Neuronal Circuits in the Cholinergic Basal Forebrain, Amygdala, and Medial Prefrontal Cortex Contributing to Noxious Stimulus Processing

PhD thesis

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1. Introduction

Recognizing threats is essential for animal survival. Therefore, it is not surprising that many brain circuits contribute to the detection of dangerous situations, leading to the elevation of attention and promoting affective brain state linked to the threat. This danger-triggered mental state helps generate the most appropriate behavioral responses, aiming to avoid, or at least, reduce any potential harm as well as forming memory that can guide future avoidance of similar threats. The complex brain processes involved in recognizing and responding to threat are regulated at different levels in the nervous system. Top-down control is provided by cortical circuits located in the basolateral amygdala (BLA) and medial prefrontal cortex (mPFC). These two structures can be parceled based on their connectivity and role playing e.g., in the control of fear and negative emotional states.

As a general principle, cortical function is efficiently and rapidly affected by subcortical inputs in a brain state-dependent manner. One of the subcortical afferent systems contributing critically to cortical network operation originates from the basal forebrain (BF), a heterogeneous structure located in the medial-ventral part of the brain. In this brain region, there are three neuron types: cholinergic, GABAergic, and glutamatergic cells that are known to project to cortical areas. Interestingly, cholinergic cells in the nucleus basalis of Meynert of the BF have been proposed to project to cortical regions, e.g., to frontal and posterior cortical areas, that are functionally interconnected with each other. These results suggest that BF cholinergic inputs may orchestrate activity in functionally related cortical areas, promoting interaction between regions and ultimately, enhancing neural computation. Whether the mPFC and BLA networks can be simultaneously or differentially regulated by cholinergic afferents conveying salient information has yet to be determined.

Furthermore, the cholinergic system also plays a pivotal role in shaping cortical network operation in various conditions, including salient information transmission and processing. By modulating neuronal excitability, acetylcholine (ACh) influences arousal, attention, and sensory computation. Acting on nicotinic and muscarinic receptors found on both excitatory pyramidal cells and inhibitory interneurons, it enhances signal-to-noise ratios, sharpens sensory discrimination, and supports synchronous neuronal activities, including theta and gamma oscillations. Its ability to facilitate disinhibition and plasticity further proves its importance in adapting cortical activity to environmental cues, suggesting that cholinergic afferents could dynamically regulate cortical network operations across different behavioral states. Importantly, cholinergic neurons in the BF have been shown to rapidly convey salient information, including noxious signals to the cortical structures, showing their critical contribution to painful stimulus processing.

2. Objectives

In this thesis we aimed to reveal the structural basis of BF cholinergic control over the interconnected BLA-mPFC fear state -regulation circuits. In addition, we studied the effects of salient/ noxious stimulation on the activity in BF cholinergic cells and spiking dynamics in frontal cortical neurons with a focus on interneuron firing. To address this, we asked the following questions:

1) What are the projection patterns of cholinergic neurons originating from the HDB and VP/SI?

2) Do specific subregions of the amygdala and mPFC receive distinct cholinergic projections from different BF nuclei?

3) Can individual cholinergic neurons simultaneously innervate both the amygdala and mPFC?

4) Are cholinergic neurons in the HDB and VP/SI activated during noxious stimulation?

5) How do specific types of cortical inhibitory interneurons respond to noxious stimulation, and is BF cholinergic input necessary for shaping their activity?

Our results advance our understanding of how painful or noxious stimuli alters cortical network dynamics by linking cholinergic signaling to cell-type-specific interneuron activity, bridging a critical gap in salient information processing.

3. Methods

To address our research questions, we employed a combination of anatomical tracing, in vivo calcium signal detection (fiber photometry), electrophysiological recordings, and behavioral assays in transgenic mouse models.

Animals:

Experiments were performed on adult mice expressing Cre recombinase in cholinergic (ChAT-Cre) and interneuron subtype-specific lines (e.g., PV-Cre, SST-Cre, VIP-Cre). Animals were maintained under standard laboratory conditions.

Tracing and Immunohistochemistry:

Cre-dependent viral vectors and retrograde tracers were used to label cholinergic projections from the HDB and VP/SI to the mPFC and BLA. Immunostaining protocols targeted markers such as ChAT, VGLUT3, and GABA to define neurochemical identity.

Fiber Photometry:

Optic fibers were implanted in the HDB and VP/SI. Population calcium activity in cholinergic neurons was recorded in response to noxious electrical stimuli.

In Vivo Juxtacellular Recordings:

We performed visually guided juxtacellular recordings from genetically identified interneurons in the M2 cortex during noxious stimulation. Neurobiotin labeling and immunostaining were used for post hoc identification.

Behavioral Assay:

Pavlovian fear conditioning was used to assess defensive behavior. The freezing response was automatically quantified in response to conditioned and unconditioned stimuli.

Data Analysis:

Statistical analysis was performed using appropriate parametric or non-parametric tests. Significance was accepted at $p < 0.05$. Data visualization was carried out using standard scientific graphing tools.

4. Results

4.1 Cholinergic innervation of the amygdala region by two basal forebrain areas, the HDB and VP/SI.

First, we aimed to investigate the overall cholinergic innervation of the amygdala region given rise by the HDB and VP/SI. To this end, we injected a high volume of AAV5.Ef1a.DIO.eYFP, a Cre-dependent adeno-associated virus vector (AAV) into the HDB and VP/SI of ChAT-Cre mice. After analyzing the cholinergic projection patterns in multiple coronal planes within the amygdala region, we found that the BA received the strongest cholinergic innervation – based on normalized fluorescence intensity – from these two BF areas. Cholinergic innervation extended also to the BMA, the medial division of the CeM, the anterior- and posteromedial cortical amygdaloid nucleus (ACo, PMCo), the anterolateral portion of the amygdalohippocampal area (AHiAL), and the piriform cortex (Pir) with comparable innervation levels among them. Conversely, the anterior and posterior sections of the medial amygdala (MeA, MeP), the lateral nucleus of the LA, the lateral division of the central amygdala (CeL), and the posteromedial part of the amygdalohippocampal area (AHiPM) received only sparse cholinergic innervation. Taken together, these findings demonstrate that the amygdala region, particularly the BA and BMA, receives robust cholinergic innervation from the HDB and VP/SI.

4.2 Localization of BLA-projecting cholinergic neurons shows separation within the BF.

To distinguish the sources of cholinergic innervation invading the BLA between the VP/SI and HDB, retrograde tracer Fast Blue (FB) or

Cholera Toxin B subunit (CTB) was injected into the three distinct nuclei of the BLA: LA, BA and BMA. In coronal sections obtained from these injected mice, we determined the location of the somata of BLA-projecting neurons in the BF. We found that BA-projecting BF neurons were located primarily in the VP/SI region. In contrast, BMA-projecting BF neurons were found dominantly in the HDB (but also partially in the lateral preoptic area (LPO), median preoptic nucleus (MnPO), medial preoptic area (MPA)), forming a separate neuronal population, the location of which overlapped minimally with BA-projecting BF neurons. Injecting a retrograde tracer into the LA resulted in only a small number of labeled neurons located in the HDB, a finding consistent with the low number cholinergic fibers observed in anterograde tracing. After performing immunostaining against choline acetyltransferase (ChAT), the enzyme, responsible for the acetylcholine synthesis, we evaluated the cholinergic content of the amygdala-projecting BF neurons. We found that more than 60% of BA-projecting BF neurons were cholinergic, while this ratio was around 25% among BMA-projecting BF neurons. Altogether, these results show that neurons in the VP/SI preferentially innervate the BA, while those neurons located in the HDB project predominantly to the BMA and to a lesser extent to the LA. The difference in the ratio of BF cholinergic neurons innervating the distinct amygdala nuclei is in accord with our anterograde labeling, showing a more profound presence of cholinergic fibers in the BA in comparison with the surrounding areas.

4.3 Cholinergic cells from the HDB and VP/SI project to the amygdala region in a mutually exclusive manner.

To confirm that cholinergic neurons in separate BF regions innervate different amygdala nuclei, we injected a small amount (30 nl) of AAV8.CAG.Flex.GFP either into the HDB or into the VP/SI of ChAT-Cre mice. This approach enabled us to specifically investigate the cholinergic axonal projections originated from the HDB and VP/SI within the amygdala region. In line with our retrograde tracing data, we observed that ChAT+ neurons in the HDB projected mostly to the BMA and other surrounding areas, such as the MeA, ACo, dorsal endopiriform nucleus (DEn), Pir, but largely avoiding the BA. Although, there were some labeled axons in the LA, this connection appeared to be weak compared to other neighboring fields, an observation, which is in accord with our previous findings. On the other hand, ChAT+ neurons in the VP/SI almost exclusively innervated the BA, with marginal projections into the CeM, BMA and Pir. These results show that cholinergic fibers from the HDB and VP/SI parcel the amygdala region in a mutually exclusive manner.

4.4 Cholinergic inputs from the HDB and VP/SI overlap in the mPFC, with VP/SI showing a preference for dorsal and HDB for ventral innervation of the PFC.

Previous studies uncovered that the mPFC and BLA are reciprocally interconnected cortical regions playing a role in similar cognitive processes. Therefore, we analyzed the cholinergic projections from the HDB and VP/SI towards the PFC in the same mice as we did for the innervation of the amygdala region. Upon analyzing the cholinergic projection patterns towards the PFC, we observed that cholinergic fibers covered the cingulate cortex area 1 and 2 (Cg1, Cg2), as well as the PL and IL cortices and the medial orbital cortex (MO) in a similar manner. Additionally, these fibers exhibited a slightly weaker

innervation to the secondary motor cortex (M2). Based on small injections targeted separately into the HDB or VP/SI, we found that both BF areas projected to the mPFC, namely the ACC/Cg1, PL and IL cortices. However, the HDB tended to project to the vPFC, including the MO, lateral orbital cortex (LO), ventral orbital cortex (VO), dorsal peduncular cortex (DP), Cg2, while the VP/SI innervated the dPFC (including the M2, Cg1 and PL). The most conspicuous differences were found at the level of 2.4 mm from the bregma, where the HDB predominantly innervated the MO, while the VP/SI had prominent projections to the PL, Cg1 and M2. At the bregma level of 1.1 mm, there was a similar exclusive projection pattern: cholinergic cells in the HDB innervated the Cg2, but not the M2, while cholinergic cells in the VP/SI gave rise to projections to the M2, but not to the Cg2. Altogether, these results show that cholinergic innervation originated from the HDB and VP/SI terminates in the differential parts of the PFC and amygdala regions. Namely, ChAT+ neurons in the HDB send axons to the vPFC and mPFC as well as to the most amygdala areas, apart from the BA, while ChAT+ neurons in the VP/SI prefer to terminate in the dPFC, mPFC and BA.

4.5 Significant portion of BF cholinergic neurons exhibit dual projections to both the mPFC and BLA.

The next question we asked was whether the same BF neurons send axonal collaterals into the mPFC and BLA, or separate populations of neurons innervate these two brain structures. To reveal the logic underlying the cholinergic control of these regions, first we performed retrograde-anterograde virus tracing in ChAT-Cre mice by injecting AAV5.Ef1a.DIO.eYFP into the mPFC. This approach reveals whether neurons projecting to a given area have axonal collaterals in other brain regions, too. Thus, AAV injection into the mPFC may visualize axons in the BLA if a portion of BF cholinergic neurons

simultaneously project to these regions. Our retrograde-anterograde virus tracing demonstrated the presence of ChAT+ neurons in the BF that innervate both the mPFC and BLA. The somata of retrogradely labeled ChAT+ cells were found in both the HDB and VP/SI along the BF, revealing the area where the dual projecting cholinergic neurons were located. Based on overlaid images, extracted from 5 animals, we found that BA received a substantial cholinergic innervation from dual projecting neurons, with the strongest innervation found at the level of -1.5 mm from the bregma. BMA also received axon collaterals from the dual projection, while there were barely any fibers in the LA. Our results provide evidence that the mPFC-BLA circuit receives dual cholinergic innervation from both the HDB and VP/SI.

To further validate the observation that cholinergic neurons simultaneously innervate the mPFC and BLA, we injected different retrograde tracers (FG and FB) into the mPFC and BA, areas receiving the strongest innervation from cholinergic cells with dual projections. In line with our previous tracing results, retrogradely labeled neurons from the mPFC were located both in HDB and VP/SI, while BA-projecting cholinergic cells were mostly found in the VP/SI. Importantly, we identified a population of dual-projecting cells in the VP/SI, accounting for 30.07±12.5% of the retrogradely labeled cells, the vast majority of which (92.7%) was immunopositive for ChAT, leaving a small portion (2.2±1.2%) of dual-projecting cells immunonegative for ChAT. These results confirm that BF cholinergic cells can regulate the interaction between the mPFC and BLA simultaneously and/or independently.

4.6 Cholinergic cells in the HDB and VP/SI show increased activation upon noxious stimulation

After investigating the projection characteristics and neurochemical properties of BF cholinergic cells, next we wanted to see if these cell groups have a role in the transmission of noxious signals towards the amygdala and cortical areas. To this end, we used fiber photometry, a technique which involves using optical fibers to deliver light into brain regions where neurons express fluorescent indicators suitable to monitor changes in intracellular Ca^{2+} concentrations. These indicators emit fluorescence signals when neurons are active, proxied by Ca^{2+} elevation, allowing researchers to measure and analyze neural activity by detecting changes in light collected through the optic fiber. In our experiments ChAT-cre mice were injected with AAV2/5.CAG.Flex.GcaMP6f virus, then an optical fiber was implanted 100 μm above the HDB or VP/SI. After the recovery period, mice received mild electrical shocks, while fluorescent signals in cholinergic cells were measured. After analyzing the results, we observed that cholinergic cells were activated upon noxious stimulation. These findings, consistent with earlier reports, indicate that BF cholinergic neurons are strongly activated by foot shocks, highlighting their important role in processing salient stimuli.

4.7 Firing in perisomatic region targeting inhibitory neurons upon noxious stimuli

To obtain recordings in the different types of interneurons, we used *in vivo* visually guided juxtacellular recordings in transgenic mice expressing fluorescent proteins in defined GABAergic cells. First, we wanted to understand how the three types of inhibitory cells giving rise to perisomatic inhibition - PVBCs, AACs, and CCKCB1BCs - react to noxious stimulation.

PVBCs were measured in the offspring of Pvalb-IRES-Cre x CAG-LSL-ZsGreen1 mice. We found two populations of PVBCs based on their responses to noxious stimulation. Type 1 PVBCs (60.35 %) showed prolonged inhibition, lasting for 2-4 seconds upon electrical stimulation, while Type 2 PVBCs (39.65%) had a more complex response, starting with a fast-short inhibition, then the activity of the cells returned to baseline (302.62 ± 89 ms), with a portion of the cells reaching higher activation, followed by a decrease in activity lasting for 1-2 seconds.

AACs were measured in the offspring of Nkx2.1-CreER x LSL-Flpo mice. The Nkx2.1 marker is commonly used to identify certain subtypes of inhibitory interneurons, particularly in the cortex. Nkx2.1 is a transcription factor that is crucial for the development of several cell types, including specific interneurons derived from the medial ganglionic eminence (MGE) during embryonic development. The genetical construction of Nkx2.1-CreER x LSL-Flpo allowed the tamoxifen inducible expression of Flpo in interneurons in a specific time window (P0), when development/migration of a group of AACs starts. In P60, mice were injected with AAV5.EF1a.eYFP.fDIO to the frontal cortex (M2) to trigger Flpo dependent eYFP expression in AACs. These neurons showed a fast (391.08± 359 ms), strong and long-lasting spike increase (2-4 s) upon stimulation.

CCKCB1BCs were sampled in both BAC-CCK-dsRed and the offspring of BAC-VGLUT3-iCre x BAC-CCK/gfp-coIN_sb mice. Overall, identified CCKBCs were immunopositive for CB1 recorded in both types of mice and these cells showed delayed activation, starting at 1 second after noxious stimulation and lasting for 4-5 seconds.

4.8 Dendrite-targeting and Interneuron selective interneurons

Next, we examined two other groups of interneurons, 1) interneurons which are responsible for dendritic inhibition, and 2) VIP ISIs. In the first group, we measured the activity of NPY-positive NGFCs (NPY/NGFCs) and SST INs. NPY/NGFCs cells were sampled in the offspring of NPY-IRES-Cre x BAC-CCK/gfp-coIN_sb and NPY-IRES-Cre x CAG-LSL-ZsGreen1. Among all the interneurons these cells fired with the shortest latency upon noxious stimulation (262.42± 97 ms). Interestingly, SST INs recorded in SST-Cre mice injected with AAV5.EF1a.DIO.eGFP were typically silent and discharged no spikes upon electrical stimulation.

A subset of ISIs in cortical structures expresses VIP and preferentially inhibits other interneuron types. Here we found three functionally different types of VIP ISIs, showing distinct activation patterns to electrical stimuli. In the offspring of VIP-IRES-Cre x BAC-CCK/gfp-coIN_sb, VIP/CCK ISIs (Type 1 VIP ISIs) were activated with a short latency but slightly later than NPY/NGFCs (291.02± 105 ms). Using Nkx2.1-CreER x LSL-Flpo mouse line, we discovered that if the tamoxifen is administered at P1 or P2, most virus infected cells will be, instead of AACs, VIP ISIs, confirmed by morphological features and immunostaining against VIP. This allowed us to investigate another group of VIP ISIs, VIP/Nkx2.1 ISIs (Type 2 VIP ISIs), which to our surprise exhibited a different activation pattern to stimulation in comparison to VIP/CCK ISIs. All VIP/Nkx2.1 ISIs responded with a long-lasting activation upon electrical shocks, but the start of their activation varied, contrary to other groups. Some of the cells were activated immediately -, while the majority of the cells peaked their spiking at 2 seconds after shocks, reaching an average peak amplitude between 2.5-3 seconds. Lastly, we measured the response of VIP ISIs

in ChAT-Cre mice. Based on our immunolabeling at least 80% of ChAT neurons were positive for VIP in the frontal cortex. These VIP/ChAT ISIs (Type 3 VIP ISIs) had a low baseline firing rate and no observable response to noxious stimulation.

5. Conclusions

Our study provides a detailed characterization of cholinergic innervation patterns in the mPFC and BLA, revealing two parallel cholinergic pathways that may allow independent as well as simultaneous regulation of these interconnected cortical structures. The presence of dual-projecting cholinergic neurons suggests a structural arrangement optimized for coordinated neuromodulatory influence over circuits involved in fear, reward processing, and social behaviors.

In addition, our research sheds light on how noxious stimuli engage cholinergic circuits to influence cortical network dynamics. We identified distinct activation patterns across multiple interneuron types, expanding current models of cortical processing during aversive experiences. Notably, our findings challenge prior assumptions about homogenous VIP interneuron populations by identifying three genetically distinct subtypes with different activation profiles upon noxious stimulation. Pharmacological interventions demonstrated that both cholinergic and glutamatergic transmission can play critical roles in modulating the firing in VIP ISIs, suggesting a complex interplay of subcortical influences on cortical inhibition and excitation.

Overall, our findings advance the understanding of BF cholinergic function, revealing the structural basis of regulating cortical and amygdala circuits involved in emotional and sensory processing. This work provides a foundation for future research into the functional implications of cholinergic co-transmission and its relevance to neuropsychiatric conditions involving altered cholinergic signaling, such as anxiety, depression, and neurodegenerative diseases.

6. Bibliography of the candidate's publications

Publications related to this thesis

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Other publication

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