as well as in the auditory system have been studied in some detail.

Product name	Host species	Catalogue number	Dilution
Primary antibodies			
Anti-calbindin D-28k Antibody	Mouse	Cat. #: C9848, clone CB-955, lot #031M4859 (Sigma). RRID: AB_476894	1:500 (fluorescent staining in rat)
Anti-mCherry Antibody	Chicken	Cat. #: AB205402 (Abcam) RRID: AB_2722769	1:1,000 (fluorescent staining and immunoperoxidase reaction)
Anti-GFP Antibody	Goat	Cat. #: AB5450 (Abcam) RRID: AB_304897	1:1,000 (fluorescent staining)
Anti-NeuN Antibody	Mouse	Cat. #: MAB377 (Merck Millipore), RRID: AB_2298767	1:500 (fluorescent staining)
Anti-Parvalbumin Antibody	Mouse	Cat. #: P3088, clone PARV-19, lot #100M4797 (Sigma) RRID: 477329	1:2,500 (fluorescent staining),

Anti-PTH2R Antibody	Rabbit	Gift from Ted B. Usdin, RRID: AB_2315229	1:20,000 (fluorescent staining)
Anti-PTH2 Antibody	Rabbit	Gift from Ted B. Usdin, RRID: AB_2315466	1:3,000 (fluorescent staining)
c-Fos Antibody	Rabbit	Cat. #: SC-166940 (Santa Cruz Biotechnology) RRID: AB_10609634	1:500 and 1:750 (fluorescent staining) and 1:3000 (immunoperoxidase reaction)
Secondary antibodies			
Alexa Fluor 594 AffiniPure Donkey Anti-Rabbit IgG	Donkey	Cat. #: 711-585-152 (Jackson ImmunoResearch) RRID: AB_2340621	1:500
Alexa Fluor 594 AffiniPure Donkey Anti-Chicken IgY	Donkey	Cat. #: 703-585-155 (Jackson ImmunoResearch) RRID: AB_2340377	1:500
Alexa Fluor 594 AffiniPure Donkey Anti-Mouse IgG	Donkey	Cat. #: 715-585-150 (Jackson ImmunoResearch) RRID: AB_2340854	1:500
Cy5 AffiniPure Donkey Anti-Mouse IgG	Donkey	Cat. #: 715-175-151 (Jackson ImmunoResearch) RRID: AB_2340820	1:300
Biotin-SP (long spacer) AffiniPure Donkey Anti-Chicken IgY (IgG)	Donkey	Cat. #: 703-065-155 (Jackson ImmunoResearch) RRID: AB_2313596	1:1,000
Biotin-SP (long spacer) AffiniPure Donkey Anti-Goat IgY (IgG)	Donkey	Cat. #: 705-065-003 (Jackson ImmunoResearch) RRID: AB_ 2340396	1:1,000
Solutions			
VECTASTAIN® Elite ABC-HRP Kit, Peroxidase	-	PK-6100 (Vector Laboratories, Burlingame, CA, USA)	1:500 (1:300 for visualization of BDA and immunolabeling in human brain sections)
DAB Substrate Kit, Peroxidase (HRP), with Nickel, (3,3'-diaminobenzidine)	-	SK-4100 (Vector Laboratories, Burlingame, CA, USA)	-
Aqua-Poly/Mount	-	87001-902 (VWR)	-
DePeX mounting medium	-	06522 (Sigma Aldrich)	-

Table S3. Antibodies and histological reagents used in the study.