



Maternally activated connections of the ventral lateral septum reveal input from the posterior intralaminar thalamus

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Abstract

The lateral septum (LS) demonstrates activation in response to pup exposure in mothers, and its lesions eliminate maternal behaviors suggesting it is part of the maternal brain circuitry. This study shows that the density of pup-activated neurons in the ventral subdivision of the LS (LSv) is nearly equivalent to that in the medial preoptic area (MPOA), the major regulatory site of maternal behavior in rat dams. However, when somatosensory inputs including suckling were not allowed, pup-activation was markedly reduced in the LSv. Retrograde tract tracing identified various brain regions potentially influencing LSv neuronal activation through their projections. Among all, anterograde tract tracing confirmed that the posterior intralaminar thalamic nucleus (PIL), implicated in processing touch-related stimuli, targets the pup-activated region of the LSv. Moreover, nerve terminals containing the maternally induced PIL neuropeptide parathyroid hormone 2 (PTH2), were found to form synaptic connections with c-Fos activated LSv neurons using electron microscopy. Confirmation of PTH2 + PIL fibers projecting to LSv was achieved by retrograde tract tracing methods. Furthermore, double retrograde injections revealed that neurons within the PIL can project to both LSv and MPOA, suggesting their simultaneous regulation by PIL input. We also established that septal neurons activated by the pups in the mother are GABAergic and send inhibitory projections to the MPOA and other components of the maternal brain circuitry. This implies that the LSv and MPOA form an interconnected subcircuit in the maternal brain network, which is primarily driven by somatosensory input from the pups via the PIL PTH2 + neurons.

Keywords mother · suckling · parathyroid hormone 2 · tuberoinfundibular peptide 39 · medial preoptic area

Abbreviations

3rd Third ventricle
 AAV Adenoassociated virus
 ac Anterior commissure

ACo Anterior cortical amygdaloid nucleus
 AHA Anterior hypothalamic area, anterior part
 AHiPL Amygdalohippocampal area, posterolateral part
 AHiPM Amygdalohippocampal area, posteromedial part
 AM Anteromedial thalamic nucleus
 APir Amygdalopiriform transition area
 APTV Anterior pretectal nucleus, ventral part
 ArcN Arcuate nucleus
 AVPe Anteroventral periventricular nucleus
 BDA Biotinylated dextran amine
 BLA Basolateral amygdaloid nucleus, anterior part
 BMA Basomedial amygdaloid nucleus, anterior part
 BMP Basomedial amygdaloid nucleus, posterior part
 BNST Bed nucleus of the stria terminalis
 BSA Bovine serum albumin
 CA1 Field CA1 of the hippocampus
 CA3 Field CA4 of the hippocampus
 cc Corpus callosum

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CeA	Central amygdaloid nucleus	ml	Medial lemniscus
CL	Centrolateral thalamic nucleus	MnPO	Median preoptic nucleus
Cl	Clastrum	MnR	Median raphe nucleus
CM	Central medial thalamic nucleus	MPB	Medial parabrachial nucleus
CnF	Cuneiform nucleus	MPOA	Medial preoptic area
CnFD	Cuneiform nucleus, dorsal part	MPOL	Lateral part of the medial preoptic nucleus
cp	Cerebral peduncle	MPOM	Medial part of the medial preoptic area
Cpu	Caudate putamen	MPT	Medial pretectal nucleus
CTB	Cholera toxin beta subunit	MS	Medial septum
DA	Dorsal hypothalamic nucleus	MTu	Medial tuberal nucleus
DAB	3,3'-Diaminobenzidine	NAc	Accumbens nucleus
DG	Dentate gyurs	NAcS	Accumbens nucleus, shell
DLEnt	Dorsolateral entorhinal cortex	Nu	Nucleus
DLL	Dorsal nucleus of the lateral lemniscus	och	Optic chiasm
DLPAG	Periaqueductal grey, dorsolateral subdivision	OPT	Olivary pretectal nucleus
DMD	Dorsomedial hypothalamic nucleus	opt	Optic tract
DMPAG	Periaqueductal grey, dorsomedial subdivision	PaDC	Paraventricular hypothalamic nucleus, dorsal cap
DpMe	Deep mesencephalic nucleus	PAG	Periaqueductal grey
DR	Dorsal raphe nucleus	PB	Phosphate buffer
DTT	Dorsal tenia tecta	PeF	Perifornical nucleus
Ect	Ectorhinal cortex	PeFLH	Perifornical part of the lateral hypothalamus
En	Endopiriform nucleus	PFA	Paraformaldehyde
f	Fornix	PFC	Prefrontal cortex
FBS	Fetal bovine serum	PHA	Posterior hypothalamic area
fi	Fimbria of the hippocampus	PIL	Posterior intralaminar thalamic nucleus
GP	Globus pallidus	PLCo	Posterolateral cortical amygdoid nucleus
GrDg	Granular layer of the dentate gyrus	PLd	Paralambdoid septal nucleus
Hb	Habenular nuclei	PMCo	Posteromedial cortical amygdoid nucleus
hif	Hippocampal fissure	PMnR	Paramedian raphe nucleus
IAD	Interanterodorsal thalamic nucleus	PN	Paranigral nucleus of the ventral tegmental area
IC	Inferior colliculus	Pn	Pontine nuclei
IG	Indusium griseum	PnRe	Pontine reticular nucleus
KF	Kölliker-Fuse nucleus	PO	Periolivary area
Ld	Lambdoid septal zone	PoT	Posterior thalamic nuclear group, triangular part
LDTg	Laterodorsal tegmental nucleus	PP	Peripeduncular nucleus
LPAG	Periaqueductal grey, lateral subdivision	PrCnF	Precuneiform area
LPB	Lateral parabrachial nucleus	PRh	Perirhinal cortex
LPO	Lateral preoptic area	PS	Parastrial nucleus
LS	Lateral septum	PT	Paratenial thalamic nucleus
LSd	Dorsal subdivision of the lateral septum	PTg	Pedunculopontine tegmental nucleus
LSi	Intermediate subdivision of the lateral septum	PTH2	Parathyroid hormone 2
LSv	Ventral subdivision of the lateral septum	PVA	Paraventricular thalamic nucleus, anterior part
LT	Lateral terminal nucleus of the accessory tract	PVN	Paraventricular hypothalamic nucleus
LTDg	Laterodorsal tegmental nucleus	PVP	Paraventricular thalamic nucleus, posterior part
lv	Lateral ventricle	REth	Retroethmoid nucleus
MBP	Medial parabrachial nucleus	Sag	Sagulum nucleus
ME	Medial eminence	SC	Superior colliculus
MeAD	Medial amygdaloid nucleus, anterodorsal part	SCh	Suprachiasmatic nucleus
MeAV	Medial amygdaloid nucleus, anteroventral part	Sfi	Septofimbrial nucleus
MePD	Medial amygdaloid nucleus, posterodorsal part		
MePV	Medial amygdaloid nucleus, posteroventral part		
MG	Medial geniculate nucleus		

SFO	Subfornical organ
SG	Supragenulate nucleus
SHi	Septohippocampal nucleus
SHy	Septohypothalamic nucleus
SI	Substantia innominata
sm	Stria medullaris of the thalamus
SN	Substantia nigra
SON	Supraoptic nucleus
SPF	Subparafascicular thalamic nucleus
STh	Subthalamic nucleus
STIA	Bed nucleus of the stria terminalis, intraamygdaloid nucleus
Su5	Supratrigeminal nucleus
SuM	Supramammillary nucleus
TB	Tris buffer
TuLJH	Tuberal region of the lateral hypothalamus
VDB	The nucleus of the vertical limb of the diagonal band of Broca
Ve	Vestibular nucleus
VGAT	Vesicular GABA transporter
VLPA	Periaqueductal grey, ventrolateral subdivision
VLPO	Ventrolateral preoptic nucleus
VMDM	Ventromedial hypothalamic nucleus dorsomedial part
VMH	Ventromedial hypothalamic nucleus
VMHC	Ventromedial hypothalamic nucleus, central part
VMHVL	Ventromedial hypothalamic nucleus, ventrolateral part
vMPO	Ventromedial preoptic nucleus
VOLT	Vascular organ of the lamina terminalis
VP	Ventral pallidum
VS	Ventral subiculum
vsc	Ventral spinocerebellar tract
ZI	Zona incerta

Introduction

Maternal care is a special form of prosocial behavior, which includes a set of behaviors to increase the offspring's survival. Indeed, the behavior of female rats changes profoundly when they become mothers. They begin caring for the offspring and protect them while demonstrating reduced anxiety and stress hyporesponsiveness (Woodside 2016). These behavioral alterations are governed by the so-called maternal circuitry of the brain. The medial preoptic area (MPOA) in the anterior hypothalamus is known to play a pivotal role within the maternal circuitry as its role is well-defined in the development, maintenance, and execution of proper maternal behavior through a subset of its GABAergic and galanin-expressing neurons (Dimén et al. 2021; Kohl et al. 2018; Wu et al. 2014). However, a network of additional

interconnected brain centers is also involved in the control of maternal motivation and behaviors (Dulac et al. 2014).

The lateral septum (LS) is a member of the maternal circuitry implied in the regulation of maternal care based on lesion studies (Flannelly et al. 1986; Fleischer and Slotnick 1978). The maternal function of lateral septal neurons is also supported by their activation in response to pup exposure. The density of pup-activated lateral septal neurons is very high in rodent mothers, comparable to that in the MPOA (Lin et al. 1998; Lonstein et al. 1997; Oláh et al. 2018). In addition, gene expressional alterations in the LS were also found to be associated with the different states of motherhood (Eisinger et al. 2013). The functional examinations reported on the maternal role of the LS implicated the area in maternal social fear response and maternal aggression (D'Anna and Gammie 2009; Lee and Gammie 2009; Menon et al. 2018; Scotti et al. 2011). Thus, the involvement of the LS in maternal adaptation is widely accepted (Numan 2007; Olazábal et al. 2013; Sheehan et al. 2004), however, its circuit-level incorporation in the maternal brain network has not yet been performed and our knowledge on the pup-activated LS neurons is also rudimentary.

The pathway through which somatosensory (e.g. suckling) stimuli from the pup reaches the MPOA and other brain regions, like the LSv, exhibiting heightened activity in response to pup exposure, has long remained elusive. The MPOA has a broad connection network with various brain areas involved in maternal adaptation (Dimén et al. 2021; Kohl et al. 2018; Simerly and Swanson 1986, 1988), including a recently intensively studied thalamic region, the posterior intralaminar thalamic nucleus (PIL). The PIL has been shown to function as an integrative area of somatosensory cues from both adult conspecifics (Keller et al. 2022; Leithead et al. 2023) and pups (Dobolyi et al. 2018) while it may also be essential in relaying pup ultrasonic vocalization to upper brain centers (Valtcheva et al. 2023). An elevated activation level of PIL neurons was described when mothers interacted with their pups freely, while a reduced but not fully diminished level was found when mothers were separated from their pups by a barrier preventing somatosensory contact (Cservénák et al. 2013). Based on this activation pattern as well as its inputs from the spinal cord dorsal horn and outputs towards social and maternal brain regions (Cservénák et al. 2017b, a), the PIL was suggested as a thalamic relay providing somatosensory information for social and maternal behaviors (Dobolyi et al. 2018). Therefore, in the present study, we addressed to determine the precise distribution of the pup-activated LS neurons as well as their neurochemical characterization. The inputs, by which they are activated in response to pup exposure, as well as their projections by which they affect other brain regions to exert their maternal regulatory functions were also investigated. While hormonal actions induce maternal

adaptation during pregnancy, maternal motivation is maintained by the pups in the postpartum period (Bridges 2015). Mothers receive a combination of sensory cues from the pups. To determine the role of the direct contact somatosensory inputs from the pups in the activation of LS neurons, pups were added to their mothers following a period of separation with or without a physical barrier. The potential neuronal pathways providing innervation to the pup-activated LS neurons were determined by injecting retrograde tracer to the ventral subdivision of the lateral septum (LSv) where suckling-activated neurons were detected at high density. Among the retrogradely labeled brain regions, those activated by pup exposure were further evaluated, and the posterior intralaminar thalamic nucleus (PIL) emerged as a brain region relaying pup-related information to the LSv. Therefore, anterograde labeling of the PIL was carried out to establish if PIL neurons approach pup-activated LSv neurons. Additional double retrograde tracer experiments were performed to find out if PIL neurons projecting to the LSv also project to the MPOA. While suckling has been reported to activate PIL neurons (Cservénák et al. 2013), a maternally induced neuropeptide, parathyroid hormone 2 (PTH2) has also been described in this nucleus (Cservénák et al. 2010, 2013). In the present study, we also addressed whether PTH2-containing neurons project to the LSv and if they innervate pup-activated neurons within this region. For that purpose, double immunoelectron microscopy was performed. Subsequently, the neurochemical characteristics of pup-activated LSv neurons were determined using a reporter mouse line where GABAergic neurons are fluorescently labeled. In this experiment, we also addressed whether LSv neurons are similarly activated in mice as we found in rats. Finally, the projection pattern of the LSv was determined using the injection of an anterograde tracer into the LSv. The target areas of the LSv projections were discussed in relation to the extent of their activation in response to pup exposure.

Material and methods

Experimental model and subject details

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/568-7/2020). All 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.

A total of 41 experimental animals were used in this study: 10 adult female mice and 31 adult female rats (Wistar, Charles Rivers Laboratories, Hungary). Maternally activated c-Fos expression patterns were examined in 18 rats: 6 rats per group (mothers with and without physical contact with their pups, control mothers). One of these mother rat brains was applied for fluorescent immunohistochemistry and two additional virgin rats were mated and then used for correlative light and electron microscopic experiment. Mother rats who delivered fewer than 8 pups or whose pups died were excluded from the study. All of the pregnant and mother rats used in this study were individually housed. The number of pups was adjusted to 8 within 3 days of delivery. An additional 9 rats were used for single tract-tracing (two virgin female rats for retrograde tracing of the LSv, three mother rats for retrograde tracing of the LSv, one mother rat for anterograde tracing of the PIL, two mother rats for anterograde tracing of the LSv, and one mother rat for retrograde tracing of the MPOA). After recovery, rats involved in maternal experiments were mated and sacrificed on the 9th day of postpartum. For double neuronal tracer injection, 2 virgin female rats were used. Five VGAT-ZsGreen mice (offspring of VGAT-IRES-Cre and GtROSA26Sor_CAG/ZsGreen1 mice obtained from Jackson Laboratory) were mated and sacrificed for c-Fos immunolabeling. Five C57BL/6 J mice obtained from Jackson Laboratories were used for c-Fos immunohistochemistry. All rats and mice were 90–120 days old when sacrificed.

Animals were kept group-housed under standard laboratory conditions (12/12 light/dark cycle, light on at 6.00 am, food, and water ad libitum) in transparent polycarbonate cages unless described otherwise. Animals were anesthetized with an intramuscular injection of a mixture containing 0.4 ml/300 g body weight ketamine (50 mg/ml) and 0.2 ml/300 g body weight xylazine (20 mg/ml) for stereotaxic injections and perfusions.

Neuronal tracers

The retrogradely spreading cholera toxin B subunit (CTB; List Biological Laboratories, Campbell, CA) was used to reveal the afferent neuronal connections of the LSv and MPOA of mother rats. We performed biotinylated dextran amine (BDA, 10,000 MW, Molecular Probes) injection into the PIL to confirm its PTH2 + LSv projection. pAAVrg-CAG-tdTomato (codon diversified) was a gift from Edward Boyden (Addgene plasmid # 59462; <http://n2t.net/addgene:59462>; RRID: Addgene_59462) and was used for double retrograde tract tracing. Adeno-associated viral vector expressing mCherry spreading anterogradely (pAAV-hSyn-mCherry, a gift from Karl Deisseroth; Addgene plasmid # 114472; <http://n2t.net/addgene:114472>; RRID:

Addgene_114472) was used for brain mapping of LSV outputs.

Pup-induced c-Fos activation experiments

For examining c-Fos activation changes in dams, mothers were examined on the 8–9th postpartum day. In all cases, dams were deprived of their pups for 22 h. This separation was necessary for lowering the c-Fos expression to the baseline level, thus, control dams did not get their pups back to establish standard c-Fos expression for analysis. After deprivation, dams received their pups back for 2 h to induce proper pup-promoted c-Fos expression. During these 2 h, dams could freely interact with their pups including suckling. To investigate the effects of other non-somatosensory inputs arriving from the pups, pups were enclosed in a circle-based wire-mesh box with a diameter of 10 cm and placed into their mothers' cage for 2 h. Owing this experimental protocol, dams could see, smell, and hear their pups, but no physical interaction could take place between them. In all cases, mothers were sacrificed following the 2 h direct or indirect interactions with their pups.

In the case of tract-tracing experiments, animals were allowed to recover from the stereotaxic injection procedure for two weeks before receiving a male conspecific for breeding. 8–9 days after parturition, mothers were deprived of their pups for 22 h to reduce c-Fos expression to baseline. Then, dams received their pups back for 2 h before sacrificing them to establish pup-induced c-Fos protein expression.

Retrograde tracing experiments

Injection of cholera toxin B subunit into the LSV and MPOA

LSV and MPOA were targeted by the retrogradely transporting CTB, respectively. For the Nanoinjector, glass capillaries with 1.14 mm diameter (World Precision Instruments) were filled with mineral oil (Sigma-Aldrich) and placed on the Nanoinjector. Then 1 μ m of pAAV-hSyn-mCherry was taken up into the capillary. The rats were positioned in a stereotaxic apparatus with the incisor bar set at -3.3 mm high. A hole of 2 mm diameter was drilled into the skull above the target coordinates. The following stereotaxic coordinates (Paxinos and Watson 2006) were used for targeting the LSV: AP: $+0.36$ mm from bregma, LM: -1.2 mm from midline, and DV: -5.6 mm from the surface of the dura mater. For the MPOA: AP: -0.48 mm from bregma, LM: -0.6 mm from midline, DV: -7.8 mm from the surface of the dura mater. 100 nl of CTB was injected into the LSV and MPOA at a speed of 50 nl/min. The capillary was left in place for 5 min and then withdrawn slowly. After the surgery, animals were allowed to recover for two weeks and then sacrificed.

Visualization of CTB was achieved in the way described under histological methods.

Injection of tdTomato-tagged viral vector into the LSV and cholera toxin B into the MPOA

In the case of double neuronal tracing, the LSV and MPOA of the same rats were targeted by a tdTomato-tagged viral vector (pAAVrg-CAG-tdTomato) and CTB at the same time unilaterally, respectively. First, the animals were placed into the stereotaxic apparatus in the previously described manner and approximately 2 mm diameter holes were drilled into the skull above the following coordinates for LSV: AP: $+0.36$ mm from bregma, LM: -1.2 mm from midline, and DV: -5.6 mm from the surface of the dura mater, and for MPOA: AP: -0.48 mm from bregma, LM: -0.6 mm from midline, and DV: -7.8 mm from the surface of the dura mater. The Nanoinjector was tilted by a 16.5° angle. For the tracer injections, we used a Nanoinjector with the same parameters as written under CTB injection. After injecting pAAVrg-CAG-tdTomato into the LSV, the glass capillary was changed to a new one filled with mineral oil and CTB was taken up without removing the animals from the apparatus. 100 nl of tdTomato and CTB was injected into the LSV and MPOA respectively. The animals were sacrificed after three weeks of recovery.

Anterograde tracing experiments

Injection of biotinylated dextran amine into the PIL

PIL was targeted by the anterograde tracer biotinylated dextran amine (BDA, 10,000 MW; Molecular Probes, $n=5$). Rats were placed in a stereotaxic apparatus with the incisor bar set at -3.3 mm and above the target coordinates ca. 2 mm diameter holes were drilled into the skull. 10% BDA dissolved in saline was loaded into glass micropipettes of 15–20 μ m internal diameter and positioned to the following stereotaxic coordinates (Paxinos and Watson 2006): AP = -5.2 mm from bregma, ML = $+2.6$ mm from the midline, DV = -6.8 mm from the surface of the dura mater. Following the proper positioning of the micropipette, the BDA was injected into the area by iontophoresis. For this process, a constant current source (51,413 Precision Current Source, Stoelting, Wood Dale, IL) was used which conveyed a current of $+6$ μ A with a 7 s on and 7 s off pulsation for 15 min. Following the injection, the pipette remained in place for 10 min without a current, then it was withdrawn under a negative current. After the surgery, the animals were allowed to recover and were sacrificed 7 days later. BDA was visualized as elaborated under histological procedures for Ni-DAB (nickel-intensified 3,3'-diaminobenzidine) labeling. To identify and verify the injection site and the projection

sites' relation to the location of PTH2 neurons and fibers, double fluorescent immunolabeling was used in a manner described under histology methods.

Injection of mCherry-tagged viral vector into the LSv

An adeno-associated viral vector expressing mCherry (pAAV-hSyn-mCherry) was injected into the LSv unilaterally. pAAV-hSyn-mCherry was injected into the area by using a Nanoinjector (Nanoinject 2010 by World Precision Instruments). The stereotaxic injections were done the same way as previously described under injection of CTB into the LSv. The capillary was placed above the following stereotaxic coordinates (Paxinos and Watson 2006): AP: +0.36 mm from bregma, ML: − 1.2 mm from midline and DV: − 5.6 mm from the surface of the dura mater. 50 nl of the viral vector was targeted unilaterally into the area at a speed of 50 nl/min. The capillary was left in place for 5 min and then withdrawn slowly. After the surgery, animals were allowed to recover for three weeks and then sacrificed. mCherry was immunolabeled for an enhanced signal intensity. The procedure is detailed under the description of the histological process.

Histology

Animal brain tissue collection and sectioning for light microscopy

Animals were deeply anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) solution in 0.1 M phosphate buffer (PB, pH 7.4). Brains were post-fixed overnight in the same solution. After washing with PB, brains were placed into 20% sucrose-containing PB solution and then sectioned at a 50 µm thickness using a cryostat (CM1520, Leica).

Peroxidase-based single c-Fos immunohistochemistry

Every fourth free-floating section was incubated in 0.1% hydrogen-peroxide containing PB buffer for 15 min to block endogenous peroxidase activity, which was followed by an hour-long incubation in blocking buffer which is PB buffer containing 1% bovine serum albumin (BSA), 10% fetal bovine serum (FBS) and 0.5% TritonX-100 to reduce non-specific labeling and to enhance antibody penetration. Sections were treated with c-Fos primer antibody (1:5000, rabbit, Abcam, Cat. #: ab190289) diluted in 0.5% BSA, 5% FBS, and 0.25% TritonX-100 containing PB buffer overnight (Table 1). Biotinylated secondary antibody (1:500, donkey anti-rabbit, Jackson ImmunoResearch) was applied for an hour followed by an hour-long incubation of the sections in ABC solution (1:1000, Vector Laboratories). For

Table 1 Antibodies and histological reagents used in the study

Reagent	Source	Catalog number	RRID
Primary antibodies			
Rabbit anti-c-Fos antibody	Abcam	ab190289	AB_2737414
Anti-PTH2 antibody	Gift from Ted B. Usdin		AB_1235466
Anti-mCherry antibody	EnCor biotechnology	CPCA-mCherry	AB_2572308
Anti-CTB antibody	List biological laboratories	703	AB_2314252
Secondary antibodies			
Biotin-SP (long spacer) AffiniPure Donkey Anti-Rabbit IgG (H + L)	Jackson ImmunoResearch	711-065-152	AB_2340593
Alexa Fluor® 594 AffiniPure Donkey Anti-Rabbit IgG (H + L)	Jackson ImmunoResearch	711-585-152	AB_2340621
Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG (H + L)	Jackson ImmunoResearch	711-545-152	AB_2313584
Alexa Fluor® 488 AffiniPure Donkey Anti-Goat IgG (H + L)	Jackson ImmunoResearch	705-545-147	AB_2336933
Alexa Fluor® 594 AffiniPure Donkey Anti-Chicken IgY (IgG) (H + L)	Jackson ImmunoResearch	703-585-155	AB_2340377
Other			
VECTASTAIN elite ABC-HRP Kit, peroxidase	Vector laboratories	PK-6100	AB_2336819
DAB substrate kit, peroxidase (HRP), with Ni-3,3'-diaminobenzidine	Vector laboratories	SK-4100	AB_2336382
NeuroTrace™ 435/455 blue fluorescent nissl stain	Thermo fisher scientific	N21479	
DAPI (4',6-diamidino-2-phenylindole dihydrochloride)	Sigma Aldrich	D9542	
Aqua-poly/mount	Polysciences	18,606-5	
DePeX mounting medium	Sigma Aldrich	06522	
Durcupan™ ACM	Sigma Aldrich	44,610	

visualization, sections were treated with 0.1% 3,3'-diaminobenzidine (DAB) enhanced by nickel-(II)-sulfate and 0.01% hydrogen peroxide solution in Tris buffer, pH = 7.6 (TB) for 7 min and were mounted by DePeX mounting medium (Sigma Aldrich).

Fluorescent c-Fos immunolabeling

Sections were incubated in a blocking buffer for an hour to reduce non-specific labeling and to enhance antibody penetration. Sections were treated with c-Fos primer antibody-containing solution (1:2000, rabbit, Abcam) overnight, which was followed by the application of the fluorescent secondary antibody, donkey anti-rabbit Alexa 594 (1:500, Jackson ImmunoResearch) for 1.5 h. Sections were mounted using an anti-fade medium (Aqua PolyMount, Polysciences Inc.).

Fluorescent PTH2 immunolabeling

Free-floating sections were treated with 0.1% hydrogen-peroxide-containing PB buffer for 15 min and with the previously described blocking buffer for an hour. First, the PTH2 primary antibody (1:3000, Research resource identifier, AB_1235466, rabbit) was applied to the sections for 48 h, which was followed by the incubation in donkey anti-rabbit biotinylated secondary antibody (1:500, Jackson ImmunoResearch) for an hour. Then, sections were washed in PB and incubated in ABC solution (1:500, Vector Laboratories) for an hour. Finally, A568-tyramide or FITC-tyramide solution containing 0.0015% H₂O₂ was used for 7 min to develop a fluorescent signal.

Fluorescent c-Fos and PTH2 double immunolabeling

Fluorescent immunolabeling of PTH2 was performed as described above. Next, another hour-long incubation in the blocking buffer was applied before incubating the section in c-Fos primer antibody (1:2000, Abcam, rabbit) overnight. Then, sections were incubated in TB containing the fluorescent secondary antibody, donkey anti-rabbit Alexa 488 (1:500, Jackson ImmunoResearch), and were mounted using anti-fade medium (Aqua PolyMount, Polysciences Inc.).

PTH2 and CTB fluorescent double immunolabeling

Following the already described endogenous peroxidase blocking, permeability enhancing, and reduction of non-specific labeling steps, the PTH2 primary antibody was first applied to the sections and visualized in the previously described method under 'Fluorescent PTH2 fluorescent double immunolabelling'. This was followed by the application of CTB primary antibody (1:10,000, List Biological

Laboratories, #703, goat) overnight. CTB was visualized by incubating the sections in TB containing the fluorescent donkey anti-goat secondary antibody, Alexa 488 (1:500, Jackson ImmunoResearch). Sections were mounted using Aqua PolyMount mounting medium.

BDA-PTH2 and BDA-c-Fos fluorescent visualization

For double fluorescent BDA-PTH2 and BDA-c-Fos labeling, sections were incubated in 0.5% Triton X-100 containing 0.1 M PB solution for 1 h, which was followed by a 2 h incubation in ABC solution (1:500, Vector Laboratories). Then, sections were treated with a biotin-tyramide solution in 1:1000 dilution with 0.003% hydrogen peroxide for 20 min and with another ABC solution (1:1000) for 1 h. Next, the sections were incubated in Alexa Fluor 594-tyramide or FITC-tyramide (1:5000) and 0.003% hydrogen peroxide-containing TB solution for 7 min to visualize the BDA signal. Following the BDA visualization, sections were incubated in PTH2 primary antibody solution (1:3000, Research resource identifier, AB_1235466, rabbit) for 48 h or c-Fos primary antibody solution (1:2000, Abcam, rabbit) for 24 h, as described previously. Finally, sections were mounted using an anti-fade medium (Aqua PolyMount, Polysciences Inc.).

c-Fos and CTB immunolabeling

As described previously, sections were incubated in blocking solution for an hour, which was followed by washing them in PB solution. Then, c-Fos primary antibody (1:2000, Abcam, rabbit) was applied to the sections overnight, followed by fluorescent secondary antibody, donkey anti-rabbit Alexa 594 (1:500, Jackson ImmunoResearch) for 1.5 h. Next, the sections were treated with the other primary antibody, CTB (1:10,000, List Biological Laboratories, #703, goat) for 24 h. CTB signal was visualized by the application of a secondary antibody, donkey anti-goat Alexa 488 (1:500 Jackson ImmunoResearch) for 1.5 h. Mounting happened with Aqua PolyMount medium (Polysciences Inc.)

mCherry and c-Fos immunolabeling

After the hour-long blocking, permeability enhancing, and reduction of non-specific labeling, sections were treated with mCherry containing solution (1:3000, EnCor Biotechnology, chicken, Cat. #: CPCA-mCherry) overnight for enhancing the mCherry signal. A secondary antibody, fluorescent donkey anti-chicken Alexa 594 (1:500, Jackson ImmunoResearch) was applied to the section for 1.5 h following washing steps in PB. Then, sections were incubated in c-Fos solution (1:2000, Abcam, rabbit) for 24 h. c-Fos signal was visualized using a fluorescent secondary antibody, donkey

anti-rabbit Alexa 488 (1:500, Jackson ImmunoResearch) for 1.5 h. As a final step, sections were mounted with an anti-fade medium (Aqua PolyMount, Polysciences Inc.).

Light microscopy and image acquiring

Microscopy and image acquiring of peroxidase-based c-Fos immunolabeled sections

Images were taken using a Nikon Eclipse light microscope equipped with a SPOT RT3 camera (Diagnostics Instruments). For the representative low magnification images, we used 2× (Plan UW objective, NA0.06) or 4× (Plan Fluor objective, NA0.13) objectives, while for higher magnification representative images and images used for statistical analysis, we applied 20× (Plan Fluor objective, NA0.50) or 40× (Plan Fluor objective, NA0.75) objectives. Representative images were adjusted using Adobe Photoshop CS6.

Microscopy and image acquiring of immunofluorescent labeled sections

Fluorescent images were captured using a Zeiss LSM 800 laser scanning confocal microscope. Lower magnification images were acquired using the 10× (Plan-Apochromat 10×/0.45) objective and the ‘Tiles’ option of the Zen software. Higher magnification images were made with 20–40× (Plan-Apochromat 20×/0.8 and EC Plan-Neofluar 40×/NA0.75) objectives. Images were adjusted using the Zen Blue Edition software.

Quantification and statistical analyses

Cell counting of c-Fos positive neurons

Analyses of images for c-Fos examination were performed using QuPath (QuPath, University of Edinburgh, Edinburgh, UK), a free bioimage analysis software (Bankhead et al. 2017). Peroxidase-based DAB immunolabeled sections were evaluated by the cell detection tool of the analysis software. In the case of VGAT-ZsGreen brain slices, first, cell nuclei were identified by the object detection algorithm, StarDist (Schmidt et al. 2018), then the Object Classifier, a supervised machine learning algorithm, was trained to identify ZsGreen, c-Fos+, and double immunolabeled cells using our own set of training images.

Statistical analyses

Cell counting data were imported to SPSS Statistics 20 (IBM) for analysis. c-Fos-positive cell density in the three distinct pup-interacting mother groups was analyzed by the

Kruskal–Wallis test and Dunn’s multiple comparison tests. Diagrams were made using GraphPad Prism8.0.2.

Semi-quantitative analysis of retrogradely labeled cell densities after CTB injection

Whole brain area mapping was performed to determine input regions of the LSv based on CTB neuronal tract-tracing experiment. Brain areas containing retrogradely labelled cell bodies were captured by 20× objective of Zeiss to clearly distinguish cell bodies and background noise (Suppl. Figure 1). Semi-quantitative scoring distinguished three level of CTB + cell densities: low number of sparsely distributed retrogradely labeled cell bodies in the area (less than 25 CTB + cell bodies per 0.1 mm²), (2) moderate number of retrogradely labeled cell bodies (25–100 CTB + cell bodies per 0.1 mm²), (3) high number of retrogradely labeled cell bodies (more than 100 CTB + cell bodies per 0.1 mm²).

Analysis of neuronal projections by anterograde tracing

After mCherry and c-Fos immunolabeling of brain sections of AAV-hSyn-mCherry injected rats, sections were captured by Zeiss LSM800 confocal microscope using a 20× objective. We used a free bioimage analysis software QuPath (QuPath, University of Edinburgh, Edinburgh, UK) (Bankhead et al. 2017) to evaluate the number of c-Fos positive cells and mCherry-related fluorescent immunoreactivity signal. A cell detection tool for c-Fos positive cell counting was used. A machine learning-based pixel classification feature was applied to automatically measure the area density of the anterogradely labeled mCherry-positive nerve fibers. Whole brain mapping was applied to reveal the target sites of LSv neurons. A diagram of the evaluated data was created with GraphPad Prism 8.0.2.

Correlative light and electron microscopy

Tissue preparation

Two female rats on the 9th postpartum day were deeply anesthetized and transcardially perfused with saline followed by 500 ml of fixative made of 4% formaldehyde, 0.05% glutaraldehyde, 0.2% picric acid in 0.1 M PB (pH 7.4). 50 µm thick sections were cut with a vibratome (VT1000S, Leica) and collected in 0.1 M PB.

Pre-embedding double immunolabeling for c-Fos and PTH2

We performed correlative light and electron microscopic immunolabelling to confirm the synaptic connection between PTH2+ terminals and c-Fos-activated neurons in the LSv of the mother rat. Free-floating vibratome sectioned brain slices

were washed in 0.1 M PB, then cryoprotected overnight first in 15% sucrose, then in 30% sucrose PB solution, and next freeze-thawed over liquid nitrogen for epitope retrieval three times. 1% sodium-borohydride, 50 mM ammonium-chloride, and 50 mM glycine solution was applied followed by blocking in 0.1 M PB containing 10% fetal bovine serum and 1% bovine serum albumin. Then, sections were incubated with anti-PTH2 primary antibody at a dilution of 1:1000 for 48 h. After washing with saline containing PB, biotin-conjugated donkey anti-rabbit secondary antibody (1:500, Jackson Immunoresearch) for 24 h, then ABC solution (1:500, Vector Laboratories) for 6 h was used. Peroxidase-based nickel-sulfate enhanced 3,3'-diaminobenzidine (Ni-DAB) (Ni-DAB; DAB peroxidase substrate kit, SK-4100, Vector Laboratories, Burlingame, CA, USA) reaction was performed to develop PTH2-related signal. After washing with 0.1 M PB, sections were processed for a second immunolabeling. The same protocol was carried out by using an anti-c-Fos primary antibody (rabbit, Abcam, ab190289) at a dilution of 1:5000. The c-Fos-related immunoreaction was revealed by DAB reaction.

Durcupan embedding of immunolabeled sections

Brain slices were postfixed in 0.5% osmium-tetroxide solution containing 3.5% glucose in 0.1 M PB for 20 min. After washing with distilled water, sections were stained with 1% uranyl acetate for one hour. Following dehydration steps in a series of increasing concentrations of ethanol and acetonitrile, sections were embedded in DurcupanTM ACM (Sigma-Aldrich) resin on slides and polymerized for 48 h at 60 °C. We identified previously selected c-Fos-positive neurons surrounded by PTH2-ir terminals by light microscope (Nikon Eclipse light microscope equipped with SPOT RT3 camera, 100×immersion oil objective NA1.3). After re-embedding the area of interest, 70 nm ultrathin sections were cut with a Reichert-Jung ultramicrotome. Sections were collected on formvar film-coated copper slot grids and contrasted with 2% uranyl acetate and lead citrate.

Electron microscopic image acquisition and image processing

Electron microscopic examination was prepared using a Jeol Jem 1011 transmission electron microscope operating at 60 kV connected with a Morada camera. Brightness and contrast were adjusted by using Adobe Photoshop CS6.

Results

c-Fos activation in the ventral subdivision of the lateral septal nucleus

The maternal activation pattern of the ventral subdivision of the lateral septum (LSv) was determined with c-Fos induction experiments in rats. The LSv was defined based on the rat brain atlas by Paxinos and Watson (Paxinos and Watson 2006) (Fig. 1) which corresponds to the traditional division of the rat LS (Risold and Swanson 1997).

Nurturing related maternal action is the major factor inducing c-Fos activation in the LSv

Mother rats were examined following 22 h of pup deprivation in three different scenarios: (i) without receiving back their pups (Fig. 2a), (ii) receiving back their pups without the possibility of physical contact with them (Fig. 2b), and (iii) receiving their pups back in a way that physical contact was possible, which always resulted in suckling within 3 min of returning the pups (Fig. 2c). We found intense c-Fos activation in the LSv in response to returning of the pups ($p < 0.001$) (Fig. 2d). The elevation was markedly higher in the group of mothers, which were allowed to suckle and nurture the pups compared to the groups where physical contact was prevented ($p = 0.002$) (Fig. 3d).

The input areas of the LSv

The brain regions that project to the part of the LSv where the highest pup-induced activation takes place in mothers were investigated next. We injected the retrogradely transporting cholera toxin B subunit (CTB) into the LSv of female rats and revealed the distribution of retrogradely labeled cells in numerous brain regions such as the medial preoptic area, paraventricular nucleus of the thalamus, the ventromedial hypothalamic nucleus, medial amygdaloid nucleus, basomedial amygdaloid nucleus, ventral subiculum, ventral hippocampus, posterior intralaminar nucleus of the thalamus (PIL), and lateral parabrachial nucleus (Fig. 3). All the input areas of the LSv are summarized in Table 2. Predominantly ipsilateral labeling of cells was distributed throughout the brain.

The LSv receives input from pup-activated neurons of the posterior intralaminar thalamic nucleus also projecting to the medial preoptic area

Among the pup-activated brain regions projecting to the LSv, the PIL has been suggested to distribute suckling

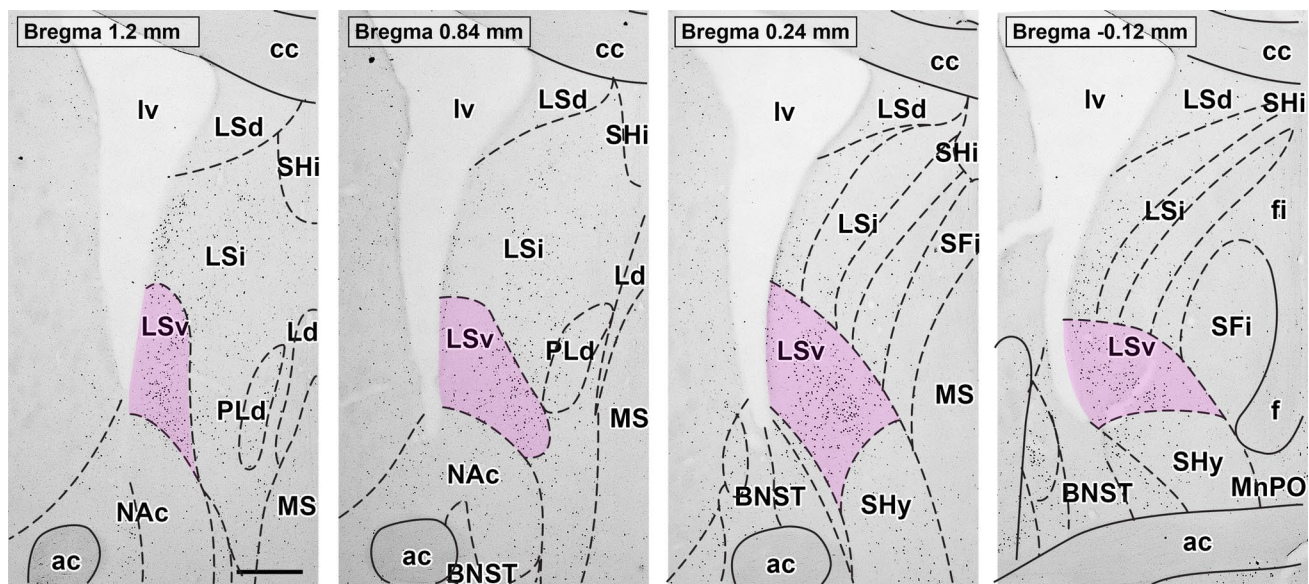


Fig. 1 Distribution of the pup-induced activation pattern in the lateral septum (LSv) in mother rat. The representative images show coronal brain sections of a mother rat after pup exposure followed by c-Fos immunolabeling. The sections demonstrate four distinct anatomical levels as indicated in the left upper corners of the panels. Magenta-colored dashed lines border the area of LSv. Templates indicating the boundaries of the brain regions were adapted from rat brain atlas by Paxinos and Watson (Paxinos and Watson 2006). Scale bar: 250 μ m.

ac anterior commissure, *BNST* bed nucleus of the stria terminalis, *cc* corpus callosum, *f* fornix, *fi* fimbria of the hippocampus, *Ld* lambdoid septal zone, *lv* lateral ventricle, *LSd* dorsal subdivision of the lateral septum, *LSi* intermediate subdivision of the lateral septum, *LSv* ventral subdivision of the lateral septum, *MS* medial septum, *MnPO* median preoptic nucleus, *NAc* accumbens nucleus, *PLd* paralambdoid septal nucleus, *SFi* septofimbrial nucleus, *SHi* septohippocampal nucleus, *SHy* septohypothalamic nucleus

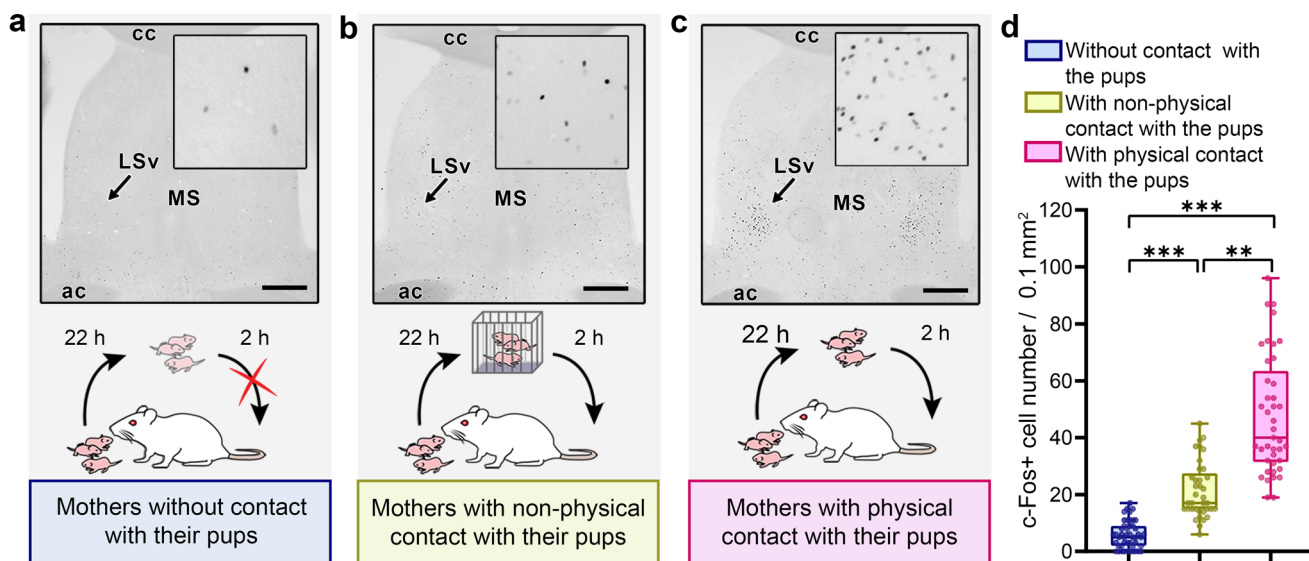


Fig. 2 Pup-induced activation of the ventral subdivision of the lateral septum (LSv) in response to pup exposure with or without physical contact. **a–c** Three experimental groups were examined to reveal the effect of pup-related stimuli on the activation of the lateral septum: mother rats after 22 h pup separation without receiving their pups back (**a**), with receiving their pups back without (**b**) and with physical contact allowed (**c**). Representative images demonstrate the c-Fos activation pattern in the LSv in the pup-exposed experimental groups.

Scale bars: 500 μ m. **d** Results of the quantification of the density of activated LSv neurons are shown. Statistical analysis was performed by Kruskal–Wallis followed by Dunn's multiple comparison tests ($n=6$ mothers per group). Horizontal lines indicate that p value is significant ($**0.001 < p < 0.001$, $***p < 0.001$). Individual values are represented by dots. Whiskers indicate minimum and maximum values. *ac* anterior commissure, *cc* corpus callosum, *LSv* ventral subdivision of the lateral septum, *MS* medial septum

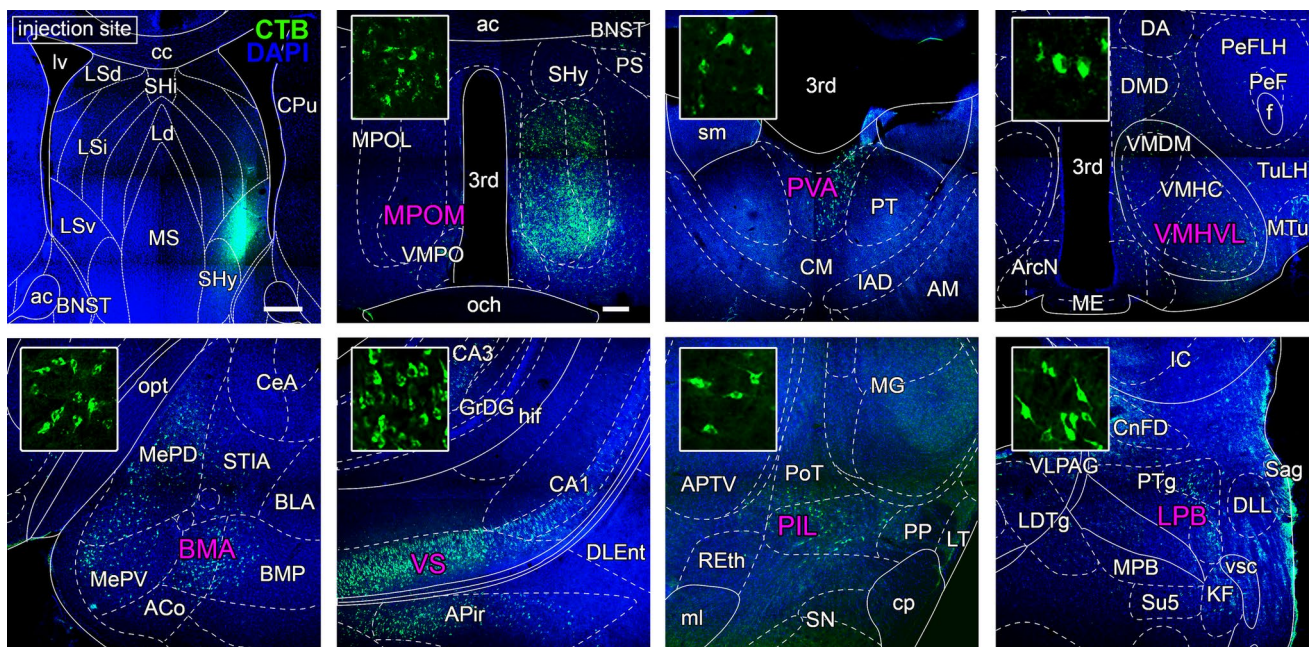


Fig. 3 Input brain regions of the ventral subdivision of the lateral septum (LSv). Retrogradely transporting cholera toxin B was used for mapping the brain areas projecting to the LSv. A representative confocal microscopic image of the injection site in the LSv is demonstrated in the left upper panel. Two female rats were used in this neuronal tract-tracing experiment resulting in the same retrogradely labelled cell distribution. Scale bar: 500 μ m. The other panels show some of the brain areas projecting to the LSv as indicated by the presence of retrogradely labeled cells. Inset panels show magnified parts of the brain regions indicated by magenta color. Scale bars: 200 μ m. 3rd third ventricle, ac anterior commissure, ACo anterior cortical amygdaloid nucleus, AM anteromedial thalamic nucleus, APir amygdalopiriform transition area, APTV anterior prepectal nucleus, ventral part, ArcN arcuate nucleus, BLA basolateral amygdaloid nucleus, anterior part, BMA basomedial amygdaloid nucleus, anterior part, BMP basomedial amygdaloid nucleus, posterior part, BNST bed nucleus of the stria terminalis, CA1 field CA1 of the hippocampus, CA3 field CA3 of the hippocampus, cc corpus callosum, CeA central amygdaloid nucleus, CM central medial thalamic nucleus, CnFD cuneiform nucleus, dorsal part, cp cerebral peduncle, CPu caudate putamen, DA dorsal hypothalamic area, DG dentate gyrus, DLEnt dorsolateral entorhinal cortex, DLL dorsal nucleus of the lateral lemniscus, DMD dorsomedial hypothalamic nucleus, dorsal part, f fornix, GrDG granular layer of the dentate gyrus, hif hippocampal fissure, IAD interanterodorsal thalamic nucleus, IC inferior colliculus, KF: Kölliker-Fuse nucleus, Ld lambdoid septal zone, LDTg laterodorsal

sal tegmental nucleus, LPB lateral parabrachial nucleus, LSd dorsal subdivision of the lateral septum, LSi intermediate part of the lateral septum, LSv ventral subdivision of lateral septum, LT lateral terminal nucleus of the accessory tract, lv lateral ventricle, ME medial eminence, MePD medial amygdaloid nucleus, posterodorsal part, MePV medial amygdaloid nucleus, posteroventral part, MG medial geniculate nucleus, ml medial lemniscus, MPB medial parabrachial nucleus, MPOL lateral part of the medial preoptic nucleus, MPOM medial part of the medial preoptic area, MS medial septum, MTu medial tuberal nucleus of the lateral hypothalamus, och optic chiasm, opt optic tract, PeF perifornical nucleus, PeFLH perifornical part of the lateral hypothalamus, PIL posterior intralaminar thalamic nucleus, PoT posterior thalamic nuclear group, triangular part, PP peripeduncular nucleus, PS parastrial nucleus, PT paratenial thalamic nucleus, PTg pedunculo-pontino tegmental nucleus, PVA paraventricular thalamic nucleus, anterior part, REth retroethmoid nucleus, Sag sagulum nucleus, SHi septohippocampal nucleus, SHy septohypothalamic nucleus, sm stria medullaris of the thalamus, SN substantia nigra, STIA bed nucleus of the stria terminalis, intraamygdaloid division, Su5 supratrigeminal nucleus, TuLH tuberal region of the lateral hypothalamus, VLPAG ventrolateral periaqueductal gray, VMDM ventromedial hypothalamic nucleus, dorsomedial part, VMHC ventromedial hypothalamic nucleus, central part, VMHVL ventromedial hypothalamic nucleus ventrolateral part, vMPO ventromedial part of the preoptic nucleus, VS ventral subiculum, vsc ventral spinocerebellar tract

information to higher-order brain areas (Dobolyi et al. 2018). To address its ability to activate LSv neurons during nurturing, CTB was injected into the LSv of mother rats (Fig. 4a) followed by pup exposure before perfusion. We found that $48.1 \pm 6.5\%$ of the LS-projecting PIL neurons are c-Fos-activated after pup exposure (Fig. 4b).

Since the MPOA is also an important output of the PIL (Keller et al. 2022), we hypothesized that both the LSv and MPOA are simultaneously affected by the input

arising from the PIL, and performed double retrograde tract-tracing (Fig. 4c). We injected tdTomato tagged retrogradely spreading AAV (AAVrg-hSyn-tdTomato) into the LSv, and CTB into the MPOA, respectively. We found double-labeled neurons in the PIL ($31\% \pm 8\%$ of LS projecting tdTomato + PIL neurons was also CTB+) confirming that afferents of the LSv may originate from MPOA-projecting PIL neurons (Fig. 4d).

Table 2 Brain areas containing retrogradely labeled cells after the injection of cholera toxin B subunit (CTB) into the ventral subdivision of the lateral septum (LSv)

Area	Density	Area	Density
Forebrain		Hypothalamus	
Cerebral cortex		Anteroventral periventricular nucleus (AVPe)	+
Prefrontal cortex (PFC)	0	Medial preoptic nucleus (MPO)	+++
Somatomotor cortex	0	Ventrolateral preoptic nucleus (VLPO)	++
Somatosensory cortex	0	Lateral preoptic area (LPO)	0
Visual cortex	0	Supraoptic nucleus (SON)	0
Auditory cortex	0	Suprachiasmatic nucleus (Sch)	0
Ectorhinal cortex (Ect)	0	Paraventricular hypothalamic nucleus, dorsal cap (PaDC)	++
Perirhinal cortex (PRh)	0	Peri-paraventricular zone	++
insular cortex (IC)	0	Anterior hypothalamic area (AH)	++
Dorsal tenia tecta (DTT)	++	Arcuate nucleus (ArcN)	++
Hippocampus		Dorsal hypothalamic area (DA)	0
Dorsal hippocampus (dHipp)	0	Ventromedial hypothalamic nucleus (VMH)	+
Ventral hippocampus (vHipp)	+++	Dorsomedial hypothalamic nucleus (DMD)	+
Ventral subiculum (VS)	+++	Medial tuberal nucleus of the lateral hypothalamus (MTu)	+++
Amygdala		Posterior hypothalamic area (PHA)	++
Central nucleus (CeA)	0	Supramammillary nucleus (SuM)	++
Anterior cortical amygdaloid nucleus (ACo)	0	Zona incerta (ZI)	0
Medial amygdaloid nuclei	0	Midbrain	
Anterodorsal part (MeAD)	0	Medial pretectal nucleus (MPT)	0
Anteroventral part (MeAV)	0	Olivary pretectal nucleus (OPT)	0
Posterodorsal part (MePD)	++	Periaqueductal grey (PAG)	
Posteroventral part (MePV)	++	Dorsomedial subdivision (DMPAG)	0
Basomedial amygdaloid nucleus (BMA)	++	Dorsolateral subdivision (DLPAG)	0
Basolateral amygdaloid nucleus (BLA)	0	Lateral subdivision (LPAG)	+
Cortical amygdaloid nuclei		Ventrolateral subdivision (VLPAG)	0
Posteromedial part (PMCo)	++	Deep mesencephalic nucleus (DpMe)	0
Posterolateral part (PLCo)	0	Superior colliculus (SC)	0
Amygdalohippocampal area		Inferior colliculus (IC)	0
posterolateral part (AHiPL)	+++	Precuneiform area (PrCnF)	0
posteromedial part (AHiPM)	+++	Cuneiform nucleus (CnF)	0
Amygdalopiriform transition area (APir)	++	Raphe nucleus	
Indusium griseum (IG)	0	Dorsal raphe nucleus (DR)	++
The nucleus of the vertical limb of the diagonal band of Broca (VDB)	+	Median raphe nucleus (MnR)	+
The bed nucleus of stria terminalis (BNST)	+++	Paramedian raphe nucleus (PMnR)	0
Subfornical organ (SFO)	0	Paranigral nucleus of the ventral tegmental area (PN)	++
Vascular organ of lamina terminalis (VOLT)	0	Pons	
Basal nuclei		Medial paralemniscal nucleus (MPL)	+
Caudate-putamen (CPu)	0	Pedunculopontine tegmental nucleus (PTg)	++
Globus pallidus (GP)	0	Laterodorsal tegmental nucleus (LTDg)	+
Endopiriform nucleus (En)	0	Parabrachial nuclei	
Clastrum (Cl)	0	Lateral parabrachial nucleus (LPB)	++
Accumbens nucleus, shell (NAcS)	++	Medial parabrachial nucleus (MPB)	0
Ventral pallidum (VP)	+	Kölliker-Fuse nucleus (KF)	+
Substantia innominata (SI)	0	Vestibular nuclei (Ve)	0
Diencephalon		Periolivary area (PO)	0
Thalamus		Pontine reticular nucleus (PnRe)	0
Paraventricular thalamic nuclei		Pontine nuclei (Pn)	0

Table 2 (continued)

Area	Density	Area	Density
Paraventricular thalamic nucleus, anterior part (PVA)	++	Medulla oblongata	0
Paraventricular thalamic nucleus, posterior part (PVP)t	++	Cerebellum	0
Habenular nuclei (Hb)	0		
Posterior intralaminar thalamic nucleus (PIL)	+++		
Subparafascicular thalamic nucleus (SPF)	+		
Peripeduncular nucleus (PP)	+		
Suprageniculate nucleus (SG)	0		
Medial geniculate nucleus (MG)	0		
Subthalamic nucleus (STh)	0		

Brain regions where we did not observe labeled cells are not included or are marked with 0 density

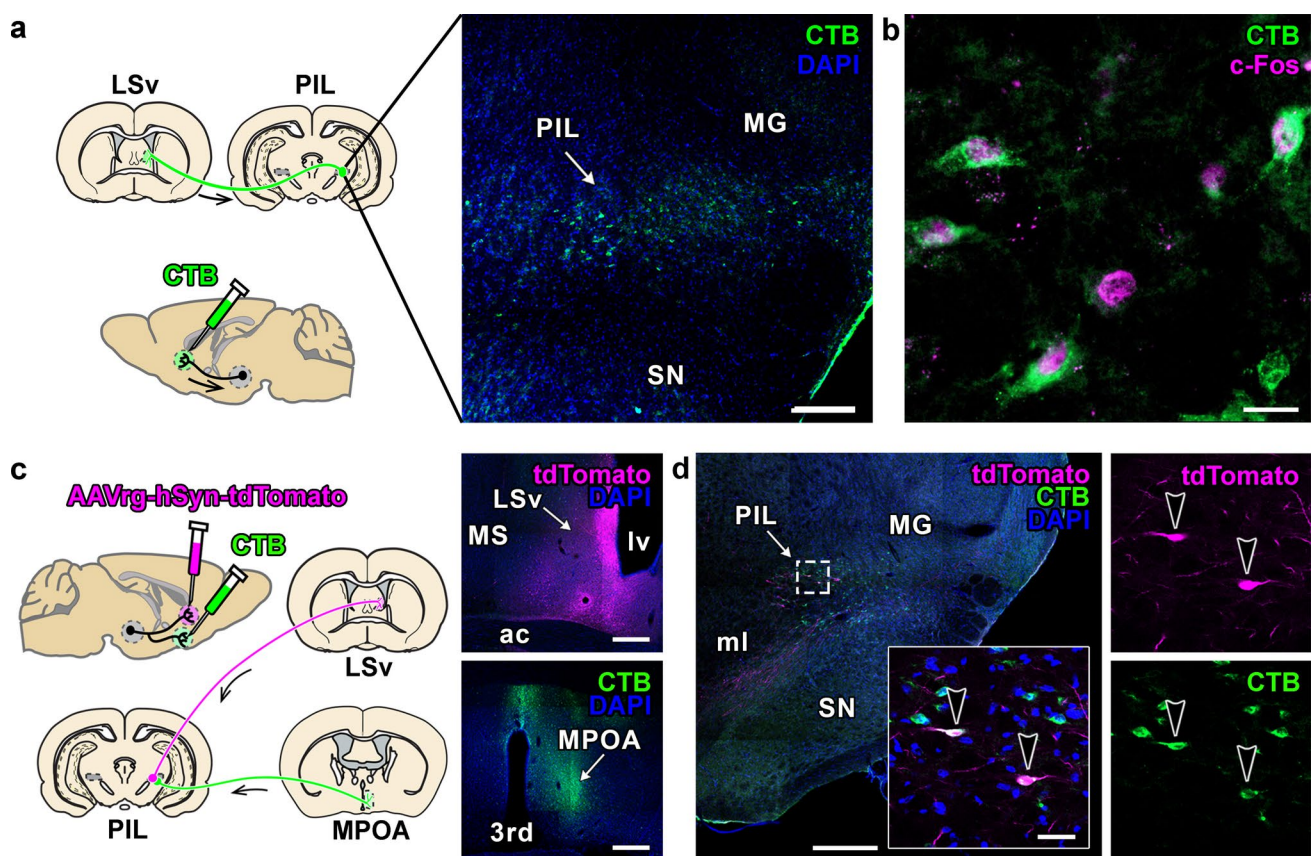


Fig. 4 Neurons of the posterior intralaminar thalamic nucleus (PIL) are activated following pup exposure and project to the ventral subdivision of the lateral septum (LSv) and the medial preoptic area (MPOA). **a** Illustration of the retrogradely transporting neuronal tracer, cholera toxin B subunit (CTB) injection into the LSv. The fluorescent image shows retrogradely labeled neurons in the PIL. Scale bar: 250 µm. **b** High magnification confocal image shows LSv-projecting PIL neurons expressing pup-induced c-Fos. Three tracer injected female rats were mated and then examined in this experiment. Scale bar: 20 µm. **c** Double retrograde tracing of the

PIL to the LSv and the MPOA was performed of two virgin female rats using an adeno-associated virus tagged with tdTomato (AAVrg-hSyn-tdTomato) introduced into the LSv and CTB into the MPOA, respectively. Confocal images demonstrate the two injection sites in the same rat. Scale bars: 500 µm. **d** The PIL contains retrogradely double-labeled neurons indicated by arrowheads in the inset panel. Scale bars: 500 µm and 50 µm for the inset. **MG** medial geniculate nucleus, **ml** medial lemniscus, **PIL** posterior intralaminar thalamic nucleus, **SN** substantia nigra

The LSv receives massive input from the parathyroid hormone 2 neurons of the PIL

As many retrogradely labeled maternally activated neurons were identified in the PIL, we further investigated whether PTH2, a neuropeptide, which is known to show a marked induction in pup-activated PIL neurons (Cservenák et al. 2010, 2013), is involved in the PIL to LSv pathway. After CTB injection into the LSv (Fig. 5a), we revealed that a portion of the retrogradely labeled CTB+ neurons in the PIL contained PTH2 (42% of the PTH2+ cells contained CTB signal and 15% of the CTB+ cells expressed PTH2) (Fig. 5b).

The projection from the PIL to the LSv was confirmed by anterograde tracing. The anterogradely transporting

biotinylated dextran amine (BDA) was injected into the PIL. We found a high number of PTH2+ /BDA+ double-labeled neurons at the injection site, confirming the precise targeting of the PIL PTH2+ cell group (Fig. 5c). Anterogradely labeled fibers were abundant in the LSv but not in adjacent brain regions suggesting a specific pathway from the PIL to the LSv (Fig. 5d, left panel). The same localization of PTH2+ and BDA-labelled fibers in the LSv suggests a PIL origin of PTH2+ fibers in the LSv. Furthermore, the PTH2+ fiber network originating in the PIL showed a similar distribution as the pup-induced c-Fos+ neurons (Fig. 5d, right panel) suggesting their contribution to the pup-induced activation in the LSv.

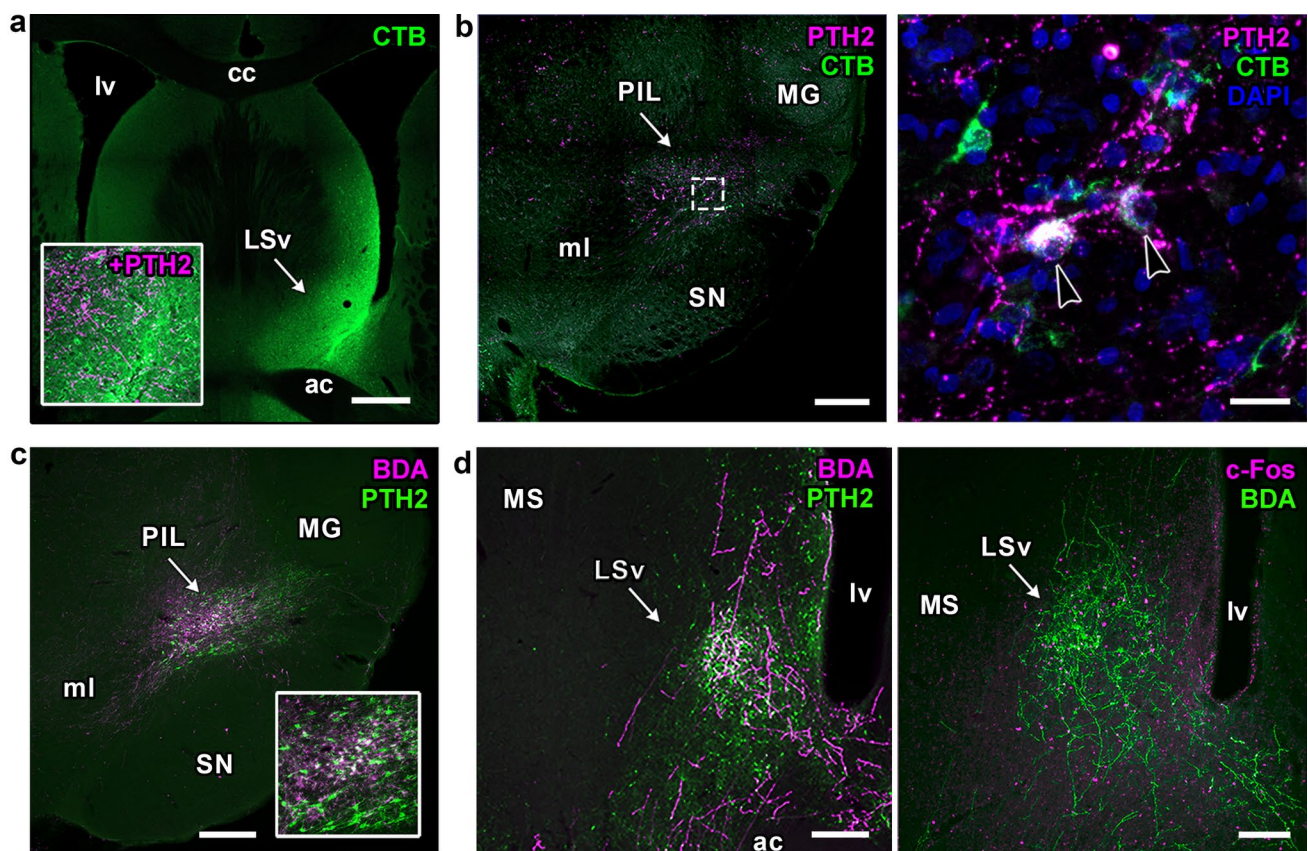


Fig. 5 PTH2-expressing neurons of the posterior intralaminar thalamic nucleus (PIL) project to the ventral subdivision of the lateral septum (LSv). **a** Retrogradely transporting neuronal tracer, cholera toxin B subunit (CTB) was injected into the LSv. A fluorescent image of the injection site with an inset shows PTH2+ fibers in the area of the same rat showed in Fig. 4a, b. Scale bar: 500 μ m. *ac* anterior commissure, *cc* corpus callosum, *LSv* ventral subdivision of the lateral septum, *lv* lateral ventricle. **b** Retrogradely labeled LS-projecting PIL neurons show PTH2 positivity. Scale bars: 500 μ m for the left and 20 μ m for the right panel. *MG* medial geniculate nucleus, *ml* medial lemniscus, *PIL* posterior intralaminar thalamic nucleus, *SN* substantia nigra. **c** Representative image of the injection of antero-

gradely transporting biotinylated dextran amine (BDA) tracer into the PIL demonstrates the injection site is where the PTH2+ cells are located in the mother rat. The inset panel shows that several BDA-containing neurons express PTH2 (white cell bodies). Scale bar: 500 μ m. **d** BDA-labelled fibers are selectively found in the LSv. These fibers are double labeled with PTH2 (left panel). BDA+ fibers are localized in the same area as c-Fos-activated neurons in the LSv (right panel). Scale bars: 500 μ m for the left and 250 μ m for the right panel. *ac* anterior commissure, *LSv* ventral subdivision of the lateral septum, *lv* lateral ventricle, *MG* medial geniculate nucleus, *ml* medial lemniscus, *MS* medial septum, *PIL* posterior intralaminar thalamic nucleus, *SN* substantia nigra

PTH2-containing terminals innervate maternally-activated LSv neurons

We further examined the contribution of the PTH2 signal to maternal adaptation in the LSv. First, we confirmed the distributional overlap between PTH2 + fibers and activated neurons by double PTH2/c-Fos immunolabelling. PTH2 + fibers were found in the close proximity of several c-Fos activated neurons implying synaptic contact between them (Fig. 6a). Therefore, we performed a correlative light and electron microscopic study applying a visualization method using the precipitation forming reaction of 3,3'-diaminobenzidine (DAB) and its NiSO_4 -based color modification (Fig. 6b). We showed that PTH2 + terminals form axosomatic and axodendritic synapses with c-Fos activated LSv neurons (Fig. 6c).

Pup-activated neurons in the ventral subdivision of the lateral septal nucleus are GABAergic

A transgenic mouse strain expressing the gene of the fluorescent reporter protein, ZsGreen driven by the promoter of vesicular GABA transporter (VGAT) (VGAT-ZsGreen mice) was used to visualize GABAergic neurons. We examined whether the neurons activated following pup exposure are GABAergic in the LSv using VGAT-ZsGreen mothers, which were exposed to pups, and then fluorescently labeled c-Fos in their brain sections. It was found that the density of pup-induced c-Fos activated neurons in the mouse LSv is comparable to that of the rat (129 ± 28 for mice and 117 ± 24 for rats based on the peroxidase-based c-Fos immunolabelling of the caudal part of the LSv). Furthermore, we also established that c-Fos + neurons are almost exclusively VGAT + (Fig. 7a). We calculated the number of VGAT +, c-Fos +, and VGAT +/c-Fos + double labeled neurons in the LSv and determined the percentage of VGAT + cell in c-Fos + population, and the percentage of c-Fos + cells in VGAT + population. Nearly all ($95.82 \pm 8.06\%$) c-Fos activated neurons were GABAergic, while $4.74 \pm 3.14\%$ of the VGAT + GABAergic neurons were c-Fos + in the LSv of mother mice (Fig. 7b).

LSv neurons send projections to widespread brain regions including the MPOA

To reveal maternally activated LSv neuronal targets, whole brain mapping following viral tract tracing was performed. Anterogradely transporting mCherry expressing AAV vector was injected into the LSv where pup-activated neurons are localized (Fig. 8a). Then, the density of mCherry-immunopositive neuronal fibers was determined. Before confocal microscopic investigation, the examined rats were perfused on the 9th day of the postpartum period following pup exposure to reveal pup-induced c-Fos activation. We focused on

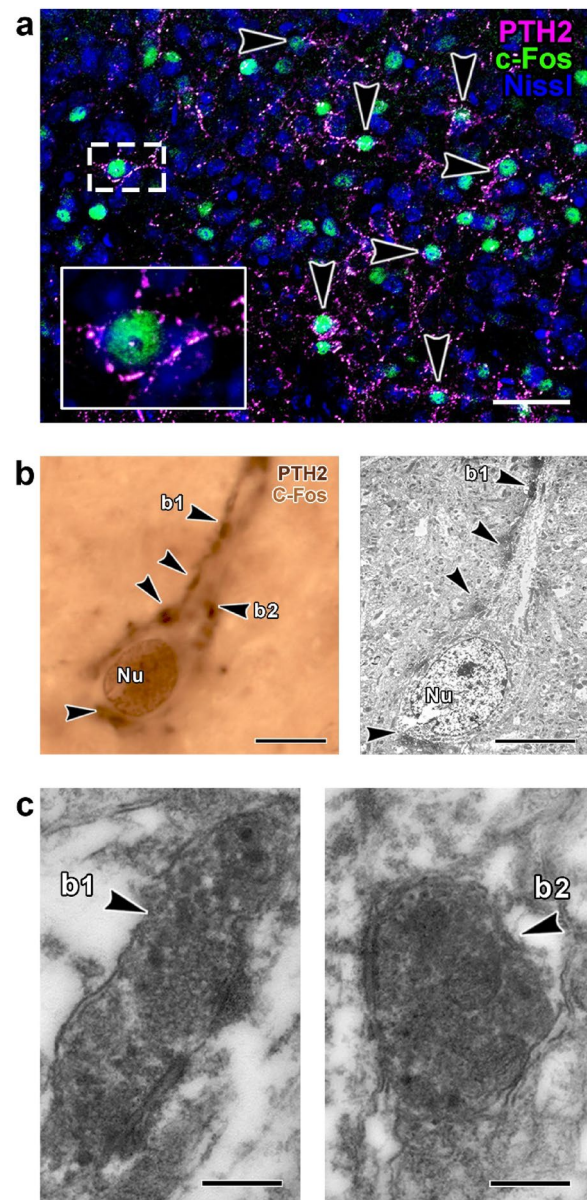


Fig. 6 PTH2-innervated pup-activated neurons in the ventral subdivision of the LSv. **a** Fluorescent double immunolabeling of PTH2 and c-Fos was performed of a rat mother also used for peroxidase-based c-Fos immunolabelling following pup exposure. PTH2 + fibers are densely distributed around maternally activated, c-Fos + neurons (black arrowheads) in the LSv as shown in the higher magnified image of a selected neuron. Scale bar: 20 μm . **b** (left panel), Peroxidase-based double immunolabeling of a brain slice of one of the two mother rats used in this examination after pup exposure demonstrates a neuron with a c-Fos + nucleus closely surrounded by several PTH2 + terminals (arrowheads). Scale bar: 5 μm . **b** (right panel), A low magnification electron micrograph (left panel) of the same neuron as in the left panel is shown. Scale bar: 5 μm . **c**, PTH2 + fibers closely appose the neuronal soma and the proximal dendritic process forming synapses as shown in the high magnification microscopic images. Two boutons are labeled as b1 and b2 in panels b and c. Scale bars: 250 nm. Nu nucleus, PTH2 parathyroid hormone 2

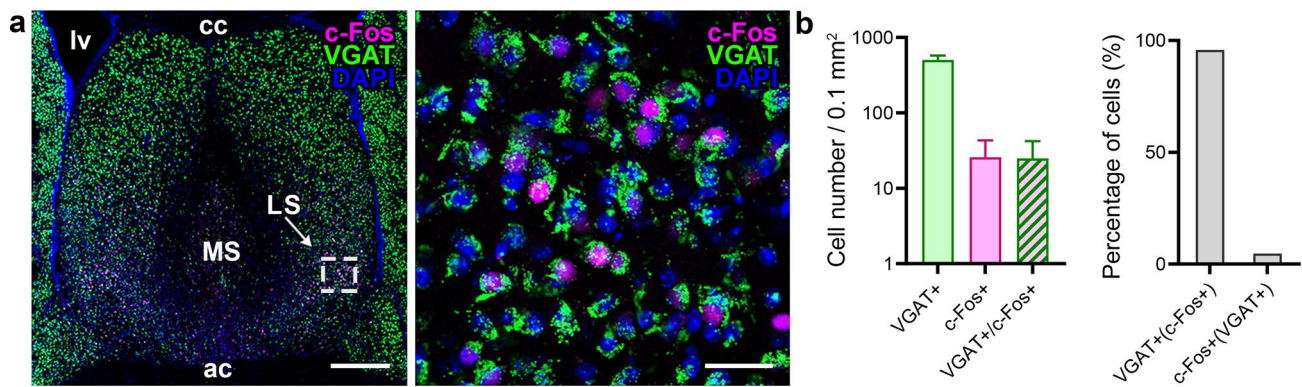


Fig. 7 PTH2-innervated GABAergic neurons in the ventral subdivision of the lateral septum (LSv) are activated in response to pups. **a** Activated neurons are GABAergic as it is shown by c-Fos immunolabeling of reporter mice that express ZsGreen fluorescent protein driven by the promoter of vesicular GABA transporter (VGAT). Scale bars: 200 μ m for the left and 20 μ m for the right panel. *ac* anterior commissure, *cc* corpus callosum, *lv* lateral ventricle, *LS* lateral

septum, *MS* medial septum. **b** The density of c-Fos+, VGAT+ and double labelled (VGAT+/c-Fos+) neurons and the quantification of the percentage of VGAT+ cells in the c-Fos+ population (VGAT+(c-Fos+)) and c-Fos+ cells in the VGAT+ population (c-Fos+(VGAT+)) in the LSv. This evaluation involved the examination of five mother mice after 22 h of pup deprivation followed by 2 h of pup exposure

ipsilateral regions to the injection site, as contralaterally located mCherry+ fibers were only sparsely distributed in some target areas. mCherry immunolabeled fibers were present in several brain regions, however, only a few of them are known to be involved in the regulation of maternal care. We found a high density of mCherry+ fibers in the MPOA overlapping the distribution of maternally activated neurons in the area (Fig. 8b). In turn, we did not find any LSv to PIL projecting fibers (Fig. 8c). Among the maternally implicated brain regions, the MPOA was found to be the major output of the LSv and also showed the highest pup-induced activation (Fig. 8d). We found mCherry+ fibers in the MePD and only a few mCherry+ fibers in the PAG where c-Fos activated neurons are not distributed. Next, we injected CTB into the MPOA (Fig. 8e) and confirmed that MPOA-projecting LSv neurons show the same distribution as maternally activated c-Fos neurons (Fig. 8f). Moreover, activated LSv neurons project to the MPOA (Fig. 8f inset panel).

Discussion

The brain region of interest in this study was defined by pup-induced c-Fos activation in the LS. This transcription factor appears in the nucleus of neurons when they undergo sustained neuronal activation. Thus, the appearance of c-Fos-containing neuronal nuclei following pup exposure indicated that pups evoked elevated activity in some neurons in the LS of their mothers. Pup exposure-activated neurons were detected within the ventral subdivision of the lateral septum (LSv). While the vast majority of the activated neurons were within the borders of the LSv, only a restricted part of the LSv contained a considerable number of activated neurons

as early studies reported on the elevated activation of the LS in response to pup exposure (Li et al. 1999; Lin et al. 1998).

c-Fos-positive neurons within the LSv were very low when pups were separated from their mothers for 22 h, indicating that this time is sufficient to eliminate c-Fos from neurons that would be present due to the pups. The almost complete absence of c-Fos in the LSv following the removal of the pups also indicated that craving for the pups or the anxiety potentially present from the loss of the pups did not activate the LSv. When the litter was returned to the mothers in a way that contact was not allowed, the mothers still were able to see, hear, and smell their pups, which was sufficient to activate several neurons in the LSv. When pups were allowed to fully access their mothers, the number of activated neurons increased further on. Although mothers perform some licking of their pups potentially leading to the activation of their gustatory system, and the olfactory activation may also be stronger than when the pups are at some distance, it is likely that suckling as the dominant somatosensory input, which is required for the full activation of the LSv. However, the impact of other nurturing behaviors such as crouching and retrieving of the pups on septal activation cannot be excluded. The distribution of the newly recruited neurons did not differ from those activated by non-somatosensory inputs suggesting that the same neuronal population is available in the LSv for processing pup-related information. It is, however, not possible to determine from our data if the neurons activated already by the sight, voice, and smell of pups are more sensitive than the rest of the LSv neurons or receive different inputs than neurons activated only by suckling and other pup-directed behaviors.

The results of the retrograde tracing suggest that many brain areas project to the part of the LSv where the

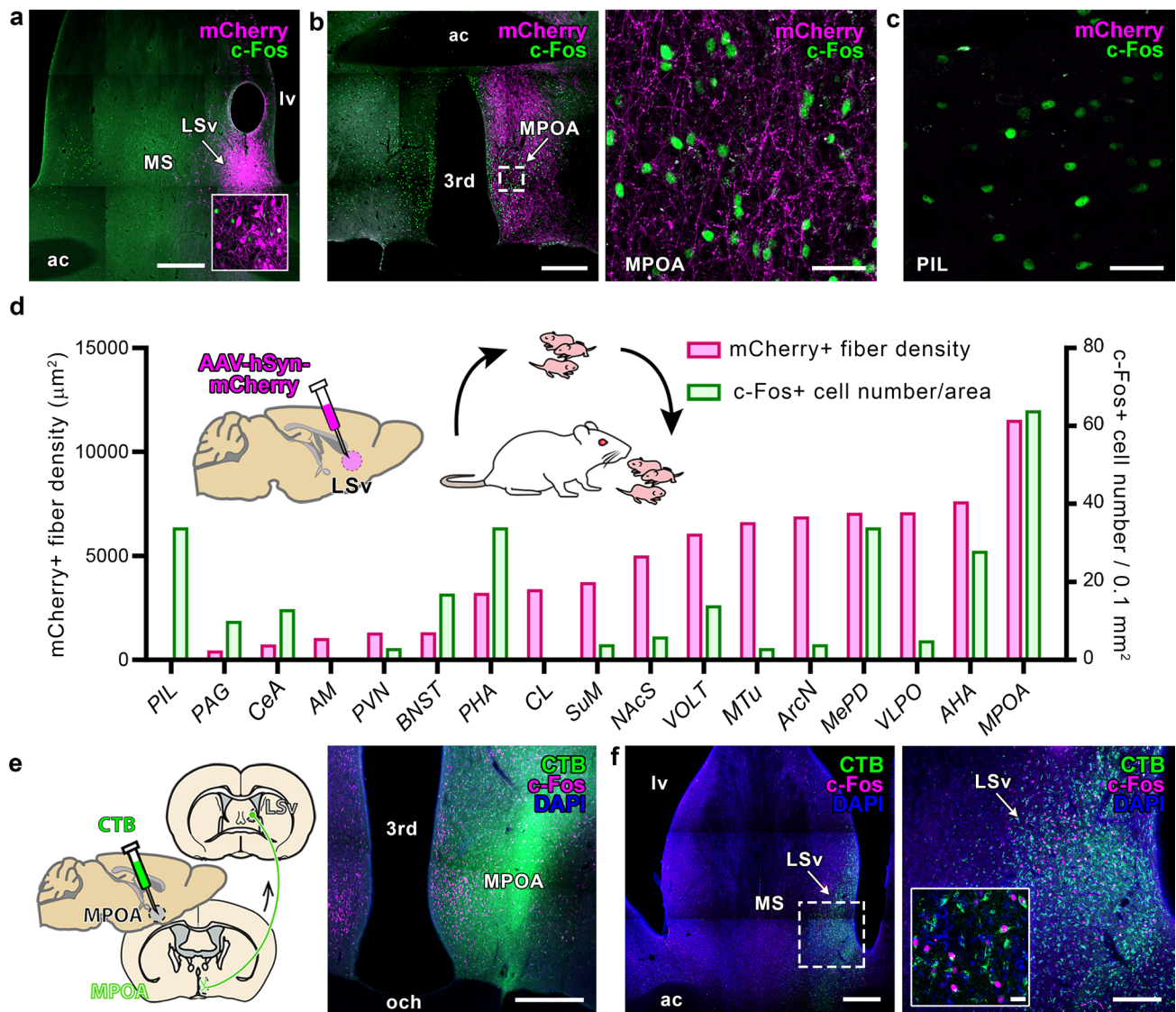


Fig. 8 Efferent connections of the ventral subdivision of the lateral septum (LSv). **a** Injection of an adeno-associated viral vector expressing anterogradely transporting mCherry (AAV-hSyn-mCherry) was injected into the LSv of a female rat. A low-magnification image of the injection site is shown after c-Fos immunolabeling the sections of a pup-exposed mother rat. The inset shows that mCherry-positive neurons are localized in the same area as the maternally activated neurons. Scale bar: 500 μm . *ac* anterior commissure, *lv* lateral ventricle, *LSv* ventral subdivision of the lateral septum, *MS* medial septum. **b** A low magnification image of the septal input in the medial preoptic area (MPOA) of the same rat. The right panel shows a higher magnified region of the same area. Scale bars: 500 μm for the left and 50 μm for the right panel. *3rd* third ventricle, *ac* anterior commissure, *MPOA* medial preoptic area. **c** LSv neurons do not project to the PIL, which is also markedly activated in mothers. Scale bar: 50 μm . *PIL* posterior intralaminar thalamic nucleus. **d** Relative fiber density and pup-induced c-Fos activated cell number were measured based on the AAV-hSyn-mCherry LSv injection of the same rat demonstrated in panels a–c. Among the projection sites of the LSv, the highest density of maternally-activated neurons and mCherry-positive fibers were found in the MPOA. Two female rats were mated after viral injections in this experiment. Further analysis was not achieved

because of the low number of infected cells in one of the two rats. *AHA* anterior hypothalamic area, anterior part, *AM* anteromedial thalamic nucleus, *ArcN* arcuate nucleus, *BNST* bed nucleus of the stria terminalis, *CeA* central amygdaloid nucleus, *CL* centrolateral thalamic nucleus, *MePD* medial amygdaloid nucleus, *MPOA* medial preoptic area, *MTu* medial tubular nucleus, *NacS* accumbens nucleus, shell, *PAG* periaqueductal grey, *PHA* posterior hypothalamic area, *PIL* posterior intralaminar thalamic nucleus, *PVN* paraventricular hypothalamic nucleus, *SuM* supramammillary nucleus, *VLPO* ventrolateral preoptic area, *VOLT* vascular organ of the lamina terminalis. **e**, The experimental design of the retrograde tract-tracing of the MPOA by using the retrogradely spreading cholera toxin B subunit (CTB). The confocal image demonstrates the injection site in the MPOA of a mother rat after CTB immunolabeling. Scale bar: 500 μm . *3rd* third ventricle, *CTB* cholera toxin beta subunit, *LSv* ventral subdivision of the lateral septum, *MPOA* medial preoptic area, *och* optic chiasm. **f**, A high number of neurons were retrogradely labeled and activated in the LSv after CTB injection of a mother rat used in this experiment. Scale bars: 500 μm for the left, 200 μm for the right panel, and 20 μm for the inset. *ac* anterior commissure, *MS* medial septum, *LSv* ventral subdivision of the lateral septum, *lv* lateral ventricle

pup-activated neurons are located. These projections are specific as judged by misplaced injections. Indeed, when the injection site was medial to the LSv, the pattern of retrogradely labeled structures differed substantially. The resolution of the method limited by the relatively large injection sites does not allow precise discrimination between different parts of the LSv. Furthermore, the retrograde tracer injected into the area of the LSv is taken up by any neurons projecting to the area. Thus, we do not know if the actual input innervates the pup-activated neurons or other neurons in the area. Still, many of the afferent connections of the LSv are well-established members of the maternal brain circuitry, such as the medial preoptic area, the bed nucleus of stria terminalis, the medial amygdala, periaqueductal grey, and the PIL. A number of these brain regions also contain pup-activated neurons and are candidates to evoke the pup-induced activation of LSv neurons. Based on the literature on the potential sensory function of these brain regions, we further investigated the PIL as a brain area specifically innervating the pup-activated LSv neurons. An anterograde tracer injected into the PIL indeed revealed projections to the part of the LSv, which contained pup-activated neurons. To further address this issue, we utilized that parathyroid hormone 2 (PTH2) neuropeptide is induced in many neurons in the PIL of mothers. Since the retrograde tracer injected into the LSv labeled PTH2-containing neurons in the PIL, we demonstrated that this subtype of PIL neurons projects to the LSv. PTH2 terminals were indeed present in the close proximity of pup-activated LSv neurons albeit a contact cannot be established with light microscopy using c-Fos and PTH2 immunolabeling due to the nuclear localization of c-Fos. Therefore, correlative light and electron microscopy using double immunolabelled material were applied, which demonstrated that PTH2-terminals form synapses on the cell bodies and proximal dendrites of pup-activated neurons. PTH2 neurons were previously shown to be glutamatergic based on their VGLUT2 content (Cservenák et al. 2017b, a) and have an excitatory effect on MPOA neurons (Keller et al. 2022). The asymmetrical synapses formed by PTH2 terminals on c-Fos-positive neurons also suggest that these neurons of PIL origin can excite LSv neurons potentially leading to their activation by pup exposure.

PTH2 is a neuropeptide which is the exclusive endogenous ligand of the parathyroid hormone 2 receptor and is induced in the early postpartum period with a peak expression at the 9th day after parturition (Dobolyi et al. 2018). PTH2 receptor antagonist administration reduced maternal care elements such as the retrieval of the pups or the preference towards the pups (Cservenák et al. 2013). Based on these findings, PTH2-expressing neurons of the PIL may provide one of the key peptidergic pathways conveying somatosensory signals to higher-order brain regions during early motherhood to maintain maternal motivation. We

have already described the PTH2 + dense fiber network both in the MPOA and LSv, the two brain areas show the most prominent PTH2 + fiber density among all the maternally implicated brain regions, however, PTH2-expressing PIL projection was previously described only in the hypothalamus (Cservenák et al. 2013; Cservenák et al. 2017b, a). Here, we determined that lateral septal PTH2 + innervation originated from PIL neurons, of which nearly half were activated in response to pups.

PTH2 neurons of the PIL may also excite other brain areas in mothers. It has been reported that they also send massive projections to the infralimbic cortex, the MPOA, the paraventricular and dorsomedial hypothalamic nuclei, and the periaqueductal gray (Keller et al. 2022). Among these, we addressed whether the same PIL neurons projecting to the LSv also project to the MPOA, the major maternal center of the rodent brain. Different retrograde tracers were injected into the LSv and the MPOA, which resulted in double-labeled neurons in the PIL providing definitive evidence that some PIL neurons project to both brain regions via collaterals. Since only a portion of projection neurons are labeled in the PIL with this technique, it cannot be established what percentage of PIL neurons project to both of these brain regions let alone other maternal centers. However, the possibility that PIL neurons have multiple projection sites within the maternal circuitry of the brain is certainly plausible by our double retrograde experiment raising the possibility of the synchronized action of PTH2 signal in these brain areas.

The neurochemical characterization of pup-activated LSv neurons was conducted in mice because we could use a reporter line in this species, where neurons expressing the vesicular GABA transporter endogenously expressed the fluorescent protein ZsGreen. This promoter is active only in GABAergic neurons, and can thus be used to visualize GABAergic cells (Vong et al. 2011). The position of the pup-activated neurons within the LSv was similar in mice as in the rat. Furthermore, the number of maternally-activated neurons was also comparable between the 2 species. These experiments indicated that the vast majority of pup-activated neurons were GABAergic. We can reasonably assume that the pup-activated neurons are also GABAergic in the rat. In this study, no further neurochemical characterization of the pup-activated neurons was performed. We have previously shown that there is a significant overlap between maternally-induced c-Fos and pSTAT5 content. However, at the cellular level, the co-expression is only partial (Oláh et al. 2018). Since pSTAT5 is an accepted indicator of prolactin-sensitive neurons (Brown et al. 2010; Grattan et al. 2008), we can assume that prolactin, known to be involved in the formation of maternal motivation (Brown et al. 2017), acts on some but not all neurons, which have elevated neuronal activity in response to sucking. Furthermore, the hormone acts on other cells of the LSv, too. Since pSTAT5 and estrogen receptor

alpha co-localized in the LSv (Oláh et al. 2018), the steroid hormone pivotal in inducing maternal behavior at the end of pregnancy (Sheehan and Numan 2002; Tsukamura and Maeda 2001) also acts on a partially separate cell population than what is activated by pup-exposure.

The activated neurons in the LSv can exert their influence on the maternal brain via their projections. That is why we examined the efferent neuronal connection of the LSv. The targets include brain regions, which contain a large number of pup-activated neurons, such as the MPOA, the medial amygdaloid nucleus, and the anterior hypothalamic area. Among them the MPOA projections were verified by retrograde tracing, too. These brain areas could potentially be activated by LSv neurons, however, they all receive major inputs from the PIL and other sensory relay nuclei (Dobolyi et al. 2018; Keller et al. 2022). Several other neuronal targets of the LSv, however, contain only a small number of pup-activated neurons, including the ventrolateral preoptic nucleus, the arcuate nucleus, mamillary and supramamillary nuclei, the accumbens nucleus, and the claustrum suggesting that LSv neurons, despite projecting there and being activated by pups, they do not necessarily stimulate them. Indeed, it aligns logically, especially if the projection neurons of the LSv are indeed the GABAergic neurons that are activated by exposure to pups. While our data do not provide information in that regard, the projection neurons of the lateral septum have been described to be predominantly GABAergic (Zhao et al. 2013). Consequently, LSv neurons presumably participate in maternal brain adaptation by exerting an inhibitory effect on their efferent targets depending on the pup-related somatosensory stimuli.

Potential functional implications

The present study provided anatomical insight into the brain circuitry involved in maternal adaptation by integrating sensory inputs from the pups during nurturing including somatosensory inputs. The LSv is a markedly activated brain area following suckling and nurturing, ranking second to the MPOA among the most activated brain areas in mother rats. The high activation level of the MPOA has been widely investigated in mice (Tsuneoka 2019; Tsuneoka et al. 2013) and rats (Fleming et al. 1994; Fleming and Walsh 1994; Lin et al. 1998; Lonstein et al. 1997; Lonstein and De Vries 2000) consistent with its pivotal role in maternal care (Dimén et al. 2021; Wu et al. 2014; Zhang et al. 2021). However, the importance of the LSv and its role in social behaviors such as maternal care came into focus only in the last few years (Besnard and Leroy 2022; Menon et al. 2022; Rizzi-Wise and Wang 2021). As opposed to the MPOA, the maternal function of the LSv is not fully established yet and our results will therefore be eminent in providing in-depth knowledge of the anatomical, connectional, and

neurochemical characterization of the LSv in dams. In accordance with the literature (Geissler et al. 2013; Lonstein et al. 1997), we revealed that non-contact inputs from the pups and suckling/nurturing associated tactile stimuli from the pups contribute to LSv maternal activation changes. We also described that the pup-activated LSv neurons are GABAergic (Zhao et al. 2013) and presumably exert an inhibitory effect on their efferent targets. A major example in that regard is the MPOA where LSv fibers were distributed in the same region as pup-induced activated neurons localized in the MPOA. The high overlap between LSv fibers and c-Fos-positive MPOA neurons reflects the lateral septal contribution to MPOA-driven maternal phenotype. It has been established that MPOA glutamatergic neurons whose stimulation resulted in reduced maternal response have been shown to receive the most significant monosynaptic input from LSv neurons (Zhang et al. 2021). Based on these findings and our results, GABAergic septal neurons may contribute to maternal care by the inhibition of glutamatergic MPOA neurons. On the other hand, LSv neurons may provide inhibitory input to pup-associated GABAergic MPOA neurons (Dimén et al. 2021) exerting a modulatory effect. In addition, LSv-projecting MPOA neurons have been described to be activated in response to pups (Numan and Numan 1997). Furthermore, we previously reported that GABAergic neurons in the MPOA, which promote maternal behaviors, send prominent projections to the LSv (Dimén et al. 2021). Based on these findings, a functional interaction is likely between the MPOA and LSv in maternal adaptation (Wang and Swann 2006).

Maternal deficits in behavior resulting from lesions in the LS may stem from the elimination of pup-activated LSv neurons. The role of LSv in eliminating pup avoidance necessary for maternal behaviors is suggested by oxytocin signaling in the LS preventing social fear during lactation (Menon et al. 2018). Additionally, prior activation data propose a specific control of maternal aggression by the LSv (Gammie and Nelson 2001), with recent findings indicating sex-dependent regulation of aggression in the LS (Borland et al. 2020), hinting at a potentially similar mechanism for maternal aggression. Maternal aggression is negatively regulated by LS neurons containing CRH receptor 2 (D'Anna and Gammie 2009), indicating the involvement of a broader LS network in maternal aggression control.

Given the established known role of LSv in suppressing anxiety-like behaviors (Parfitt et al. 2017; Tong et al. 2022), pup-activated LS neurons may contribute to the anxiolytic effects of pups (Hillerer et al. 2011; Silva et al. 1997; Slattey and Neumann 2008). Furthermore, experimental activation of dorsal LS neurons inhibits feeding behavior (Azevedo et al. 2019). Given the heightened feeding during lactation, inhibiting dorsally located LS neurons may lead to disinhibition. Emerging evidence on intraseptal inhibitory actions within LS

parts (Besnard and Leroy 2022) supports the hypothesis that GABAergic LSv neurons, activated by pups in mothers, may project to more dorsally located LS subdivisions, contributing to elevated feeding in mothers. In fact, inhibition of LS GABA receptors induces rapid food intake (Gabriella et al. 2022), providing further support for this hypothesis.

In conclusion, the data indicate that the LSv is part of an interconnected subcircuit in the maternal brain network, which may be driven by somatosensory input from the pups via the PTH2-expressing neurons of the PIL. Thus, the new activation and connectional data provide useful information for future studies on the brain mechanisms controlling the maternal phenotype.

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Author contributions Gina Puska participated in the design of the experiments, performing surgical procedures and light and electron microscopic histological work and analyses, interpretation of the results and the literature, and writing of the manuscript. Vivien Szendi did neuronal tracing experiments and participated in the analysis of histological images, and writing of the manuscript. Máté Egyed and Diána Dimén participated in brain sectioning and immunolabeling. Melinda Cservenák performed tracer experiments. Árpád Dobolyi participated in the design of the experiments, data analysis, interpretation of the results and the literature, and writing of the manuscript.

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Data availability Lead contact: further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Arpad Dobolyi (dobolyi.arpad@ttk.elte.hu). Data and code availability: all data used in this manuscript are available upon request from the lead author.

Declarations

Conflict of interest There are no conflict of interest for any author.

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