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The role of the lateral septum in maternal adaptation

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1. ABSTRACT

Parental care is a special form of social behaviour, which increases the survival of the offspring by the behaviour of the parents. Our research group previously described that neurons in the posterior intralaminar thalamic nucleus (PIL) project to the medial preoptic area (MPOA), a major centre to control maternal behaviours, and that only in mothers, the PIL expresses an excitatory neuropeptide, parathyroid hormone 2 (PTH2). First we demonstrated that PIL neurons project to the lateral septum (LS), a brain area whose lesion disrupts maternal behaviour. Therefore, I further examined the involvement of LS in the maternal regulatory brain circuitry in rodent animal models. I found that inhibitory neurons in the LS are innervated by axon terminals containing PTH2, and they are activated by pup-exposure. A significant amount of the activated cells shows calbindin immunopositivity and are densely surrounded by PTH2+ fibres. Furthermore, injecting anterograde tracer into the LS revealed that inhibitory neurons project to the MPOA. I confirmed this finding by targeting retrograde tracer into the MPOA and observed that many neurons projecting to the MPOA arise from the calbindin-positive neurons of the LS. Both LS and MPOA receive prominent input from the PTH2-expressing neurons of the PIL. Thus, I applied double retrograde tracing and revealed that afferents reaching the LS and MPOA from the PIL emerge from the same neuronal group. Based on my results, I conclude the inhibitory neurons of LS take part in the control of maternal behaviour through their connections with MPOA and PIL.

2. LIST OF ABBREVIATIONS

3v	third ventricle
ac	anterior commissure
AAV	adeno-associated virus
ABC	avidin biotin complex
ant. LS	anterior lateral septum
BDA	biotinylated dextran amine
Cb	calbindin
cc	corpus callosum
CLEM	correlative light and electron microscopy
Cr	calretinin
CTB	cholera toxin B
DAB	3,3'-diaminobenzidine
f	fornix
FITC	fluorescein isothiocyanate
fs	fimbria septi
GABA	gamma-aminobutyric acid
LS	lateral septum
LSd	dorsal lateral septum
LSi	intermediate lateral septum
LSv	ventral lateral septum
MPOA	medial preoptic area
MG	medial geniculate body
Nu	nucleus
och	optic chiasm
PB	phosphate buffer
PFA	paraformaldehyde
PIL	posterior intralaminar thalamic nucleus
post. LS	posterior lateral septum
PPD	postpartum depression
PTH2	parathyroid hormone 2
AAV-rg-mCherry	retrogradely transported adeno-associated virus containing mCherry reporter gene
SHy	septohipothalamic nucleus
SN	substantia nigra
v	lateral ventricle
VGAT	vesicular gamma-aminobutyric acid transporter

3. INTRODUCTION

Parental care is a special form of social behaviour defined as a set of behaviours that increases the survival chance of offspring. Getting to know neural network behind maternal care can help us to understand and treat disorders regarding maternal behaviour, such as postpartum depression (PPD) [1]. PPD affects around 10-15% of mothers in developed countries and even more in developing ones. It disturbs the connection between the new mother and her infants even causing neglect or death of new-borns. Even though there are similarities between PPD and general depression, important distinctions should be made. However, efficient specialised psycho-and pharmacotherapies and prevention methods are underdeveloped as our knowledge regarding the physiological background of it is limited [2]. Gaining information about neural networks, cell groups and affected brain areas can help us to identify new specialised pharmaceutical and therapeutical targets.

The neural network behind maternal behaviour is very complex and made of several different brain areas. One of the most important area is the medial preoptic area (MPOA), which is necessary to develop, maintain and execute proper maternal behaviour elements [3] through its GABAergic neuronal population, as these neurons decrease anxiety-like behaviour and promote maternal caring as our laboratory recently identified [4]. MPOA has a broad projection network with various brain areas involved in maternal adaptation, including the posterior intralaminar thalamic nucleus (PIL). PIL receives direct sensory information about suckling stimulus needed for proper maternal care and sends it further to the MPOA via fibres containing a neuropeptide induced specifically in mothers [5], the parathyroid hormone 2 (PTH2) also called tuberoinfundibular peptide 39 [6].

PTH2 is a member of a small peptide family, and it is the only high affinity endogen ligand of parathyroid hormone 2 receptor. Following birth, only the neurons localized in the medial paralemniscal nucleus, the thalamic periventricular grey and the PIL express PTH2 [7]. Our research group described, the expression level of PTH2 increases for two weeks during postnatal development then decreases dramatically in all brain areas [8]. Nevertheless, an increasing expression was determined in the medial paralemniscal nucleus and PIL in female rodents during postpartum period with a peak at the 9th day (**Fig. 1 A-B, D-E**) [9]. It was also observed that PTH2⁺ neurons of PIL show prominent suckling-induced activation in mother rodents assuming PIL and PTH2 have a role in maternal adaptation (**Fig. 1 C**) [5]. A dense PTH2⁺ fibre localization can be found in various parts of the brain, such as in the bed nucleus of stria terminalis, the hypothalamic paraventricular nucleus, the MPOA and in the lateral

septum (LS), especially its ventral part, which shows one of the most prominent PTH2+ fibre density (**Fig. 1 F**) [7].

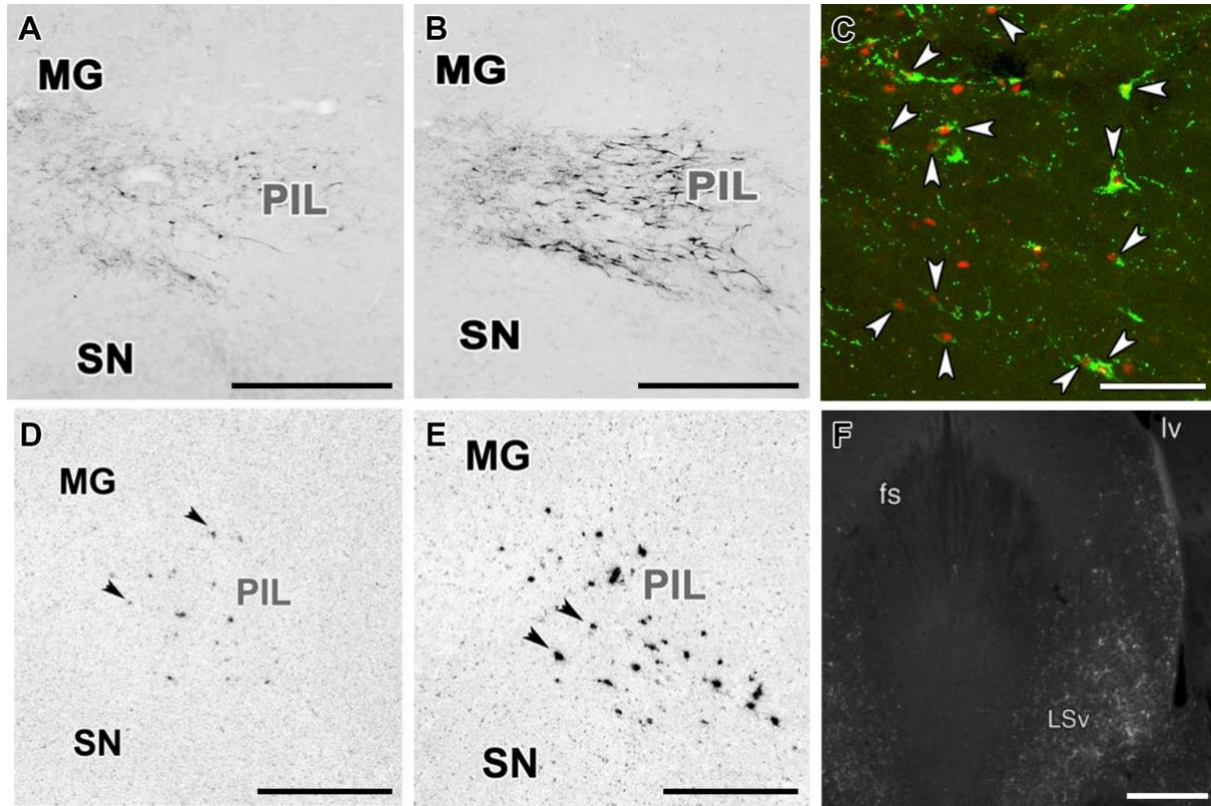


Fig. 1: Demonstration of PTH2+ neurons in the PIL of control females and lactating mothers and the dens PTH2+ fibre localization in the ventral lateral septum. A-B: Localization pattern of the PTH2-expressing neurons in PIL of control female (A) and lactating mother (B). **C:** PTH2-expressing neurons show c-Fos activation in the PIL in mothers (white arrowheads) [5]. **D-E:** Expression of PTH2 mRNA in the PIL of control female (C) and lactating mother (D) [9]. **F:** The image demonstrates PTH2+ fibres enmeshing the LSv [7]. Scales: A-B, D-E: 500 μ m; C: 1 mm; F: 250 μ m. Abbreviations: fs: fimbria septi; lv: lateral ventricle, LSv: ventral lateral septum MG medial geniculate body, PIL: posterior intralaminar thalamic nucleus, SN: substantia nigra.

LS is a subcortical forebrain structure described as a participant in regulating social behaviour including maternal care. LS is a heterogeneous brain area with distinct neuron populations and projections [10], although the majority of neurons are GABAergic [11]. Classically, LS can be divided into three regions, the dorsal (LSd), intermediate (LSi) and ventral lateral septum (LSv) [12]. LS has reciprocal connections with brain areas involved in regulating parental care, including the MPOA. Its role is to mediate proper maternal behaviour and pup avoidance [13]. Previous studies have found that LS neurons show higher activation following pup-exposure in mother rats [14,15], which was also confirmed by our laboratory in mother mice [16].

In summary, previous research confirmed the role of LS in maternal adaptation, although mechanisms behind the process are still poorly understood. Therefore, the aim of my research is to gain more information about the involvement of LS in maternal adaptation.

4. OBJECTIVES

1. To examine pup-induced c-Fos activation pattern in mother mice throughout the LS
 - a. Investigation of neurons containing the calcium-binding protein, calbindin (Cb) regarding pup-induced c-Fos activation
 - b. Studying the relevance of PTH2+ fibres located in the LS in relation to pup exposure activated neurons of the LS
2. To investigate the inhibitory system of LS by using VGAT-Cre-ZsGreen mice
 - a. Examination of inhibitory GABAergic neurons regarding c-Fos activation after exposure to pups
 - b. Verification of synaptic connection between PTH2+ terminals and lateral septal inhibitory neurons
 - c. Demonstration of the efferent connection of GABAergic neurons of the LS to maternally involved brain areas
3. To establish the connections of the LS with the major maternal centre medial preoptic area (MPOA)
 - a. Exploration if LS neurons project to the MPOA
 - b. To reveal if the same PIL neurons project to the LS and the MPOA

5. MATERIALS AND METHODS

5.1. Animals

4 wild type mice and 4 transgenic VGAT-IRES-Cre/Gt(ROSA)26Sor_CAG/ZsGreen1 (VGAT-Cre-ZsGreen) mice were used to reveal pup-induced activated distinct neuronal groups in mothers by immunohistochemistry. One VGAT-Cre-ZsGreen mouse was used for correlative light and electron microscopy, as well. Furthermore, 1 transgenic VGAT-IRES-Cre mouse was used for cell-specific neuronal tracing. In VGAT-IRES-Cre transgenic mouse, the gene of Cre recombinase enzyme is expressed in neurons containing vesicular GABA transporter (VGAT), while in VGAT-Cre-ZsGreen mice, ZsGreen fluorescent reporter gene is also expressed in VGAT⁺ neurons. All animals were sacrificed at the age of 10-14 weeks. Rats (5 Wistar females, 10-12 weeks old) were used for common neural tracing studies for accurate targeting to restrict diffusion of tracer to non-relevant brain areas. Moreover, one rat brain was used to confirm some immunohistochemical results of mice samples. Animals were maintained on a 12h light/dark cycle and food and water were available *ad libitum*.

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 were performed in accordance with the European Union directive 2010/63/EU regarding the welfare of experimental animals in the presence of person having permission for experiments on laboratory animals.

5.2. Animal surgeries and tracing

Animals were anesthetized with ketamine/xylazine-hydrochloride (mice: 16.6 mg/kg and 0.6 mg/kg respectively, in 0.9% saline, i.p., rats: 75 mg/kg and 10 mg/kg respectively, i.p.) mixture and placed in a stereotaxic frame. The following neural tracers and viruses were used for the experiments: 20 nl anterogradely transported Cre-dependent adeno-associated virus construct containing mCherry reporter gene (AAV-hSyn-DIO-mCherry) was injected to the LS of VGAT-IRES-Cre mouse at a flow rate of 50 nl/sec using nanoinjector (Nanoinject2010 by WPI). 50 nl retrogradely transported adeno-associated virus containing mCherry reporter gene (AAV-rg-mCherry) to the LS or 50 nl anterogradely transported tracer, biotinylated dextran amine (BDA) to the PIL or 50 nl cholera toxin B (CTB) to the MPOA of Wistar rats was injected at the same flow rate. After the injection was completed, the glass capillary was held still for 5

min to allow the virus to diffuse, then was withdrawn slowly. Animals were allowed to recover for 2 weeks, then sacrificed.

5.3. Pup-induced c-Fos studies

To determine maternally activated LS neurons in wild-type mother mice, we performed pup exposure-based c-Fos activation test. Mother animals were deprived from their pups for 22 hours and received them back for 2 hours on the following day to indicate proper c-Fos expression, then they were sacrificed. The control group did not interact with their pups. To reduce the number of sacrificed animals, we omitted the control group in the VGAT-Cre-ZsGreen mother mice study, as the previous results from wild-type mice sufficiently showed the difference in c-Fos activation between the two groups.

To examine the location of maternally activated neurons and fibres arising from the PIL in the LS, we performed the c-Fos activation test on the rat injected with BDA, as well. The mother rat was also deprived from their pups for 22 hours, and reunited with them for 2 hours on the following day, then was sacrificed.

5.4. Brain preparation

Animals were deeply anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) solution in phosphate buffer (PB) or 4% PFA, 0.05% glutaraldehyde and 15% cc. picric acid in PB for electron microscopy. Brains were post-fixed overnight in the same solution, then either sectioned 50 μ m using vibratome (VT1000S, Leica) or placed into 20% sucrose in PB solution until sectioning with cryostat (CM1520, Leica). After the preparation, different proper immunolabelling were applied.

5.5. Immunohistochemistry

For confocal microscopy, brain slices were permeabilised and non-specific binding sites were blocked with PB solution containing 10% fetal bovine serum, 1% bovine serum albumin and 0.5 % TRITON X-100, then incubated in appropriate primer antibody solutions for overnight or in the case of PTH2 antibody for 48 hours at room temperature. The following antibodies were used for the study: rabbit anti-c-Fos antibody (1:2000, Abcam, ab190289), rabbit (1:1000, Swant, CB38) or guinea pig (1:2000, SYSY, 214004) anti-calbindin, mouse anti-calretinin

antibody (1:1000, Swant, 6B3), anti-PTH2 antibody (1:2000, Research resource identifier, AB_1235466). For tracer visualization, cholera toxin B (1:10000, goat, List Biological Laboratories, #703) and CPCA-mCherry (1:3000, chicken, EnCor Biotechnology) antibodies were used. After washing steps, appropriate fluorescent secondary antibodies were applied (Jackson Laboratories). In case of peroxidase-based immunohistochemistry, slices were incubated in biotin-conjugated secondary antibody followed by avidin-biotin-peroxidase complex (ABC) (1:500, Vector Laboratories) solution. Developing were achieved either with incubation in 3,3'-diaminobenzidine (DAB) with nickel-sulphate or in fluorescein isothiocyanate (FITC) or Alexa 568 (A568) -conjugated tyramide solution. Brain slices were washed and mounted onto glass slides coverslipped using DePeX or PolyMount mounting medium.

5.6. Correlative light and electron microscopy (CLEM)

We performed correlative light and electron microscopy to confirm the synaptic connection between PTH2⁺ terminals and GABAergic neurons by using VGAT-Cre-ZsGreen mouse brain. As no anti-ZsGreen antibody is available for immunohistochemistry to label VGAT-expressing GABAergic cell bodies, we needed to identify the GABAergic inhibitory neurons closely surrounded by PTH2⁺ fibres in the LS by detecting the fluorescent ZsGreen signal and PTH2 immunolabelling.

After the proper brain preparation, 50 µm thin brain slices were washed, then cryoprotected for overnight first in 15% sucrose, then in 30% sucrose PB solution, next freeze-thawed over liquid nitrogen for epitope retrieval. 1% sodium-borohydride, 50 mM ammonium-chloride and 50 mM glycine solution was applied followed by blocking and incubating in PTH2 antibody for 48 hours. After washing with PB with saline, biotin-conjugated secondary antibody (1:500, Jackson Immunoresearch), then ABC solution (1:500, Vector Laboratories) was used. Developing happened using A568-conjugated tyramide solution, and then neurons showing ZsGreen expression and closely surrounded by PTH2⁺ signal was identified by fluorescent microscope. After image acquiring, peroxidase-based DAB reaction was performed to develop PTH2-related signal appropriate for electron microscopy. Brain slices were post-fixed in 1% osmium-tetroxide water solution. Following dehydration steps, sections were embedded in DurcupanTM ACM (Sigma-Aldrich) resin. We identified previously selected GABAergic neurons by light microscope. After reembedding the area of interest, it was sectioned at 70 nm

with a Reichert-Jung ultramicrotome. Sections were collected on formvar film-coated copper slot grids and contrasted with lead citrate.

5.7. Microscopy

Light microscopy images were taken using Nikon Eclipse light microscope equipped with SPOT RT3 camera. Confocal images were taken with Zeiss LSM 800 laser scanning confocal microscope using 10-40x objectives. Images were adjusted using ZEN Software.

For CLEM, Carl Zeiss AxioImager Z1 fluorescent microscope equipped with ApoTome1 unit was used first, and ultrastructural examination were prepared using Jeol Jem 1011 transmission electron microscope operating at 60 kV connected with a Morada camera.

5.8. Image analysis and statistics

Analysis of images for c-Fos examination were performed using QuPath, a free bioimage analysis software [17] with object detection algorithm, StarDist [18] and Object Classifier using own scripts or with manual cell counting tool. Data were imported to SPSS Statistics 20 (IBM). Based on Shapiro-Wilk test, non-normally distributed data was analysed by Kruskal-Wallis test and with post hoc comparison.

6. RESULTS

6.1. Neurons activated following pup-exposure in the lateral septum of mother mice contain calbindin

I used mother mice deprived from their pups for 22 hours, which were then returned to her on the following day ('pup-back' mothers) for two hours to indicate c-Fos expression. The control group did not interact with the pups on the following day ('pup-separated' mothers) (**Fig. 2 A, F**).

I observed a prominent group of activated neurons in the region of Cb⁺ cells located in the LS in 'pup-back' mothers. The c-Fos⁺ neurons are localized throughout the intermediate and ventral part of the LS in anterior sections (rostral part, bregma +1.09 to +0.74), and in the LSv in posterior sections (caudal part, bregma +0.61 to +0.14) (**Fig. 2 B-E**).

I counted the number of Cb⁺, c-Fos⁺ and double immunolabelled (c-Fos⁺/Cb⁺) neurons in the previously described areas in both groups. I revealed significantly higher amount of c-Fos⁺ neurons in the posterior part of LS ($p < 0.001$) and c-Fos⁺/Cb⁺ neurons in both examined areas ($p = 0.011$ ant., post.) in mothers in response to pups compared to 'pup-separated' mothers (**Fig. 2 K**). The percentage of Cb⁺ cells in the c-Fos⁺ population were significantly higher in 'pup-back' mothers than in controls in both areas ($p = 0.011$, ant., post.) (**Fig. 2 L**).

Because of the prominent Cb⁺ neuron population localized in the LS, I examined and showed that PTH2⁺ fibres surround septal Cb⁺ neurons suggesting a promising Cb⁺ neuron population target for further research (**Fig. 2 M**).

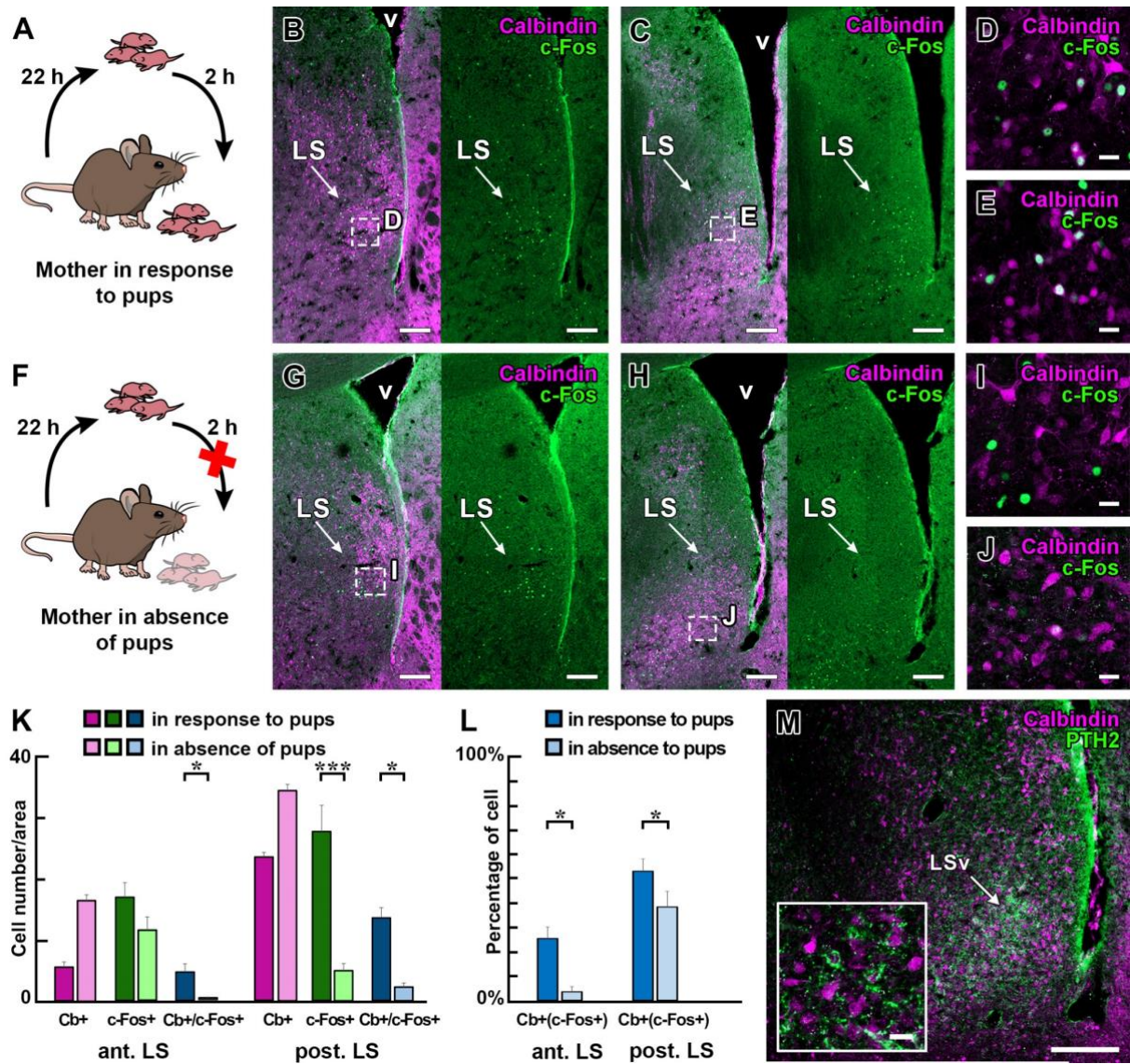


Fig. 2: Calbindin⁺ neurons are activated after interaction with pups in the lateral septum.
A: The experimental design of pup induced c-Fos detection. **B:** Confocal images of the anterior brain sections of mothers after pup-exposure, which demonstrate calbindin⁺ (Cb⁺) and c-Fos⁺ neurons of the lateral septum (LS). **C:** Posterior brain sections of the same mother. **D-E:** Higher magnification images of panel B and C. **F:** The experimental design of c-Fos detection in the absence of pups. **G-H:** Anterior (G) and posterior (H) sections of the LS of a mother in absence of pups. **I-J:** Higher magnification images of panel G and H. **K:** The density of Cb⁺, c-Fos⁺ and double labelled (Cb⁺/c-Fos⁺) cells in the examined areas. Area = 0,1 mm². Data are presented as means $\pm$ s.e.m., *** p < 0.001, 0.01 < * p < 0.05. **L:** Quantification of the percentage of Cb⁺ cells in the c-Fos⁺ population (Cb⁺(c-Fos⁺)). All data are presented as means $\pm$ s.e.m., 0.01 < * p < 0.05, *** p < 0.001. **M:** Cb⁺ neurons are surrounded by PTH2+fibres. Scales: B, C, G, H: 200 μ m; D, E, I, J: 20 μ m; M: 500 μ m and 20 μ m for inlet. Abbreviations: LS: lateral septum, LSv: ventral lateral septum.

6.2. The pup-exposure activated neurons in the lateral septum are GABAergic

Furthermore, I studied whether the neurons activated after pup-exposure are GABAergic and whether inhibitory neurons have connection with PTH2⁺ fibres. For this, I examined VGAT-Cre-ZsGreen mice brain slices. I found that PTH2⁺ axons closely appose VGAT⁺ neurons in the LS (**Fig. 3 A1, A2**). Moreover, by pup-induced c-Fos examination, I revealed that a prominent number of activated neurons in the LS are also VGAT⁺ (**Fig. 3 B1, B2**).

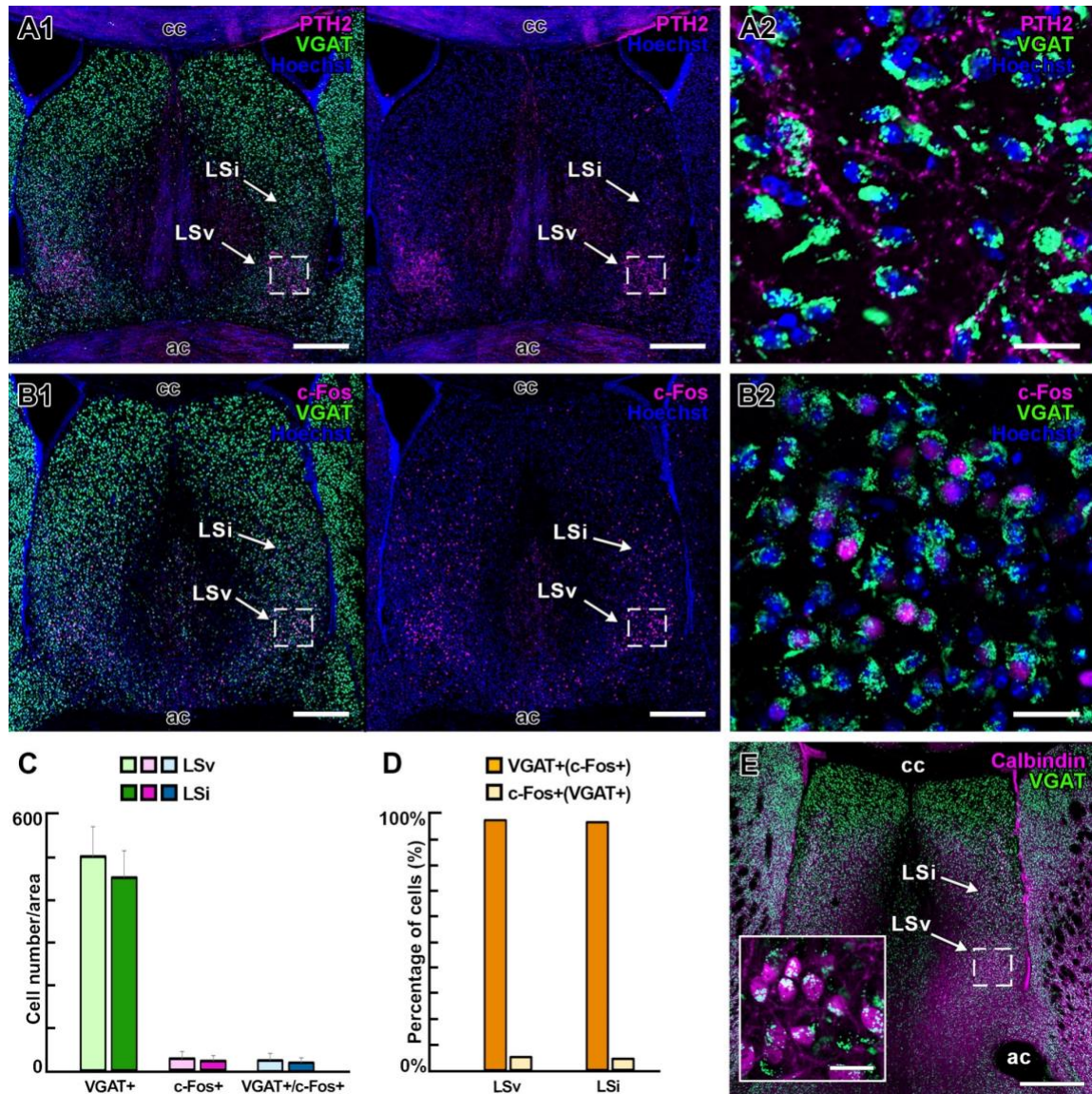


Fig. 3: PTH2⁺ fibres surround VGAT⁺ neurons activated following pup-exposure in lateral septum of mother mice. **A1:** PTH2⁺ fibres localized in the ventral part of lateral septum (LSv) of VGAT-Cre-ZsGreen reporter mother mouse. **A2:** The higher magnification image indicates VGAT⁺ neurons are closely surrounded by PTH2⁺ terminals. **B1:** Low magnification confocal image of the lateral septum (LS). **B2:** The high magnification image demonstrating c-Fos and VGAT labelled neurons. **C:** The density of c-Fos⁺, VGAT⁺ and double labelled (VGAT⁺/c-Fos⁺) neurons in the LS. **D:** 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+)). **E:** Cb immunolabelling of VGAT-Cre-ZsGreen mouse brain slice. Scales: A1, B1: 200 μ m, A2, B2: 20 μ m, E: 500 μ m, 20 μ m for inlet. Abbreviations: ac: anterior commissure, cc: corpus callosum, LSi: intermediate lateral septum, LSv: ventral lateral septum.

Next, I counted the number of VGAT+, c-Fos+ and double labelled VGAT+/c-Fos+ neurons in the LSi and LSv (**Fig. 3 C, D**). There is a high number of VGAT+ neurons in both areas and nearly all activated neurons are GABAergic (LSv: $95.82\% \pm 8.06\%$, LSi $96.41\% \pm 6.75\%$) (**Fig. 3 C**). While $4.74\% \pm 3.14\%$ of the VGAT positive neurons localized in the LSv and $4.26\% \pm 2.74\%$ of them in the LSi are c-Fos+ (**Fig. 3 D**).

Finally, I studied the overlap between Cb+ and VGAT+ neuron populations and determined that $96.07\% (\pm 2.39\%)$ of the Cb+ neurons show VGAT positivity, as well (**Fig. 3 E**).

6.3. The pup-exposure activated neurons in the lateral septum are surrounded by PTH2+ fibres in mother mice

Based on my results shown previously (**Fig. 2**), it is noticed that localization pattern of PTH2+ fibres and pup-induced activated Cb+/GABAergic LS neurons are similar (**Fig. 2 M**). Thus, I further examined the localization pattern of c-Fos activated neurons and PTH2-containing axon terminals in the LS following pup-exposure by immunohistochemistry. I found that most of the c-Fos+ neurons are closely apposed by PTH2+ fibres, although there are some non-c-Fos-activated neurons also approached by PTH2+ axons (**Fig. 4**).

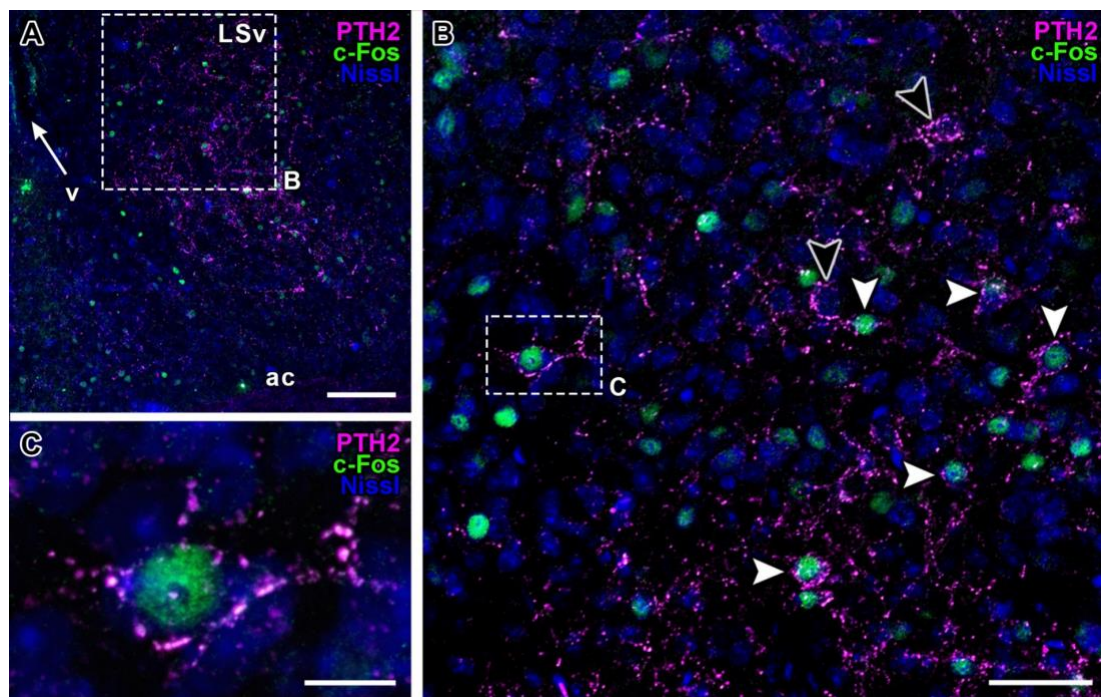


Fig. 4: The localization of activated neurons and PTH2+ fibres in the ventral lateral septum of mother mouse. **A:** Low magnification image showing dens PTH2+ input in the ventral lateral septum (LSv). **B:** Higher magnification image of the area indicated on panel A, showing PTH2+ fibres border c-Fos activated neurons (white arrowheads). Black arrowheads sign non c-Fos-activated neurons surrounded by PTH2+ terminals. **C:** Magnified image of the activated neuron signed on panel B, shows PTH2+ terminal. Scales: A: 100 μ m, B: 20 μ m, C: 10 μ m. Abbreviations: ac: anterior commissure, LSv: ventral lateral septum, v: lateral ventricle.

6.4. Synaptic connections between PTH2+ fibres and VGAT+ neurons in LSv in mother mice

Based on my confocal microscopic results showed previously, we supposed synaptic connection between PTH2+ terminals and c-Fos-activated VGAT+ neurons. Therefore, CLEM technique was used on mother VGAT-Cre-ZsGreen brain slices (**Fig. 5**).

VGAT+ neuron surrounded by PTH2+ terminals was identified first using fluorescent immunohistochemistry of PTH2 (**Fig. 5 A1-3**). Then, I visualized the same neuron with peroxidase-based immunohistochemistry for electron microscopy (**Fig. 5 B1-3**). Based on high magnification electron microscopic images the localization of small synaptic and dense core vesicles, puncta adherentia, the synaptic cleft, and the postsynaptic density between the GABAergic neuron and the PTH2+ terminals were demonstrated (**Fig. 5 C1'-C3'**).

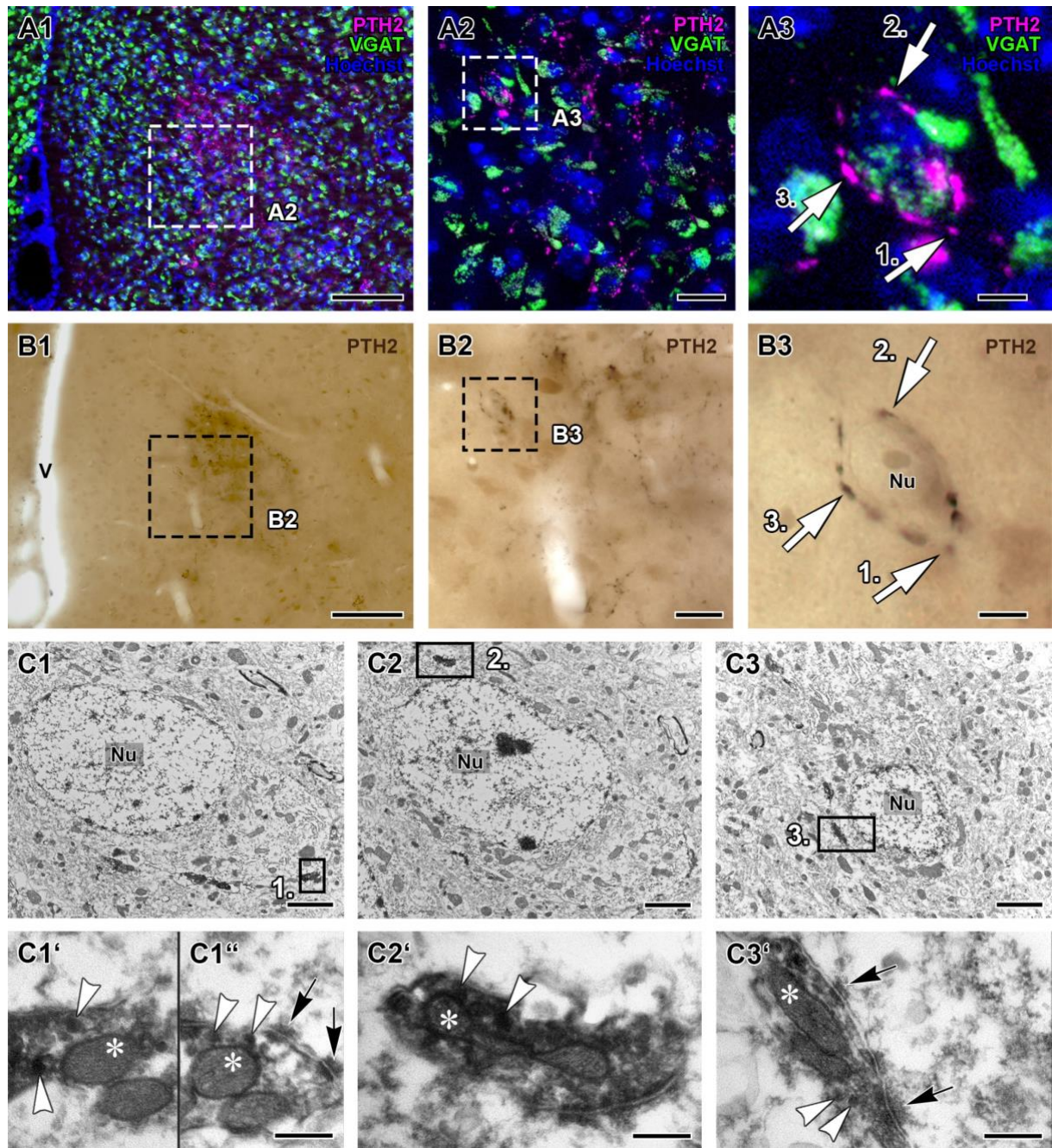


Fig. 5: Synaptic connection between PTH2-containing terminals and GABAergic neurons in the lateral septum of mother mouse. **A1:** Fluorescent microscopic image of the lateral septum (LS) following PTH2 immunolabelling of VGAT-ZsGreen mother mice. **A2:** Higher magnification image of the area indicated in panel A1. **A3:** High magnification image of a VGAT+ neuron. **B1:** The same lateral septal area showed in panel A1 following immunoperoxidase reaction. **B2:** The panel shows the same area as in panel A2. **B3:** Image of the same neuron as in panel A3. White arrows indicate PTH2+ terminals. **C1-C3:** Low magnification electron microscopic images of serial sections of the neuron showed in panel A3 and B3. The numbers indicate the terminals signed in panel A3 and B3. **C1'-C3':** High magnification images of the terminals indicated with numbers. Panel C1' is an image of the following ultrathin section of the same synaptic terminal showed in panel C1'. Synaptic dense core vesicles (white arrowheads), puncta adherentia (black arrows), mitochondria (*). Scales: A1, B1: 100 μ m; A2, B2: 20 μ m; A3, B3: 5 μ m; C1, C2, C3: 2,5 μ m; C1', C2', C3': 250 nm. Abbreviations: Nu: nucleus, v: lateral ventricle.

6.5. GABAergic neurons of the lateral septum project to the medial preoptic area

As GABAergic neurons in the LS show increased pup-induced c-Fos activation (**Fig. 3 D**) suggesting importance in maternal regulation, I further investigated their projection to reveal the neuronal circuitry, in which LS may regulate maternal adaptation. Therefore, I injected anterogradely transported Cre-dependent viral vector containing mCherry reporter gene into the LS of VGAT-IRES-Cre female mice (**Fig. 6 A, B**). I found that VGAT⁺ neurons send prominent projection to the neurons of the MPOA, a central area in maternal adaptation process (**Fig. 6 B, D**).

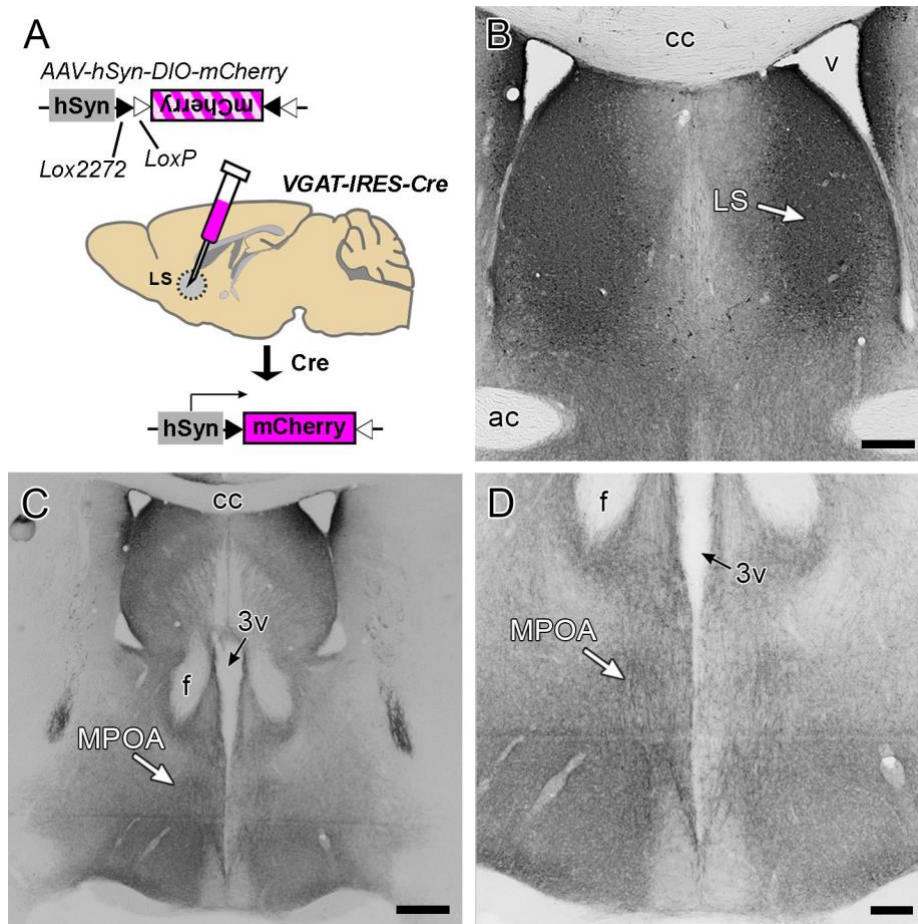


Fig. 6: GABAergic neurons of the lateral septum project to the medial preoptic area. A: Illustration of the anterograde vector tracing of lateral septal VGAT⁺ neurons. **B:** Representative image shows the injection site in the LS. **C-D:** Light microscopic images of the projection to the MPOA demonstrate dense VGAT⁺ fibre localisation. Scales: B-C: 250 μ m; D: 100 μ m. Abbreviations: 3V: third ventricle, ac: anterior commissure, cc: corpus callosum f: fornix, LS: lateral septum, MPOA: medial preoptic area, v: lateral ventricle.

6.6. Neurons of the lateral septum receive PTH2+ input from the posterior intralaminar thalamic nucleus

Based on my results of PTH2 immunohistochemistry and anterograde tracing from the LS in mice, I aimed to study the PTH2+ projections and the pathway between LS and MPOA in rats, as well.

To examine the origin of the PTH2+ afferents of the LS, an anterogradely transported neuronal tracer, biotinylated dextran amine (BDA) was injected into the PIL of mother rats (**Fig. 7 A**). We observed a prominent input from PIL neurons (**Fig. 7 B, C**) to the LS (**Fig. 7 D, E**). Moreover, c-Fos-activated neurons in the LS following pup-exposure are surrounded by BDA labelled fibres (**Fig. 7 F**).

Since Cb+ neurons showed significantly higher activation in the LS of mother mice in response to pups (**Fig. 2 L**), I further investigated whether PTH2 neurons of the PIL innervate the Cb+ neuron population in mother rats, as well. I confirmed in rat mother that many of the Cb+ neurons show pup-induced activation. Moreover, I revealed that the activated Cb+ neuronal cell bodies and dendrites are also closely surrounded by PTH2+ fibres in mother rats, in accordance with the result in mother mice (**Fig. 7 G-I**). However, synaptic connection is more than likely based on our confocal images, further electron microscopic examination will be needed for confirmation.

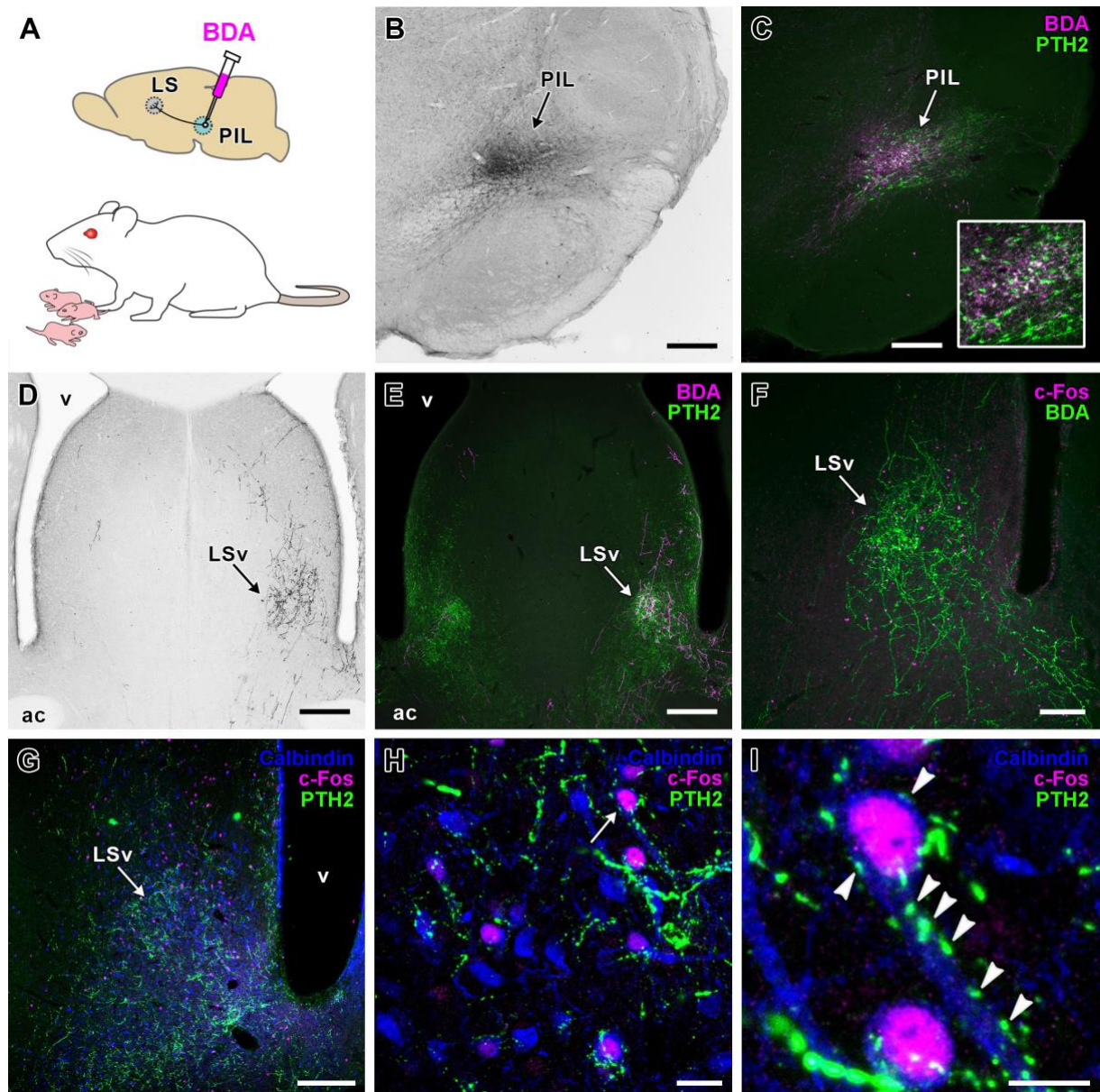


Fig. 7: The lateral septum receives input from PTH2+ neurons of the posterior intralaminar thalamic nucleus. **A:** Illustration of the injection of BDA into the PIL. **B:** Injection site in the PIL followed by peroxidase immunoreaction. **C:** Confocal image of the injection site. The inlet shows several double labelled neurons expressing PTH2 and containing BDA, as well. **D:** Representative image of the projection seen in the LS. **E:** Double labelled fibres contain both BDA and PTH2. **F:** c-Fos activated neurons in the lateral septum are surrounded by BDA containing fibres. **G, H, I:** Cb+ neurons in the LS are c-Fos+ and surrounded by PTH2+ fibres. White arrowheads indicate PTH2+ terminals. Scales: B-E: 500 μ m; F: 100 μ m; G: 200 μ m; H, I: 20 μ m. Abbreviations: ac: anterior commissure, BDA: biotinylated dextran amin, LSv: ventral lateral septum, PIL: posterior intralaminar thalamic nucleus, PTH2: parathyroid hormone 2, v: lateral ventricle.

6.7. Investigation of the connection between the lateral septum and the medial preoptic area in female rats

The pathway between LS and MPOA shows a promising role in maternal adaptation process, thus I examined this projection in female rats, as well. I injected retrograde tracer, cholera toxin B (CTB) into the MPOA of female rats (**Fig. 8 A**). I confirmed the connection between LS and MPOA seen in mice and revealed that many neurons localized in LS projecting to MPOA show Cb immunopositivity (**Fig. 8 B-E**).

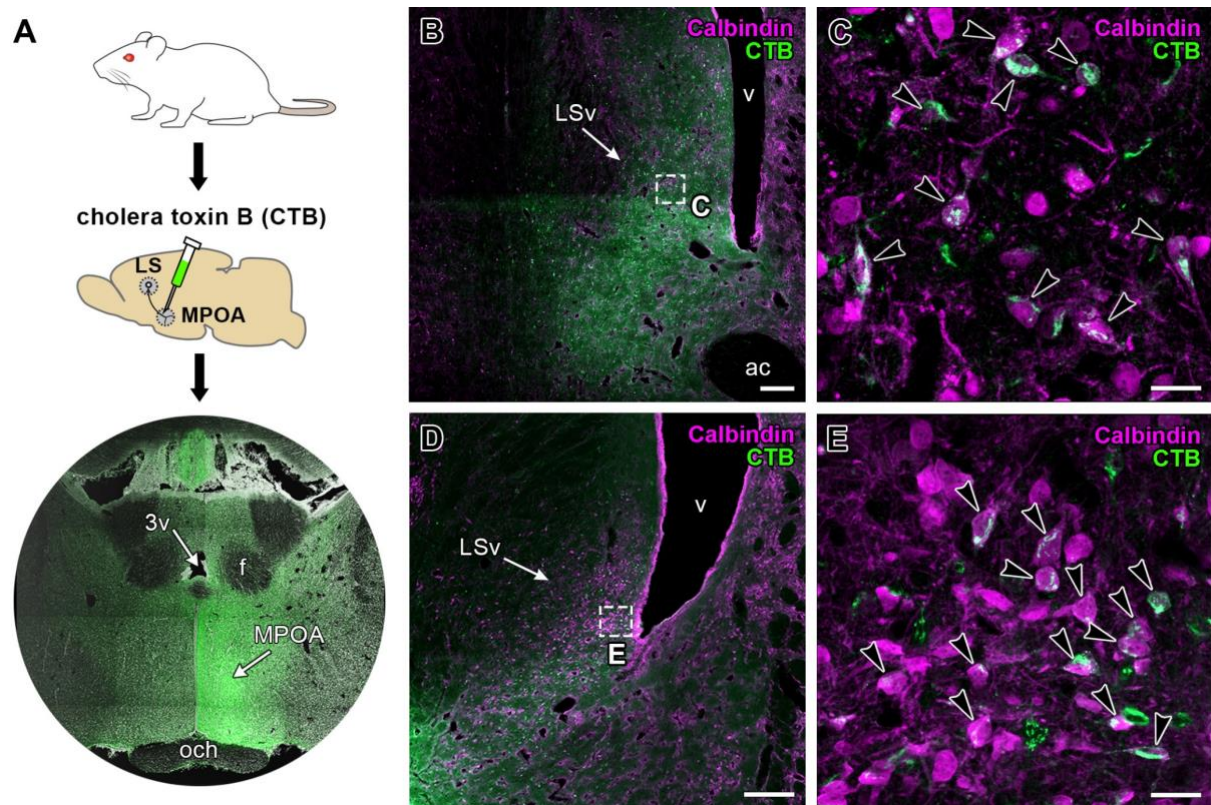


Fig. 8: Septal projection to the medial preoptic area originates mainly from calbindin+ neurons. **A:** Illustration of the retrograde tracer injection to the medial preoptic area and a representative image of the injection site. **B:** Immunolabelling of septal neurons with calbindin and CTB. **C:** Higher magnification confocal image of the area indicated in panel B. Black arrowheads signed double immunolabelled neurons. **D, E:** The same situation like in panel B and C, but on a more posterior section. Scales: B: 200 μ m; C, E: 20 μ m; D: 500 μ m. Abbreviations: 3V: third ventricle, ac: anterior commissure, CTB: cholera toxin B, f: fornix, MPOA: medial preoptic area, LSv: ventral lateral septum, och: optic chiasm, PIL: posterior intralaminar thalamic nucleus, v: lateral ventricle.

6.8. The same neurons of the posterior intralaminar thalamic nucleus project to the lateral septum and the medial preoptic area

Both LS and MPOA neurons receive prominent PTH2⁺ input from the PIL in dams. Thus, I aimed to gain more information about these pathways supposing that, these connections have importance in the septal modulation of maternal adaptation process.

To characterise the neurons of the PIL projecting to the LS and MPOA specifically, I injected mCherry-tagged retrogradely transported virus (AAV-rg-mCherry) into the LS (**Fig. 9 A**) or CTB into the MPOA (**Fig. 9 D**). Previous publication of our research group showed calcium binding protein containing PTH2-expressing neurons localize in the PIL of rat dams [19]. Therefore, I examined the distribution pattern of Cb and calretinin (Cr) markers in retrogradely labelled neurons. I demonstrated that many neurons projecting to the LS and MPOA show Cb or Cr immunopositivity, as well (**Fig. 9 B1-C2, E1-F2**).

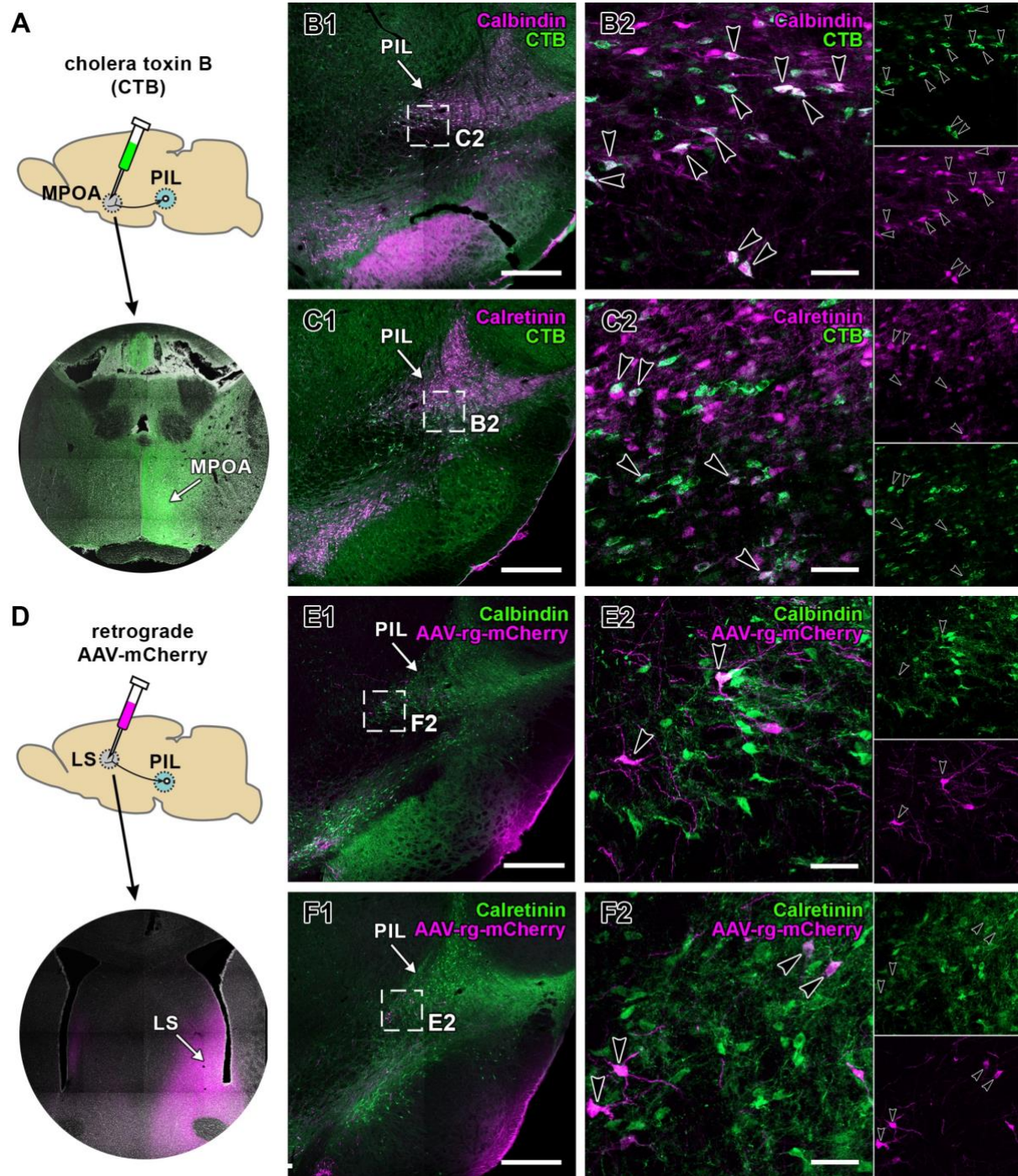


Fig. 9: Calbindin⁺ and calretinin⁺ neurons of the PIL project to the medial preoptic area and lateral septum. **A:** Illustration of the retrograde tracer injection with a representative image of the injection site in the MPOA. **B1-2, C1-2:** Cb⁺ and Cr⁺ neurons projecting to the MPOA from the PIL show CTB labelling (black arrowheads). **D:** Illustration of the retrograde viral injection, and a confocal image of the injection site in the LS. **E1-2, F1-F2:** Cb⁺ and Cr⁺ neurons that project to the LS from the PIL show mCherry labelling (black arrowheads). Scales: B1, C1, E1, F1: 200 μ m; B2, C2, E2, F2: 20 μ m. Abbreviations: AAV-rG-mCherry: retrogradely transported adeno-associated virus tagged with mCherry, CTB: cholera toxin B, LS: lateral septum, MPOA: medial preoptic area, PIL: posterior intralaminar thalamic nucleus.

As Cb⁺ and Cr⁺ neurons of the PIL project both to the LS and the MPOA, I further examined whether these afferents originate from axon collaterals of the same PIL neurons. Thus, two different retrograde tracers, AAV-rg-mCherry and CTB were injected into the LS and into the MPOA, respectively at the same time in a female rat (**Fig. 10 A**). I revealed that double retrogradely labelled neurons present in the PIL supporting my assumption (**Fig. 10 D1-2**).

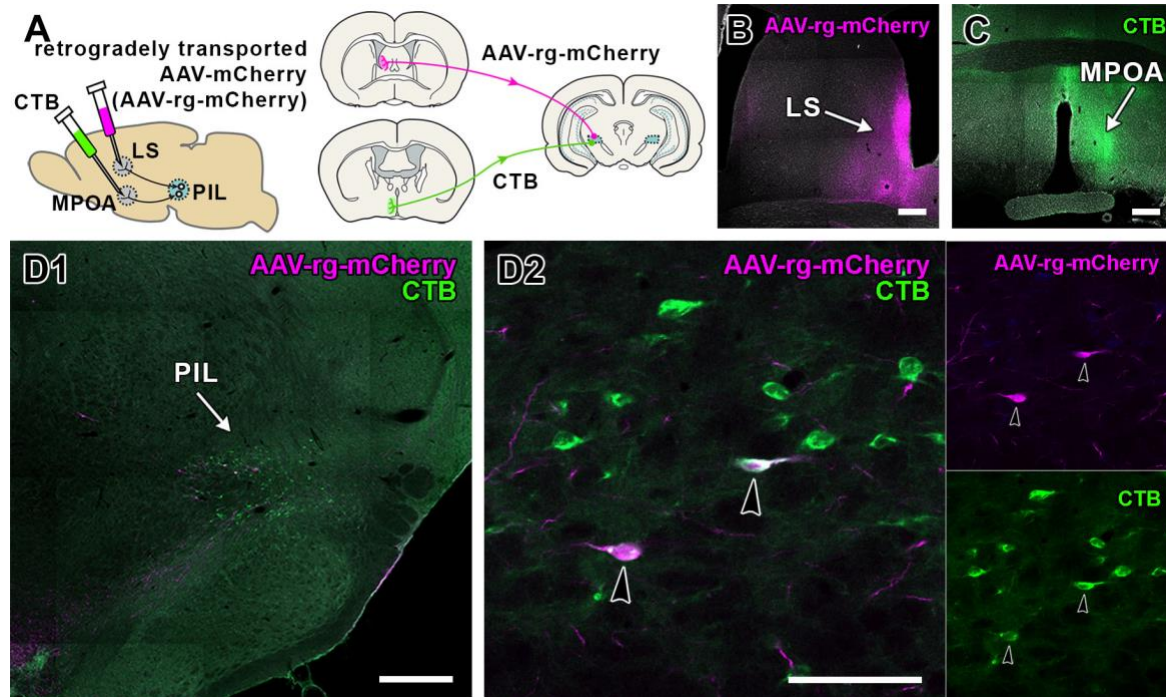


Fig. 10: The lateral septum and the medial preoptic area receive input from the same neurons of the posterior intralaminar thalamic nucleus. **A:** Illustrations of the double retrograde tracing of the LS and the MPOA. **B, C:** Representative images of the injection site in the LS (B) and MPOA (C). **D1:** Retrogradely labelled neurons are localized in the PIL. **D2:** Higher magnification image demonstrates neurons projecting to the LS, to the MPOA and to both areas (black arrowheads). Scales: B, C: 500 μ m; D1: 500 μ m; D2: 100 μ m. Abbreviations: AAV-rg-mCherry: retrogradely transported adeno-associated virus tagged with mCherry, CTB: cholera toxin B, LS: lateral septum, MPOA: medial preoptic area, PIL: posterior intralaminar thalamic nucleus.

7. DISCUSSION

Exposure to pups resulted in activation of Cb⁺ neurons in the LS in mother mice. In addition, the activated Cb⁺ neurons are all inhibitory cells and are innervated by fibres containing PTH2, a neuropeptide induced specifically in mothers, as it was shown in case of GABAergic neurons. Only a few research have addressed the importance of the GABAergic and Cb⁺ neuron population of LS regarding maternal care. Menon *et al.* revealed, higher level of oxytocin release in lactating mice promotes the activation of GABAergic Cb⁺ neurons in the LS causing a reduced social fear reaction. This helps to bring the protection of offspring to the fore, thus it maintains proper maternal behaviour [20]. However, we still have limited knowledge about the functional importance of the septal Cb⁺ neuron population concerning maternal care and other social interactions. Therefore, we plan to examine them functionally by using calbindin-Cre transgenic mice for chemogenetic modifications in the future.

In agreement with previous publications describing reciprocal connection between MPOA and LS [21], I determined that there is dense projection to the MPOA from the septal GABAergic neurons. Since MPOA is a central regulator of maternal behaviour, the pathway from LS to MPOA seems a promising target for future research on maternal behaviour. Previously, it has been shown that the glutamatergic neurons of MPOA receive major monosynaptic input from the LS and their activation increases anxiety-like behaviour and decreases parental care [22]. Furthermore, our research group revealed that GABAergic neurons of MPOA are activated in response to pups and enhance maternal behaviour in mice [4]. In addition, a previous publication described the role of LS in regulating anxiety response, as the activation of LS-projecting ventral hippocampal neurons decreases anxiety-like behaviour [23]. Based on these findings and my results regarding MPOA-projecting septal neurons shown Cb positivity, we suppose that GABAergic Cb⁺ neurons of LS probably inhibit glutamatergic neurons of the MPOA and take part in inducing maternal care and regulating anxiety-like behaviour during motherhood. It is also a possibility that septal neurons inhibit local GABAergic interneurons of MPOA resulting in similar output behaviour.

PTH2⁺ innervation of LS might have a role in this presumed process, as we confirmed that PTH2⁺ terminals from the PIL show the same distribution pattern with pup-activated c-Fos⁺ neurons found in the area. Thus, I further examined the connection among PIL, LS and MPOA and demonstrated that MPOA and LS projecting PIL neurons show Cb and Cr positivity. In addition, part of the inputs received by both areas originate from the same neurons of the PIL.

As PTH2-expressing neurons contain calcium binding protein in the PIL [19], I suppose, LS and MPOA-projecting PIL neurons are also part of the PTH2-expressing neuronal population.

8. CONCLUSION

Our results suggest that GABAergic Cb⁺ neurons of LS can be regulated by the PTH2⁺ input from PIL in rodent mothers. The pup-activated LS neurons further project to the MPOA, therefore LS possibly takes part in the regulation of maternal behaviour via the PIL-LS-MPOA pathway.

9. AUTHOR CONTRIBUTION

Hereby, I, Vivien Szendi, declare, that:

- I carried out stereotaxic surgeries for tracing and the perfusion of the animals including anaesthesia under the supervision of a person with permission to experiment with laboratory animals
- I performed all parts of immunohistochemistry procedures including perfusion, brain sectioning and immunolabelling.
- I took the confocal images and examined them using QuPath, then carried out the statistical analysis with SPSS.
- During electron microscopic procedures, I performed the immunolabelling, the embedding of sections and identified the area of interest with light microscope.

Hereby I declare that I have not been awarded any prize at previous OTDK conferences in Hungary.

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