Final report

The overall goal of our proposal was to define neural circuits carrying critical information into the amygdala that are necessary for associative fear learning. We defined three objectives, accomplishment of which may uncover the source(s) of teaching signals indispensable for associative learning at the amygdala levels.

Objective 1. We aimed to uncover the role of vasoactive intestinal polypeptide (VIP) expressing neurons in the periaqueductal gray (PAG) that project directly into the central amygdala (CeA) and bed nucleus of stria terminalis (BNST) in fear conditioning. Using optogenetics combined with slice recordings, we revealed that PAG VIP neurons are glutamatergic and give rise to excitatory postsynaptic responses in their postsynaptic partners located in the CeA and BNST. Their postsynaptic targets expressed somatostatin or PKC delta, neurochemical markers that are present in the two main neuron types in the CeA and BNST. Using electron microscopy, we observed that the axon terminals of PAG VIP neurons form asymmetric synaptic contacts preferentially with spines (65%), but also with dendritic shafts (35%). In addition, the presence of the tyrosine hydroxylase could have been shown in 25% of PAG VIP neurons revealed by immunocytochemistry, indicating that at least a portion of these PAG neurons can be dopaminergic. In line with this observation, injection of 6-OH-dopamine into the PAG resulted in a 50% reduction in the number of VIP neurons, strengthening the hypothesis that PAG VIP neurons, at least a portion of them, may be indeed dopaminergic in addition to their glutamatergic nature. These results were presented on a poster (Muller et al., 2017).

In collaboration with Prof. Francesco Ferraguti (Innsbruck Univesrity, Austria), we determined the inputs of PAG VIP neurons using monosynaptic tracing with rabies virus. These investigations showed that neurons innervating PAG VIP cells are present mainly in the oval nucleus of the BNST, CeA, hypothalamus and brainstem. Finally, we assessed the role of PAG VIP neurons in associative fear learning. To this end we teamed up with the group of Dr. Éva Mikics (ELRN IEM). In a first set of investigations, we expressed tetanus toxin light chain (TeLC) specifically in PAG VIP neurons by infecting them locally using viral vectors, a treatment that leads to blocking the neurotransmitter release from axon terminals. In comparison to control mice, mice expressing TeLC in PAG VIP neurons showed reduced fear memory

acquisition and recall in both cue-dependent and context-dependent fear conditioning. In addition, there was a significant decrease in freezing upon predator odor presentation. In contrast, there was no difference in the behavior of the two mouse groups in elevated plus maze, open field and sociability tests, indicating that this pathway is not involved in controlling anxiety levels and social interactions. In the second, still on-going investigations, we applied a chemogenetic approach to temporarily inhibit the function of PAG VIP neurons in behaving mice. An inhibitory DREAAD (Designer Receptors Exclusively Activated by Designer Drugs) was expressed specifically in PAG VIP neurons, and the ligand of DREADD receptors (in this case, CNO, clozapine N-oxide) was injected i.p. to mice expressed the DREADD or the control construct. Our preliminary data indicate that the temporal inhibition of the function of PAG VIP neurons recapitulate those results obtained by the permanent suppression using TeLC.

These data collectively show that PAG VIP neurons terminating in the CeA and BNST play a role in associative fear learning, as both the cued and contextual fear conditioning were impaired in mice where the function of PAG VIP neurons was compromised. We currently analyze behavioral data obtained by chemogenetics. After finishing the analyses of this last set of investigations, we will prepare a manuscript including data achieved by neuroanatomical techniques, in vitro electrophysiological methods combination with optogenetics and behavioral studies with impaired function of PAG VIP neurons. We planned to submit our MS for review in this year.

Objective 2. We planned to determine the function of vesicular glutamate transporter type 3-expressing cells within and near the BNST that project into the basolateral amygdala. Our subsequent studies revealed that these neurons are cholinergic, i.e. they are a part of the basal forebrain structure. The role of cholinergic neurons was planned to be investigated in associative fear learning in Objective 3. Therefore, these two objectives were merged.

Objective 3. We aimed to uncover the role of the basal forebrain cholinergic input in the amygdala in fear learning. First, we investigated the distribution of cholinergic cells in the basal forebrain that innervate the amygdala region. Unexpectedly, we observed that there are at least three groups of cholinergic neurons located in distinct areas within the basal forebrain, giving rise to projections into largely non-overlapping regions in the basolateral amygdala complex.

These results were presented on a poster at the FENS meeting in Berlin (Barabas et al., 2018). Based on these results, we have started a collaboration with Dr. Andrew Holmes's lab (NIH, USA) to uncover the role of distinct cholinergic afferents in amygdala-related processes. As in Jiang et al., 2016 (a paper that was published after funding our project proposal), the role of cholinergic afferents in fear learning has been revealed, in our collaborative on-going work with Dr. Holmes' group, we focus on the function of these subcortical inputs in extinction learning.

To uncover the circuit mechanisms underlying the cholinergic impacts on amygdala network operation, we next examined the postsynaptic effects of neurotransmitter release from cholinergic fibers activated optogenetically in slice preparations. Here, we studied the cholinergic effects onto GABAergic cells within the circuits of the amygdala, as inhibitory neurons play a critical role in associative fear learning (Hájos, 2021). We found that neurogliaform cells, axo-axonic cells and VIP-expressing interneurons received strong inward currents upon blue light stimulation of ChR2-expressing cholinergic afferents. However, the inward currents recorded in these interneuron types showed distinct pharmacology, as different nicotinic receptor antagonists blocked the light-evoked postsynaptic responses. These results have been presented at the Conference of the Hungarian Neuroscience Society in Debrecen (Magyar et al., 2019). We plan to combine these data with our previous observations obtained by studying the postsynaptic responses of basal forebrain GABAergic afferents in amygdalar neurons in a separate manuscript.

As in other cortical regions it has been described, VIP-expressing interneurons may disinhibit pyramidal cells when a salience stimulus carried e.g. by cholinergic afferents reaches amygdalar networks (Hangya et al., 2015). Importantly, a temporal disinhibition may be a critical prerequisite for associative learning. Our in vitro data obtained by optogenetics indeed show that VIP-expressing interneurons in the amygdala can be excited via cholinergic inputs (see above). If the circuit motif of a disinhibitory network is a general principle in cortical networks, then VIP-expressing interneurons in the amygdala should also preferentially, if not exclusively target other GABAergic cells, an assumption that has not been tested. In a collaborative study with Prof. Francesco Ferraguti, we characterized the VIP-expressing interneurons in the basolateral amygdala complex and found that the vast majority of these GABAergic cells indeed target other GABAergic cells (Rhomberg et al., 2018). These results strongly imply that one of

the key circuit mechanisms that can play a role during fear learning can be the cholinergic activation of a disinhibitory circuit composed of VIP-expressing interneurons in the amygdala. This prediction has been fully supported by a subsequent study from Prof. Andreas Lüthi's group (Krabbe et al., 2019).

Linked to the research topic of this grant, we also examined the potential role of cholecystokinin (CCK)-expressing interneurons in fear learning. The function of this enigmatic interneuron group is unclear in cognitive processes. In collaboration with Dr. Andrew Holmes's group, our investigations uncovered that CCK-expressing interneurons in the basolateral amygdala display a surprising diversity, as at least 4 distinct types of GABAergic cells can be targeted under the control of CCK promoter using a genetic approach. Activation of these GABAergic cells led to a marked facilitation of extinction learning. These results imply that extinction learning is under a tight control of inhibitory cells at the amygdala level. Our observations were published in Rovira-Esteban et al., 2019.

In addition, we began to examine the firing properties of distinct neurons in the lateral and basal amygdala nuclei upon noxious stimulation using in vivo electrophysiological recordings. The aim of this study was to identify the circuit elements that receive a direct and fast excitation upon presentation of unconditioned stimuli, e.g. electrical shocks, which serve as a teaching signal in associative fear learning. The current model of amygdala function states that inputs carrying noxious information reach the lateral nucleus of the amygdala where the association between the conditioned and unconditioned stimuli takes place during fear conditioning. This model predicts that upon noxious stimulation, neurons in the lateral amygdala should fire first, with a shorter latency in comparison to neurons located in the basal amygdala. In contrast to this prediction, we observed by means of in vivo electrophysiological recordings that in both amygdala nuclei there is a substantial portion of neurons, which spike with a similarly short latency (~ 20-30 ms) upon delivery of electrical shocks. These results, presented at the Conference of the Hungarian Neuroscience Society in Debrecen (Magyar et al., 2019), argue for the revision of the current model. As the main question of our project is to identify the source(s) of the teaching signals received by the amygdala circuits during associative learning, we have attempted to reveal the projection(s) that transmit noxious information into the basolateral amygdala. Our preliminary findings show that projections from the posterior thalamic

nuclei are in the positon to carry aversive information into the lateral amygdala. At present, it is not clear, which projection delivers noxious information into the basal amygdala, but this question will be in the focus in the next years, outreaching the period of the funding. We plan to continue this work to uncover the circuit mechanisms underlying the short-latency excitation of basal amygdala neurons upon noxious stimulation.

Many recent studies have showed that inhibitory neurons control associative fear learning and other amygdala-linked neural processes both in normal and pathological conditions. However, there is a significant gap in our knowledge as, until now, it was unknown the number and types of GABAergic cells in the basolateral amygdala. Therefore, we put a serious effort to determine these fundamental circuit parameters in the lateral and basal nuclei. Using unbiased stereology, we determined that the number of GABAergic cells in the basal nucleus (22%) is significantly larger than in the lateral nucleus (16%). Furthermore, using immunocytochemistry combined with viral strategy in transgenic mice, we revealed the ratio of the six cardinal GABAergic cell types in these two amygdala nuclei. We estimated that the following cell types together compose the vast majority of GABAergic cells in the lateral and basal amygdala: axo-axonic cells (5.5-6 %), basket cells expressing parvalbumin (17-20 %) or cholecystokinin (7-9 %), dendritic inhibitory cells expressing somatostatin (10-16 %), NPY-containing neurogliaform cells (14-15 %), VIP and/or calretinin-expressing interneuron-selective interneurons (29-38 %) and GABAergic projection neurons (5.5-8 %). Our results show that these amygdalar nuclei contain all major GABAergic neuron types with a similar ratio as found in other cortical regions. Furthermore, our data offer a basic reference for future studies aiming to reveal changes in GABAergic cell number and in inhibitory cell types typically observed under different pathological conditions, and to model functioning amygdalar networks in health and disease. The results of these experiments have been recently published (Vereczki and Muller et al., 2021).

Furthermore, upon invitation, I summarized our current knowledge about inhibitory cells and their circuits with the basolateral amygdala in a review (Hájos, 2021).

During the funded period, two papers tightly related to the aims of the project were published. Furthermore, two other papers though they link only loosely to the current project were very recently accepted. Moreover, three additional manuscripts with topics tightly linked to the objectives are planned to be finalized and submit for review within the next 2 years. Therefore,

upon publication of these yet unpublished studies we plan to request a re-evaluation of our achievements in the close future.