STRUCTURAL BASIS OF PEPTIDERGIC CONTROL OF AMYGDALA FUNCTIONS

PhD thesis

Kinga Müller

János Szentágothai Doctoral School of Neurosciences

Semmelweis University

Supervisor: Norbert Hájos, Ph.D, D.Sc

Official reviewers: Ferenc Mátyás, Ph.D,
Gergely Zachar, Ph.D,

Head of the Complex Examination Committee:
Alán Alpár, Ph.D, Habil., D.Sc

Members of the Complex Examination Committee:
Lucia Wittner Ph.D,
Tibor Zelles Ph.D,

Budapest, 2023

1. Introduction:

The amygdala is a complex structure in the temporal lobe of the brain, which plays a crucial role in recognizing emotions and detecting threats in the environment. The amygdala has 13 subdivisions, including the basolateral amygdala (BLA) and central amygdala (CeA), each with a distinct function. The BLA comprises the lateral (LA) and basal nuclei (BA). The BLA is responsible for associating threatening stimuli with neutral stimuli and long-term memory consolidation, while the CeA is involved in the expression of fear behaviour. The BLA and CeA have different developmental origins, neurochemical differences, and projection neurons, indicating their heterogeneity. The LA and BA have a similar origin to cortical structures, while the CeA is a striatal area. These parts also differ in the neurotransmitter content of their projection neurons; as the BLA principal neurons (PC) release glutamate while the CeA neurons use GABA (gamma-aminobutyric acid) as a neurotransmitter. Deep knowledge of the circuit organizations and the function of the amygdala nuclei is crucial to understand their role played in defensive behaviour, but these networks are also regulated by ascending neuromodulatory systems. One of the neuromodulatory systems contains dopamine. The dopaminergic input source of CeA is the ventrolateral part of the periaqueductal grey (vlPAG) and the dorsal raphe nucleus (DRN). Recently, several studies investigated the function of neurons expressing the tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, in the vlPAG-DRN. It

is not clear whether the TH^+ neurons expressing vasoactive intestinal polypeptide (VIP) have the same or different role in neural functions than dopaminergic neurons lacking VIP.

The BLA is a cortical structure, however, it is non-layered. Similar types of neurons are located here as in the neocortex, even the ratio between the excitatory PCs and the inhibitory interneurons was suggested to be comparable. Indeed, around 20% of all neurons are inhibitory interneurons (INs) in the mouse amygdala.

The most evident difference between the LA and BA nuclei of the basolateral amygdala (BLA) is the size of the somata of the PCs. In other aspects, like the dendritic arborization and spine density, the PCs in the LA and BA are similar. In contrast to local dendrites of PCs in BLA, their axons project remotely.

There are three major IN categories in cortical structures: perisomatic inhibitory cells, dendritic inhibitory cells, and interneuron-selective interneurons (ISIs). The BLA perisomatic inhibitory cells target preferentially the somata, proximal dendrites or axon initial segments of PCs. Three types of INs preferentially inhibit the perisomatic region: cholecystokinin and cannabinoid receptor type 1-expressing basket cells (CCK/CB_1BCs), parvalbumin (PV)-expressing BCs (PVBCs), and axo-axonic cells. These INs are crucial in controlling spiking activity of their postsynaptic partners. In contrast to the perisomatic inhibitory cells, INs targeting preferentially the PC dendrites were shown to be effective in suppressing the generation of dendritic calcium spikes. There are two main types of dendrite-

targeting INs in the BLA: somatostatin (SST)-expressing INs and the neurogliaform cells (NGF cells). VIP-expressing INs are the third major source of synaptic inhibition in the BLA, as in the neocortex or hippocampus. In line with their name, VIP^+ ISIs innervate other types of INs: PV^+ INs, SST^+ INs, CCK^+ BCs, and other VIP^+/ calretinin-expressing ISIs. A notable difference between the BLA and other cortical structures is in the ratio of the different types of INs. How these differences in the proportion of distinct neuropeptide-expressing IN types translate to the differences in circuit operation is not yet fully understood. As our knowledge about INs expressing different neuropeptides is still in infancy, we set out to investigate these GABAergic cells in the circuits of the BLA.

2. Aims

The main goals of our studies were to determine the features of the neuropeptide-expressing neurons in the amygdala and a neuropeptidergic input terminating in the amygdala. Therefore, we focused on four main topics with the following specific questions:

I. To unfold the nature of the somatostatin (SST)-expressing GABAergic neurons in the BLA circuits.
   - What are the electrophysiological and morphological properties of the distinct SST+ inhibitory neurons in the BLA?
   - How many SST^+ GABAergic neurons are in the LA and BA?

II. To determine the properties of neuropeptide Y (NPY)-containing neurogliaform (NGF) cells in the BLA.
   - How do the electrophysiological features differ between the various NPY^+ inhibitory neurons in the BLA?
   - What is the number of NPY^+ GABAergic cells of the LA and BA?

III. To probe a new strategy for the investigation of cholecystokinin (CCK)-expressing inhibitory neurons.
   - What are the electrophysiological properties of the GABAergic neurons that express CCK neuropeptide?

- What is the distribution pattern of the diverse CCK-expressing neurons in the BLA?

IV. To unravel the characteristics of the vasoactive intestinal polypeptide (VIP)-expressing inputs of the CeA.

- What are the output features of the VIP^+ neurons located in the vlPAG-DRN region?

3. Materials and methods

3.1. Experimental animals

The experimental animals used in the study were obtained from The Jackson Laboratory or Mutant Mouse Resource & Research Center. All procedures involving animals were approved by Hungarian legislation and institutional guidelines to minimize animal suffering and the number of animals used. Different mouse lines were used for specific experiments, such as Sst-IRES-Cre and Npy-Cre;Dlx5/6-Flp mice for studying the ratio or morphology of SST^+ or NPY^+ neurons and Npy-Cre x Ai14 mice for electrophysiological studies. Offspring of Cck-IRES-Cre crossed with Dlx5/6-Flpe mice for studying CCK-expressing GABAergic neurons, and Vip-IRES-Cre, Vip-IRES-Cre_ZsGreen1 or Vgat-IRES-Cre_ZsGreen1 mice for studying neurons in the midbrain. Mice were housed in same-sex groupings and in a temperature- and humidity-controlled vivarium under a 12 h light/dark cycle (lights on 06:00 h).

3.2. Slice preparation for electrophysiology

After 4-6 weeks following the injection of viral vectors mice were deeply anaesthetized with isoflurane, and acute brain slices were acquired. Depending on the project, 200 μm thick brain sections were prepared with a vibratome. After at least a 60-min-long incubation, slices were transferred to a submerged-type recording chamber and perfused with 32-34°C ACSF.

3.3. Whole-cell recordings

Recordings were performed under visual guidance using differential interference contrast microscopy and a 40x water dipping objective. Fluorescent protein expression in neurons was visualized with a mercury arc lamp and CCD camera. Whole-cell recordings were obtained using patch pipettes pulled from borosilicate capillaries and filled with K-gluconate based intrapipette solution and containing 0.2% biocytin. Recordings were performed using a Multiclamp 700B amplifier, low-pass filtered at 3 kHz, digitized at 10 kHz, and analysed using various software tools. For firing pattern analysis, neurons were recorded in current-clamp mode at holding potentials of -65 mV or -70 mV depending on the projects and tested with a series of hyperpolarizing and depolarizing square pulses of current with varying amplitudes.

3.4. Virus or tracer injections and optogenetic experiments

In this study, anaesthesia was induced and maintained with a ketamine/xylazine cocktail. Mice were secured in a stereotaxic frame during the virus injections. Adeno-associated virus (AAV)-based constructs were injected unilaterally to BLA in Sst-Cre, Npy-Cre;Dlx5/6-Flp, and Cck-Cre;Dlx5/6-Flp mice. AAV-based INTRSECT constructs were also applied to transfect Cre^{+}/Flp^{+} cells with ChR2 in ex vivo experiments. Finally, AAV5-EF1a-DIO-ChR2-mCherry viruses were bilaterally injected into the vlPAG of VIP-Cre

mice to evaluate the light-evoked transmitter release from the axon terminals at the projection sites. AAV5-EF1-DIO-eYFP virus was injected into the BNST to visualize antero-retrogradely the collateralization of VIP^+ neurons in the whole brain, and AAV1-CAG-FLEX-tdTomato or AAV1-Cre(on)-GFP was injected in the PAG to visualize the VIP^+ neurons. For the retrograde labelling of the CeA or BNST projecting neurons, we used the cholera toxin β subunit injected into the CeA and fluorogold into the BNST unilaterally.

3.5. Post hoc identification of labelled neurons

The biocytin content of recorded neurons from various transgenic mouse lines was visualized using different conjugated streptavidin molecules. Confocal images of the filled cells were obtained, and slices were subsequently immunostained with antibodies targeting specific neuronal characteristics. Finally, the images were deconvolved and analysed using ImageJ software. Image analysis was performed using Neurolucida Explorer.

3.6. Section preparation for anatomy

After being anaesthetised with ketamine/xylazine, adult tracer-, virus-injected or non-injected transgenic mice (P56-P70) were transcardially perfused with 0.9% NaCl for 1-2 minutes followed by a fixative solution containing 4% PFA in 0.1 M PB, pH 7.4, for 30 min. Coronal sections were prepared from the tissue blocks containing the entire amygdalar or midbrain region using a Leica VT1000S

vibratome (Leica Microsystems). Sections were stored in a cryoprotectant antifreeze solution at -20°C until further processing.

3.7. Human tissue processing

Brain tissue samples from control subjects were collected and perfused by the Human Brain Research Laboratory at the ELRN Institute of Experimental Medicine. The experiments were validated by the Regional and Institutional Committee of Science and Research Ethics of Scientific Council of Health. The brains were washed with a solution containing an anticoagulant before perfusion with a fixative solution. The amygdala and midbrain blocks or 50 μm thick sections were used for analysis. The sections were immunostained with VIP, TH, and neuronal nuclei antigen and autofluorescence was reduced using a cupric sulphate solution.

3.8. Statistical analysis

Data are presented as mean ± SEM, if not indicated otherwise. Statistical significance ($p<0.05$) was assessed by t-test for comparison of data with a normal distribution, whereas Kruskal–Wallis ANOVA, Dunn's test, Mann-Whitey (MW) *U* test, and Kolmogorov–Smirnov test were used for datasets with a non-normal distribution.

4. Results:

4.1. Interneurons and projecting inhibitory cells expressing SST in the BLA

The distribution and targets of SST-containing inhibitory cells in the mouse basolateral amygdala (BLA) were studied using immunostaining and intracellular labelling. We found that EYFP-expressing boutons that formed close appositions with the intracellularly labelled PCs were evenly distributed along their dendritic trees in the BLA. Our study confirmed that SST is expressed in two types of GABAergic cells: one is the basal forebrain- or entorhinal cortex-projecting SST-expressing cells, which mostly showed immunoreactivity for neuronal nitric oxide synthase (nNOS); the other type is the local SST^+ interneurons were mainly nNOS immunonegative. The dendritic reconstruction of labelled cells from both types revealed that the structure of their dendrites was different.

Next, we aimed to estimate the ratio of SST^+ GABAergic cells in two amygdalar nuclei by labelling them in Sst-Cre mice using an eYFP-containing viral vector. We found that the ratio of SST^+ inhibitory cells was significantly different between LA and BA. We observed that nNOS negative and thus, the dendrite-targeting SST^+ INs were more abundant than SST^+ GABAergic projection cells.

4.2. NPY^+ inhibitory neurons in the BLA

We aimed to estimate the fraction of different types of GABAergic cells expressing NPY in two amygdalar nuclei. To ensure that we study only NPY^+ GABAergic neurons in the amygdala double-transgenic mice were generated by crossing Npy-Cre mice with Dlx5/6-Flpe mice. Three main GABAergic cell groups were identified among randomly sampled neurons expressing reporter proteins based on single-cell electrophysiological features. The largest number of recorded neurons (52%) were NGF cells with late-spiking phenotype, the second largest group (33%) were PVBCs showing a fast-spiking phenotype, and the third group (15%) were SST^+ inhibitory cells that had relatively narrow spikes and showed accommodation in spiking.

In a subsequent experiment, the fraction of NPY^+ inhibitory cells in the LA and BA was determined. The ratio of NPY^+ inhibitory cells was significantly different in the two amygdalar nuclei, and approximately 25% of NPY^+ GABAergic neurons were immunoreactive for PV, while a similar portion of neurons showed immunoreactivity against SST. Based on the observations that almost half of all NPY^+ GABAergic cells are NGF cells, we calculated that these INs make up 1.8% and 3.5% of all neurons in the LA and BA, respectively.

4.3. CCK^+ inhibitory neurons in the BLA

We aimed to investigate the properties of INs containing cholecystokinin (CCK) in the BLA. Previous results clearly showed

that CCK is also present in subpopulations of PCs. To assess selectivity, we applied an intersectional targeting strategy injecting channelrhodopsin (ChR2) containing virus in the BLA of Cck-Cre;Dlx5/6-Flp mice. The combined optogenetic and whole-cell recordings showed that blue light illumination of ChR2-expressing neurons evoked outward currents with distinct fast and slow components. The fast component was blocked by the $GABA_A$ receptor antagonist, gabazine, while the remaining slow outward current was eliminated by the $GABA_B$ receptor antagonist, CGP 5699A. No inward current was observed, indicating that photostimulation exclusively evoked inhibitory, GABA receptor-mediated synaptic currents in BLA neurons, consistent with the selective targeting of INs and not PCs in this region.

We next asked whether the targeted BLA INs were CB_1R-positive. Our results showed that a significant component of synaptic currents in BLA neurons evoked by light illumination in slices originated from GABAergic cells expressing CB_1R. Accordingly, immunostaining of BLA sections containing transfected cellular processes revealed that ~40% of EYFP-expressing axonal varicosities were positive for CB_1R, indicating that a significant proportion of the targeted cells exhibit a defining feature of CCKBCs.

These observations suggested that other IN populations might also be present. This led us to perform immunolabelling for the presence of various IN neurochemical markers in EYFP-expressing cells. We found that a subset of EYFP-expressing cells also expressed NPY

(29%) and PV (17%), while none of them expressed SST or nNOS. Finally, none of the EYFP-expressing cells were immunopositive for CaMKII, a glutamatergic neuronal maker.

Our immunolabelling results thus suggest that in addition to CCK/CB_1R-expressing BCs, three IN subtypes: PVBCs, PV^+ axo-axonic cell and NPY^+, likely NGF cells, were targeted in Cck-Cre;Dlx5/6-Flp mice using intersectional strategy. Immunolabelling and intracellular labelling were used to classify the infected interneurons into three main subcategories: fast-spiking cells, which included PVBCs and PV^+ axo-axonic cells, CCK/CB_1-expressing BCs, and late-spiking NGF cells. The majority of the recorded and labelled cells (~60%) were NGF cells, while the remaining cells were equally distributed among the other two classes. These findings suggest that in addition to CCK/CB_1-expressing BCs, PVBCs, PV^+ axo-axonic cells, and NPY^+ NGF cells were also infected using the intersectional strategy.

Previous chapters presented our findings about the neuropeptide-expressing inhibitory neurons in the BLA. The last chapter will show the properties of PAG VIP^+ neurons connected with the extended amygdala.

4.4. Characterization of VIP^+ neurons projecting to the CeA

At the beginning, we investigated the specificity of the transgenic mice, VIP-Cre line used in the subsequent studies. In virus injected VIP-Cre mice, VIP immunostaining was applied. The results showed that 80% of virus labelled neurons were also marked by immunostaining, and 92% of immunolabeled neurons were visualized with viral vectors. There were no significant difference between males and females in the proportion of labelled neurons.

The study aimed to confirm whether VIP^+ neurons in the PAG are dopaminergic. Using VIP-Cre transgenic mice, we quantified the co-expression of VIP and the dopamine marker, tyrosine hydroxylase (TH), in vlPAG-DRN neurons in both male and female mice. They found that around 26% of VIP neurons expressed TH, and a similar proportion of TH neurons co-expressed VIP. We found no significant difference in the number of VIP^+/TH- and VIP^+/TH^+ neurons between the sexes.

Using Vip-Cre::tdTomato mice to locate the somata of VIP^+ neurons we found that they were distributed along the anteroposterior axis of the ventral PAG and DRN. We also visualized the axons of VIP^+ neurons and found that they were restricted to the dorsolateral part of the BNST and the lateral nucleus of the CeA. No axon terminals were observed in any other brain regions.

Antero-retrograde viral and retrograde tracing were performed to investigate whether VIP^+ neurons in the PAG-DRN collateralize and project to both the BNST and CeA bilaterally. Our results indicating that there are VIP^+ cells that can control these two nuclei bilaterally and simultaneously. These findings provide new insights into the characteristics of VIP^+ neurons in the PAG/DRN.

So far, we used mice to study VIP^+ neurons, but as a further step, we tested the hypothesis of whether these cells are present in humans as well. To this end, we made immunohistochemistry in post-mortem human brain sections. In most cases, the VIP^+ and TH^+ labelling was colocalized in the human PAG (83% of VIP^+ neurons were TH^+). In addition, VIP^+ axon fibres in the CeA were numerous, especially in the lateral part. These results highlight the relevance of our study obtained in mice suggesting VIP^+ neurons present in humans may have a similar function as in rodents.

We aimed to reveal the membrane characteristics of VIP^+ neurons, to this end the membrane properties of VIP^+ and GABAergic neurons in the vlPAG-DRN were compared using whole-cell patch-clamp recordings in acute brain slices from Vip-Cre and Vgat-Cre mouse lines. The results showed that VIP^+ neurons had different membrane properties than $VGAT^+$ neurons in this region, including a larger current step to evoke the first action potential, lower input resistance, shorter membrane time constant, and a larger relative sag amplitude. Optogenetic experiments were also performed to determine the nature of the neurotransmitter molecules released from the axon terminals of

VIP$^+$ neurons in their target areas. The results showed that PAG VIP$^+$ neurons could release glutamate from their axon terminals and excite neurons in the extended amygdala nuclei via activation of ionotropic glutamate receptors.

5. Conclusion

The balance of inhibitory and excitatory inputs arriving on PCs is critical for the proper function of the amygdala. We described three types of neuropeptidergic inhibitory neurons in the basolateral amygdala (BLA) and a neuropeptidergic excitatory input to the extended amygdala. We quantified the proportions of the given inhibitory cell types in the BLA, which may provide a solid ground for future studies aiming to clarify the changes in a different pathological operations linked to the amygdala.

The dendrite targeting SST$^+$ or NPY$^+$ INs are morphologically or electrophysiologically diverse. In Sst-Cre mice, we separated two types of GABAergic neurons based on nNOS content and morphology. However, their electrophysiological features were found to be similar.

In Npy-Cre mice, both electrophysiological and anatomical features of the 3 subtypes of amygdala INs were distinct. We found that NGF cells had a late spiking firing phenotype and gave rise to dense local axons and short dendrites. The next group was the fast-spiking PV$^+$ INs with spine-free dendrites, and axons resembling BCs or axo-

axonic cells. The last NPY^+ subgroup was SST^+ sparsely spiny neurons. The proportion of both SST^+ and NPY^+ GABAergic neurons was higher in the BA compared to LA, though the ratio of subgroups of NPY^+ neurons was not different.

In Cck-Cre;Dlx5/6-Flp mice 40% of virus labelled boutons expressed CB_1 cannabinoid receptor, whereas the other INs expressing CCK belonged to the NGF cells, PV^+ axo-axonic cells and BCs.

Using Vip-Cre mice we found that a portion of VIP^+ neurons in the PAG expressed the TH, a marker for dopaminergic neurons both in mice and humans. We proved that these VIP^+ neurons can provide control in the networks of the CeA and BNST bilaterally by releasing glutamate.

6. Bibliography of the candidate's publications

Publications related to this thesis

Viktória K. Vereczki[1], Kinga Müller[1], Éva Krizsán, Zoltán Máté, Zsuzsanna Fekete, Laura Rovira-Esteban, Judit M. Veres, Ferenc Erdélyi, Norbert Hájos; *Total Number and Ratio of GABAergic Neuron Types in the Mouse Lateral and Basal Amygdala;* Journal of Neuroscience 26 May 2021, 41 (21) 4575-4595; DOI: 10.1523/JNEUROSCI.2700-20.2021

[1] equal contribution

Laura Rovira-Esteban, Ozge Gunduz-Cinar, Olena Bukalo, Aaron Limoges, Emma Brockway, Kinga Müller, Lief Fenno, Yoon Seok Kim, Charu Ramakrishnan, Tibor Andrási, Karl Deisseroth, Andrew Holmes, Norbert Hájos; *Excitation of Diverse Classes of Cholecystokinin Interneurons in the Basal Amygdala Facilitates Fear Extinction;* eNeuro 21 October 2019, 6 (6) ENEURO.0220-19.2019; DOI: 10.1523/ENEURO.0220-19.2019

Other publications

Vereczki VK, Veres JM, Müller K, Nagy GA, Rácz B, Barsy B and Hájos N; *Synaptic organization of perisomatic GABAergic inputs onto the principal cells of the mouse basolateral amygdala;* Frontiers in Neuroanatomy, 2016, Volume 10 Article 20, doi:10.3389/fnana.2016.00020