Organization and functional effects of perisomatic inhibition on principal cell activity in the basolateral amygdala

Ph.D. thesis

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Budapest

2016

I. INTRODUCTION

Information processing in cortical networks relies on the precise, spatiotemporally coordinated interaction of billions of neurons. The accurate activity of neurons is ensured and controlled by various mechanisms at subcellular, cellular, synaptic and network levels. At network levels, the intricate wiring and precisely orchestrated excitatory and inhibitory interactions between the components are crucial for proper cortical functions, and even seemingly slight changes in the connections can lead to severe pathological states like epileptic discharges or schizophrenia. Unravelling the basic principles of connectivity patterns and the effects of different connections on the activity of the network components is indispensable for understanding cortical information processing in health and disease. The basolateral amygdala (BLA) belongs structurally and developmentally to cortical areas and this region is known to play a critical role in fear and extinction memory learning. Examination of the network build-up and the interactions between the elements in this region therefore can contribute to understanding of information processing and learning mechanisms in cortical areas.

Cortical ensembles consist of two main neuronal cell types: 80-90% of the neurons are excitatory principal cells (PCs) and 10-20% are inhibitory interneurons (INs). Local inhibitory circuits in the amygdala have been shown to play major roles in controlling the acquisition, expression and extinction of fear memories. Among the different IN types, perisomatic region targeting interneurons (PTIs) have received special attention in the last decades. This cell type predominantly innervates the cell body, proximal dendrites and axon initial segment (AIS) of excitatory neurons - membrane domains of PCs where the inputs are finally integrated and the action potentials are generated. Therefore, these interneurons are in the critical position to powerfully regulate the output of individual PCs as well as to synchronize the spiking activity of large neural ensembles, and can have a major influence on the output of the BLA. PTIs can be further subdivided into axo-axonic cells (AAC) and at least two types of basket cells (BC). AACs are specialized to innervate the AIS of PCs by multiple synapses, whereas BCs predominantly target the soma and proximal dendrites of PCs and also other INs. BCs can be separated into two groups based on their neurochemical content: one type expresses parvalbumin (PVBC), whereas the other expresses cholecystokinin (CCK) and type 1 cannabinoid

receptors (CCK/CB_1BC). There is growing evidence supporting the concept that in cortical areas the two type of basket cells have distinct inhibitory roles during network activities: PVBCs can effectively synchronize PC activity generating rhythmic patterns, while CCK/CB_1BCs appear to have a modulatory effect on network dynamics conveying information from subcortical areas about emotional, motivational and general physiological states. In the BLA only few data is available on the contribution of the different type of INs to network activities. However, it has been proved that the activity of BLA PV+ cells is indispensable for fear memory formation, indicating a crucial role for this cell types in information processing. Despite the recognition of the importance of PTIs in the BLA it is still uncertain how effective the regulation of PC firing by single INs is, how they can influence the timing of PC firing and how many presynaptic INs have to discharge synchronously to veto PC action potential generation. Moreover, the basic morphological features of individual IN-PC connections, and the convergence and divergence of INs on the PC population are also unknown. Investigation of how these cell types are affiliated in the local network and their effect on the output of the BLA can help us

to understand the mechanisms of fear memory processes in health and disease.

II. OBJECTIVES

The main goal of our studies was to investigate the organization and impact of the perisomatic inputs of BLA PCs. Therefore, we focused on three main topics:

I. Uncovering the density and source of the perisomatic inputs, and the ratio of the innervation emerging from different cell types.

II. Uncovering the electrophysiological and anatomical properties of the AAC input on the AIS and its effect on PC activity.

III. Comparing the electrophysiological and anatomical properties of the input of PCs emerging from the two major types of basket cells and their effects on PC activity.

III. METHODS

Electrophysiology experiments

All experiments were approved by the Committee for the Scientific Ethics of Animal Research (22.1/360/3/2011) and were carried out according to the guidelines of the institutional ethical code and the Hungarian Act of Animal Care and Experimentation (1998. XXVIII. section 243/1998, renewed in 40/2013.). Transgenic mice of both sex (P18-24) expressing enhanced green fluorescent protein (eGFP) under the control of the PV promoter (Meyer et al., 2002) or expressing red fluorescent protein under the control of cholecystokinin (CCK) promoter (BAC-CCK-DsRed) were used. Horizontal amygdalar slices (200 μm) were prepared after decapitation under isoflurane anesthesia. Slices were kept in an interface type chamber for at least one hour before recordings, which were carried out in a submerged type chamber in artificial cerebrospinal fluid at 32^{o}C. For whole cell recordings K-gluconate based intrapipette solution was used with 4 mM [Cl^-], and biocytin or Alexa488 dye for cell labelling. Presynaptic INs were held in current clamp at -65 mV and action potentials were evoked with brief square current pulses (2 ms, 1.5–2 nA). Postsynaptic responses were monitored in voltage clamp mode

at -45 mV or in current clamp mode at -55 mV. For perforated patch recordings 100 mg/ml gramicidin was used. After recordings, slices were fixed overnight in 4 % paraformaldehyde (PFA), then biocytin and Alexa488 content of the cells were revealed with fluorescent immunocytochemistry. Morphological properties of the recorded cells were analyzed with confocal microscope, and several pairs were further processed for electronmicroscopic validation of the synaptic contacts.

Anatomical experiments

C57Bl/6J mice were deeply anaesthetized and transcardially perfused with 2% PFA in 0.2 M Na-acetate buffer (pH 6.0) or with 2.5% acrolein with 4% PFA in 0.1 M phosphate buffer (pH 6.8). To assess the inhibitory inputs of PCs, we carried out immunostainings using guinea pig anti-vesicular ɣ-amino butyric acid transporter (VGAT) IgG (Frontier Institute Co.Ltd 1:1000) and goat anti- glutamic acid decarboxylase, both isoforms (panGAD) IgG (Frontier Institute Co.Ltd 1:500) primary antibodies, visualized by Cy3 (donkey anti-guinea pig and donkey anti-goat, 1:200). To label PC perisomatic region mouse anti-Kv2.1 (1: 1000, 75-014, Neuromab) was used, and visualized with an A488-conjugated donkey anti-mouse

antibody. To label AISs mouse anti-ankyrin G IgG (Santa Cruz 1:200) or rabbit anti-Nav 1.6 (1:500, Alomone Labs) was used. To analyze the source of the inhibitory inputs slices were incubated in a mixture of primary antisera of rabbit anti-CB_1 (1:1000, Cayman Chemical Company, Ann Arbor, MI), guinea pig anti-VGAT (1:1000, Frontier Institute Co. Ltd.,), goat anti-PV (1:5000) visualized by DyLight 405-conjugated donkey anti-rabbit (1:500, Invitrogen, Carlsbad, CA), Alexa 488-conjugated donkey anti-guinea pig (1:500, Invitrogen) and Alexa 649-conjugated donkey anti-goat (1:500, Invitrogen) secondary antibodies. Confocal images were collected using a Nikon A1R microscope fitted with an oil immersion apochromatic lens (N.A.: 1.4, z step size: 0.13 μm, xy: 0.06 μm/pixel), colocalization studies and reconstruction of the cells were carried out using the Nikon Imaging System and Neurolucida 10.53 software.

IV. RESULTS

Part I: Organization of perisomatic inhibitory inputs

The perisomatic region of cortical PCs consists the cell body, the AIS and the proximal dendrites, however, its border

along the dendrites is not defined yet. A functional approach to determine the spatial extent of the perisomatic region of amygdalar PCs is to reveal the amount and ratio of excitatory and inhibitory inputs along the proximal dendritic branches depending on the distance from the soma. By reconstructing the dendritic spines of PCs and the density of GABAergic inputs along the dendrites we found that the extent of the perisomatic region along the individual dendrites of amygdalar PCs shows variability (5-50 μm), which can be predicted by the diameter of the dendrites at their somatic origin. We also found that the soma and proximal dendrites belonging to the perisomatic region of BLA PCs can be visualized by immunostaining against the type 2.1 voltage gated potassium channel (Kv2.1).

By reconstructing immunolabeled GABAergic terminals we found that BLA PCs receive ~53 inhibitory inputs on the AIS, ~160 on the soma and ~70 on proximal dendrites belonging to the perisomatic region. The GABAergic input of the AIS on the first 10 μm derives from PVBCs, which constitutes 6-7% of the input of the AIS, whereas the vast majority of the inhibitory terminals arrives from AACs covering the first 60 μm of the axon. We found that the majority of GABAergic input on the soma and proximal

dendrites of PCs emerges from PVBCs (~40%) and CCK/CB_1BCs (~27%). By analyzing the target distribution of *in vitro* filled INs, we found that ~50% of the terminals of both cell types innervate the somata and proximal dendrites belonging to the perisomatic region, defining these cells as basket cells. Moreover, we found that individual PVBCs innervate the perisomatic region of single PCs with more terminals than CCK/CB_1BCs (5.8 vs. 3.9, respectively), but similar number of BCs from both types converges on a PC (16-17 CCK/CB_1BCs and 15-16 PVBC). In the next parts of the study, we investigated the electrophysiological and morphological properties of the connections between PTIs and PCs in details and the functional effects of these INs on PC spiking.

Part II: Electrophysiological and morphological properties of the output synapses of AACs

First, we aimed to reveal the synaptic effects of AACs onto PCs without disturbing the ionic milieu. Therefore, we carried out paired recordings, when the postsynaptic PC was recorded in perforated patch mode using gramicidin in the recording pipette. By measuring the reversal potential of the

evoked postsynaptic responses and comparing it to the resting membrane potential of the PC, we found that AACs inhibit PCs in the BLA. In the next set of experiments, we explored how inhibitory input from an AAC can control action potential generation in a postsynaptic PC. In paired recordings we found that AACs can efficiently inhibit PC firing (~80% reduction in firing probability) or postpone the spike generation with up to 30 ms. To get deeper insights into the properties of synaptic organization underlying the potent inhibition between AACs and PCs, we first determined the number of putative synaptic sites between the recorded pairs using multichannel high resolution confocal microscopy. We found that individual AACs formed ~8.4 contacts on their postsynaptic partner on average, and that those cells, which contacted their partner with more synapses, could inhibit the action potential generation more efficiently. Our results showed that synchronous activity of 2-3 AACs is sufficient to completely block spike generation in PCs. By analyzing the distribution of the contacts along the AIS we found that AACs preferentially innervate the region between 20 and 40 μm from the soma, regardless of the number of the contacts they establish. To compare the innervation pattern of AACs to the site of action potential generation, we carried out dual soma and axon recordings to

measure the spike generation zone along the AIS. We found that the site of the action potential generation closely matches the area preferentially targeted by AACs, which indicates that AAC output synapses are optimized to control Na^+ channel opening and thus PC firing in the BLA.

Part III: Comparison of the physiological and morphological properties of the output synapses of CCK/CB1BCs and PVBCs

In the next part of the study we extended our investigations to the other two interneuron types targeting the perisomatic region of PCs and compared the electrophysiological and morphological properties of the inhibition provided by CCK/CB_1BCs and PVBCs in the BLA. With IN-PC paired recordings we found that the basic kinetic properties of the inhibitory connections originating from CCK/CB_1BCs and PVBCs are similar and have the same potency to control postsynaptic PC spiking. By testing the capability of these INs to inhibit or postpone PC firing we found that both cell types could inhibit PC spiking with 75% probability, or postpone the firing with up to 38 ms. These results showed that inhibitory inputs from CCK/CB_1BCs and

PVBCs have the same powerful potential to veto or control the timing of PC spiking.

Immunofluorescent labeling of the recorded pairs enabled the detailed examination of the potential contact sites along the entire somato-dendritic surface of the postsynaptic PCs and therefore the comparison of the morphological features of the connections established by the two basket cell types. We found that the number of the contacts was very variable in both IN types, ranging from 1 to 25 and targeting both the perisomatic region and more distal dendritic compartments as well. PVBCs and CCK/CB_1BCs innervated their postsynaptic partners with similar number of boutons, and the average distance of the innervation along the somato-dendritic tree and the number of the contacts established on the perisomatic region was not different either. Importantly, we found that the most determining factor of the inhibitory efficacy was the number of the contacts established on the perisomatic region of PCs. These results showed that CCK/CB_1BCs and PVBCs innervate their postsynaptic partners with similar pattern, targeting somatic, proximal- and distal dendritic compartments with multiple synapses.

The analysis of the innervation pattern of both types of perisomatic INs on single postsynaptic PCs showed that some

cells tend to target the soma and proximal dendrites, while others target predominantly the dendritic segment. This raises the question whether there are INs, which can be classified as classical basket cell, innervating predominantly the soma-near region of their postsynaptic partner in most cases, and accordingly, other cells might be classified as dendrite-targeting interneurons innervating mostly the dendritic shafts of PCs. Or, alternatively, one IN innervates some of their partners mainly perisomatically, whereas other PCs receive inputs from the same cell mainly on the dendrites. To address this question, we analyzed the target distribution of single labeled INs on the whole somato-dendritic surface of three labeled postsynaptic PCs with the same method as used in the paired recordings or in multiple Kv2.1 labelled PCs. We found that the number of the perisomatic contacts from individual INs was very variable on their different postsynaptic partners. Therefore, it is not possible to determine the characteristic innervation pattern of a given IN based on the bouton distribution on a single postsynaptic partner, rather investigation at the population level is needed. In addition, these observations strengthen our previous notion that both types of INs expressing PV or CCK/CB1 form a continuum in respect of their postsynaptic target distribution, rather than be

separated into perisomatic region- and dendrite-targeting IN types.

V. CONCLUSIONS

The main findings of the study are as follows:

- The soma and proximal dendrites of PCs are innervated primarily by two neurochemically and electrophysiologically distinct basket cell types.
- Similar number (15-17) of PVBCs and CCK/CB_1BCs converge onto single PCs.
- Both types of basket cells in the BLA innervate the perisomatic region and also the more distal dendritic segments in varying extents.
- The inhibitory efficacy of the two basket cell types is equal: they both can powerfully influence the probability and timing of action potential generation in PCs.
- The initial part of PC axons is parcelled out by PVBCs and AACs, as the majority of GABAergic inputs onto the soma-near, proximal region (between 0-10 μm)

originates from PVBCs, while the largest portion of the AISs is innervated by AACs.

- AACs in the BLA hyperpolarize their postsynaptic partners at resting membrane potential or near the spike threshold.
- AACs can effectively inhibit or delay the firing of PC, simultaneous activity of 2-3 AACs is sufficient to completely block spike generation.
- AACs concentrate their synapses onto the AIS between 20 and 40 μm from the soma, a region corresponding to the action potential initiation site. Thus, AACs maximize their inhibitory efficacy by strategically concentrating their synaptic junctions along the AISs to effectively counteract PC Na^+ channel activation and action potential generation.

Our results suggest that the three main interneuron types innervating the perisomatic region of PCs are able to powerfully modulate the firing of their postsynaptic partners with high temporal precision, a feature, which might endow them with crucial roles in amygdalar network operations during fear memory processing.

VI. LIST OF PUBLICATIONS

Publications related to the dissertation

Vereczki VK[1], **Veres JM**[1], Müller K, Nagy GA, Rácz B, Barsy B, 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

[1]equal contribution

Veres JM, Nagy GA, Vereczki VK, Andrási T, Hájos N

Strategically positioned inhibitory synapses of axo-axonic cells potently control principal neuron spiking in the basolateral amygdala. Journal of Neuroscience, 2014 Dec 3;34(49):16194-206.

Other publications

Zemankovics R, **Veres JM**, Oren I, Hájos N

Feedforward inhibition underlies the propagation of cholinergically induced gamma oscillations from hippocampal CA3 to CA1. Journal of Neuroscience, 2013 Jul 24;33(30):12337-51.

Holderith N, Németh B, Papp OI, **Veres JM**, Nagy GA, Hájos N
Cannabinoids attenuate hippocampal gamma oscillations by suppressing excitatory synaptic input onto CA3 pyramidal neurons and fast spiking basket cells. Journal of Physiology, 2011 Oct 15;589(Pt 20):4921-34.

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Nitric oxide signaling modulates synaptic transmission during early postnatal development. Cerebral Cortex, 2011 Sep;21(9):2065-74.