

# **Functional characterization of local feedback excitation onto different types of GABAergic inhibitory cells in the basal amygdala**

Ph.D. thesis  
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## **INTRODUCTION**

Computations performed by neural networks depend on the connectivity among excitatory and inhibitory neurons. Gamma-amino butyric acid (GABA) releasing basket (BCs) and axo-axonic cells (AACs) targeting the perisomatic region of cortical principal neurons (PNs) are in a key position to effectively control the firing of their postsynaptic partners. Consequently, perisomatic region-targeting inhibitory neurons (PTIs) are essential for neural computations and thus cognitive processes like learning & memory, perception, and motor control. The critical role that these inhibitory interneurons (INs) play in circuit operation is reflected in the wide variety of neurological and psychiatric diseases that have been implicated in their malfunction, including epilepsy, schizophrenia and autism. In cortical structures two distinct types of BCs expressing either parvalbumin (PV, PVBCs) or cholecystokinin (CCK, CCKBCs) give rise to the main inhibitory input onto the soma and proximal dendrites of excitatory PNs, while their axon initial segment (AIS) is innervated by PV-expressing AACs. The two BC types are markedly dissimilar in many single-cell features and are thought to generate postsynaptic

inhibition with different properties, suggesting distinct functions in cortical computation, presumably also supported by their different connectivity with local excitatory and inhibitory cells. However, the wiring motifs of cortical INs responsible for perisomatic inhibition are largely unknown.

The basal nucleus of the amygdala (BA) is a cortical structure known to be a site of plastic changes during fear learning. PNs in this region are glutamatergic excitatory neurons that give rise to local collaterals and project to remote areas. In contrast, the vast majority of GABAergic cells, which provide less than 20% of the total neuronal population, have only local axon arbor. The three previously described types of perisomatic inhibitory cells have also been recognized in amygdalar networks. However, as in other cortical regions, the synaptic organizing principles between these GABAergic cell types and PNs, a key knowledge for understanding microcircuit operation, are ill-defined.

## **AIMS**

The aim of this study was to unravel the mechanisms underlying the recruitment of the perisomatic inhibitory neurons in the BA by local collaterals of PNs. To address this question, we needed to reveal the connectivity between CCKBCs, PVBCs, AACs and PNs in the BA and the properties of these connections. In the three main parts of the study, we aimed to answer the following specific questions:

**I. Characterization of the input-output properties of the PTIs in the BA.**

**II. Investigation of the local feedback excitation received by the PTIs.**

**III. Unraveling the wiring principles of PTIs and PNs in the BA circuits.**

## **MATERIALS AND METHODS**

All experiments were approved by the Committee for the Scientific Ethics of Animal Research and were performed according to the guidelines of the institutional ethical code and the Hungarian Act of Animal Care and Experimentation in accordance with the European Directives.

Adult transgenic or double-transgenic mice of either sex expressing enhanced green fluorescent protein (eGFP) under the control of the Pvalb promoter (BAC-PV-eGFP), expressing DsRed under the Cck promoter (BAC-CCK-DsRed), or expressing both eGFP and DsRed were used in in vitro experiments. For acute slice preparation, horizontal slices of 200  $\mu\text{m}$  thickness containing the BA were prepared. To test the firing characteristics, neurons were recorded with whole-cell (WC) patch clamp method and were injected with 800-ms-long hyperpolarizing and depolarizing square current pulses. In WC paired recordings (IN  $\rightarrow$  PN, IN  $\rightarrow$  IN and PN  $\rightarrow$  IN) 3-10 APs were evoked in the presynaptic neuron by brief current pulses and IPSCs/EPSCs or IPSPs/EPSPs were recorded. The basic properties of the inhibitory/excitatory transmission were then offline analysed and calculated. For determining the activation threshold of PTIs and for constructing the connectivity map, transgenic mice were injected with adeno-associated virus (AAV) carrying channelrhodopsin 2 (ChR2) construct bilaterally into the BA in order to obtain ChR2 expression selectively in local PNs. During mapping the connection probability between PNs and PTIs, the postsynaptic IN was recorded in WC mode, while single PNs were sequentially activated by

a blue light spot and the light-evoked APs were simultaneously monitored in loose-patch mode. To investigate the excitability of the INs, firing threshold in PVBCs and CCKBCs were always simultaneously measured in loose-patch mode, while the whole area of the BA was stimulated with blue (447 nm) light with gradually increasing intensity, thereby successively activating the ChR2 containing PN population. For miniature event analysis, CCKBCs and PVBCs were recorded in WC mode in the presence of 0.5  $\mu$ M tetrodotoxin (TTX).

For morphological analysis of the recorded cells, biocytin-filled recorded cells were visualized either with Cy3, Alexa488 or Alexa647-conjugated streptavidin. Using the confocal images, the postsynaptic IN was fully reconstructed in 3D, and the putative contact sites from the presynaptic PN were labelled. For confirmation of the presence of putative contact sites determined by confocal microscopy some of the samples were further processed for EM analysis and the presence of the synapses could be clearly verified. *Post hoc* confirmation of the identity of the INs was performed based on their neurochemical content. The estimation of the density of excitatory inputs received by BCs, the number of VGluT1-immunostained boutons

forming close appositions with the biocytin-labeled dendrites, where Bassoon staining within the boutons was unequivocally present facing toward the dendrite, was counted. To estimate the content of AMPA receptors at synapses of identified IN dendrites, 3D direct stochastic optical reconstruction microscopy (3D-STORM) was performed. Localization points (LPs) were collected and 2D Convex Hull area and density of LPs was calculated. To investigate the connectivity among INs, CCK-DsRed mice and wild-type mice were used. Appositions were only identified as contacts if the postsynaptic anchoring protein of GABA<sub>A</sub> receptors, gephyrin, was localized at the side of the immunolabeled terminal, which faced the somatic membrane.

## **RESULTS**

### **I. Characterization of the input-output properties of the PTIs in the BA.**

To test the passive and active membrane characteristics of the three different types of PTIs, we performed WC patch clamp recordings. Comparison of the three cell types revealed that the intrinsic membrane

properties of PV-expressing BCs and AACs were markedly different from those expressing CCK, however PVBCs and AACs appeared to be rather similar. PVBCs and AACs typically displayed fast membrane kinetics and fast spiking phenotype with narrow AP half width and without apparent accommodation compared to CCKBCs. Further detailed analysis of the firing features of PTIs revealed differences in their input-output characteristics. In current-clamp mode, gradually increasing depolarizing and hyperpolarizing current steps were injected to the cell. Smaller current steps resulted in a similar response curve in all the three cell types, however, the injection of larger depolarizing currents clearly divided the different cell types, as AACs displayed the highest firing rate and CCKBCs showed the lowest. Our results clearly demonstrated that the passive and active membrane properties of CCKBCs markedly differ from the PV-expressing INs.

Besides the similarities or differences in the input-output features of the distinct types of PTIs, we investigated the properties of their inhibitory synaptic transmission onto PNs, which may help to understand how they impact the network operation. In order to address this question, we made dual WC recordings from PTIs and local PNs in the BA. The



statistical analysis of the results revealed that the CCKBCs give IPSCs with slower rise time and latency onto PNs compared to PV-expressing cells, although the amplitude, potency and decay kinetics were not significantly different between the cell types. The properties of the synaptic transmission from PVBCs and AACs tended to be similar except the latency of the IPSCs, which was significantly shorter in PVBCs. Next, we determined the short-term kinetics of the synaptic transmission. Our results showed that in average short-term depression could be observed at all the three IN types, however the strength of the depression was highly dependent on the firing frequency, as higher frequency resulted stronger depression. Next, we tested whether, in addition to the phasic GABA release tightly locked to the APs, a higher IN activity is capable of producing asynchronous transmitter release from the axon terminals of these cells. We found that no apparent asynchronous release could be detected at the output synapses of PV cells. In contrast, CCKBC output synapses displayed marked asynchronous release in response to an AP train. These data indicate that, in contrast to PV-expressing PTIs, CCKBCs are able to prolong their inhibitory effect on

PNs at high network activity levels, if they spike at high rates.

The similarities in the unitary events originating from the two BC types implied that these GABAergic cells might have comparable effects on the spiking of PNs. To test this prediction, we injected sinusoidal current trains into the PNs near their firing threshold and three spikes at 30 Hz were evoked in the presynaptic BCs at the peak of a sinusoidal wave, the point where PNs spiked with the highest probability. This analysis fully supported our expectation based on the unitary event properties, namely, that the two BC types inhibited the PN firing with equal efficacy.

## **II. Investigation of the local feedback excitation received by the PTIs.**

These results show that in this cortical network, PVBCs and CCKBCs provide similarly effective synaptic inhibition onto their neighboring PNs. To reveal that the distinct PTI types are activated similarly or distinctly by local PNs, simultaneous loose-patch recordings from a PV-containing IN and a CCK-expressing IN were obtained, while the intensity of light illumination was gradually increased in order to successively activate the local Chr2

containing PN population. We noticed that spikes could be detected at significantly lower light power in PVBCs in comparison to CCK-containing INs, indicating that PVBCs are driven by lower PN activity levels than CCKBCs. These observations clearly showed that the BC types are distinctly recruited by BA PNs, which can be primarily explained by the difference in their excitatory inputs.

To reveal the differences in synaptic excitation that can contribute to the distinct excitability of BCs by BA PNs, we first estimated the density of excitatory inputs received by intracellularly labeled BCs. This analysis showed that VGluT1-expressing boutons more densely covered the dendrites of PVBCs than those of CCKBCs. These results are in line with our observations that the activity of a smaller fraction of PNs should excite PVBCs more readily, if there is no substantial difference in the unitary events received by the two BC types. To test this latter assumption, we next investigated the properties of unitary events from individual PNs onto BCs. We made *in vitro* dual recordings from *post hoc* identified PN-CCKBC, PN-PVBC pairs. We found that the average potency and failure rate of uEPSCs were significantly larger and lower, respectively, in PVBCs than in CCKBCs, moreover PVBCs received EPSCs with

significantly faster kinetics. Next, we analysed the connection probability between PNs and BCs. In this experiment, we used patterned light stimulation of individual ChR2 expressing PNs and tested their synaptic connection in postsynaptic BCs. We noticed that, surprisingly, the connection probability showed distinct distance-dependence. The construction of a spatial map for PN-BC pairs uncovered that PVBCs were contacted preferentially by their neighboring PNs (<200-250  $\mu\text{m}$ ), but only rarely by more distal excitatory neurons. In contrast, PNs innervated CCKBCs with lower probability, but the chance to find a connected pair was distance-independent. Next, we performed an analysis of contact sites between PNs and BCs. In order to identify the putative appositions between the presynaptic terminals and the postsynaptic partner, we used dual color labelling of the pre- and postsynaptic neurons during the recording. This investigation has revealed that twice as many contacts could be identified from single PNs onto PVBCs on average, than onto CCKBCs.

To further strengthen the finding at the population level that excitatory synaptic inputs at individual release sites received by the two BC types are qualitatively and quantitatively different, we performed two sets of

experiments. First, we recorded miniature (i.e. quantal) excitatory postsynaptic currents (mEPSCs) in the presence of 0.5  $\mu$ M TTX and investigated the properties of single events. We found that the amplitude distribution of mEPSCs significantly differed in the two BC types. Importantly, mEPSCs in CCKBCs had small and largely uniform amplitudes, while mEPSCs in PVBCs had overall larger amplitudes and showed a more skewed distribution. In addition, we also observed a marked difference in the interevent interval distributions of mEPSCs. In CCKBCs, the time intervals between mEPSCs were significantly longer than between those events recorded in PVBCs. These data further supporting the finding that BA PNs excite CCKBCs predominantly via a few (one or two) synaptic contacts, having small and uniform EPSC amplitudes. Moreover, these results are in line with our findings, showing that the density of VGluT1-expressing axon terminals along the dendrites of CCKBCs was significantly lower than of PVBCs. To reveal a potential reason why release of single vesicles causes significantly smaller mEPSCs in CCKBCs than in PVBCs, we estimated the AMPA receptor content at individual synapses along the CCK- and PV-expressing IN dendrites using super-resolution microscopy. These investigations

uncovered that the number of LPs, representing AMPA receptors, at individual clusters apposed to bassoon labeling along the CCKBC dendrites was significantly lower than those observed along the IN dendrites expressing PV. In addition, there was a significant difference in the 2D convex hull area size of LP clusters along the dendrites of two IN types. These data collectively show that PNs innervate the two BC types via different principles and the characteristics of connections between the PNs and BCs favor the excitation of PVBCs at a lower activity level.

### **III. Unraveling the wiring principles of PTIs and PNs in the BA circuits.**

To understand the information processing properties of the microcircuits formed by PNs and PTIs, it is also necessary to reveal the connections between these GABAergic cell types. Therefore, in the final set of experiments, the connectivity among INs expressing PV or CCK was examined. First, using immunocytochemistry, we investigated the anatomical substrate for the connectivity among INs. We noticed that boutons expressing CB1, used as a marker of axon terminals of CCKBCs, often contacted the somata of CCKBCs, while PV-containing boutons often

apposed PV-immunolabeled somata, implying that both PVBCs and CCKBCs formed synaptic contacts with other INs of their own kind. In sharp contrast, we found that CB1-containing terminals avoided PVBC somata, and vice versa, CCKBC cell bodies were not contacted by PV-immunolabeled terminals. In contrast, the somata of AACs received synaptic inputs from both CB1- and PV-containing boutons as indicated by the presence of gephyrin labeling. To confirm these unexpected results, we performed paired recordings between INs in slice preparations. We found monosynaptic connections between CCKBCs and a unidirectional connectivity from CCKBCs onto AACs. No monosynaptic connection could be detected between CCKBCs and PVBCs, whereas we observed with high likelihood connectivity among PVBCs. In addition, we found that PVBCs innervated AACs, but not CCKBCs. These *in vitro* results support our morphological data, confirming that PVBCs and CCKBCs do not innervate each other, but form two independent, parallel GABAergic circuits. This active avoidance of cross-connectivity between the two BC types is strengthened by the fact that they both still innervated the intermingled population of AACs expressing PV.

## CONCLUSION

The new findings of our investigations are the followings:

- The membrane properties of the PTIs are significantly different.
- The kinetic features of the synaptic transmission from PTIs onto PNs showed some differences, however, the magnitude of the postsynaptic responses was rather similar.
- We found differences in the short-term dynamics and the degree of asynchronous release in response to AP trains in a cell-type specific manner.
- Our results clearly show that the potency to control the PN spiking by each IN group is similar.
- Lower activity levels of PNs are sufficient to excite PVBCs and AACs but not CCKBCs.
- The density of VGluT1-expressing glutamatergic inputs is significantly higher on the dendrites of PVBCs than



of CCKBCs and, in parallel, the mEPSC frequency in PVBCs is considerably higher than in CCKBCs.

- uEPSCs originating from single PNs are larger and faster in PVBCs and AACs than in CCKBCs.
- PVBCs are preferentially innervated by neighboring PNs, while CCKBCs receive excitation from PNs with a lower probability but in a distance-independent manner.
- Quantal excitatory events in CCKBCs and AMPA receptor content at individual synapses along their dendrites show a surprisingly uniform distribution, whereas a high variability characterizes both mEPSC amplitude distributions and AMPA receptor content at single clusters in PVBCs.
- BCs in the BA are mutually interconnected within their own category as well as both types innervate AACs, while the two BC types avoid innervating each other.
- The connectivity map of PTIs and PNs suggests that the two BC types form independent inhibitory networks in the BA.

In summary, as the same IN types are present in all cortical structures studied to date, the circuit motifs and synaptic organizing principles revealed here in the BA may offer a general framework for understanding the role of the three IN types in brain functions.

## LIST OF PUBLICATIONS

### **Publications related to the dissertation:**

**Andrási, T.**, Veres, J.M., Rovira-Esteban, L., Kozma, R., Vikór, A., Gregori, E., and Hájos, N. (2017). Differential Excitatory Control of Two Parallel Basket Cell Networks in Amygdala Microcircuits. *PLoS Biol* *15*, e2001421.

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