SYNAPTIC PROPERTIES OF PERISOMATIC INHIBITORY CELLS IN CORTICAL STRUCTURES

thesis book

Zsuzsanna Fekete

Semmelweis University Doctoral School
János Szentágothai Neurosciences Division

Supervisor: Norbert Hájos, DSc

Official reviewers: Zita Puskár, PhD

Attila Szűcs, PhD

Head of the Complex Examination Committee:

Alán Alpár, DSc

Members of the Complex Examination Committee:

Lucia Wittner, DSc

Tibor Zelles, PhD

Budapest
2024

1. Introduction

In cortical structures, excitatory, glutamatergic, neurons comprise the majority (80-85%) of neurons. With a rounded pyramid-shaped soma, an apical dendrite and many basal dendrites bearing numerous dendritic spines, pyramidal neurons are the most abundant among glutamatergic cells, and are also referred to as principal neurons (PNs). Functionally not less significant, the smaller portion of cortical neurons are inhibitory cells that shape and control the spatial and temporal pattern of neuronal activity. Inhibitory cells in cortical structures are comprised of diverse, specialized GABAergic cell types, and those that arborize locally are called interneurons (INs). A form of specialization is achieved by targeting certain subcompartments of postsynaptic partners, for instance a group of inhibitory cell types called the perisomatic region targeting interneurons (PTIs) preferentially innervate the soma, the proximal dendrites and the axon initial segment, collectively called the perisomatic region. The location of PTI output synapses enables these cells to efficiently control the activity of postsynaptic cells and are therefore key elements of local computations, and are also in the focus of our research. The group of PTIs includes 3 cell types introduced below.

Two of these IN types are basket cells, meaning that about half of their axonal boutons target the perisomatic region and typically surround the soma, creating a ‘basket’ and enabling potent inhibition by these cell types. Parvalbumin positive basket cells (PVBCs) are fast

spiking interneurons and their firing is crucial in the induction and maintenance of rhythmic network activities both *in vitro* and *in vivo*. PVBCs are regarded as the clockwork of neuronal circuits, while the other basket cell type, the cholecystokinin positive basket cell (CCKBC) is more suited to fine-tune local activity due to its integrative capabilities and the receptors expressed in its membrane that provide its sensitivity for subcortical inputs. CCKBCs also express type 1 cannabinoid receptor (CB1) and via these G-protein coupled receptors, endocannabinoids released from PNs upon depolarization suppress GABA release from CCKBCs, known as depolarization-induced suppression of inhibition (DSI). Chandelier cells (ChCs) are the third PTI type, with their boutons exclusively innervating PN axon initial segments where the parallelly lined up boutons create the distinctive morphology of this interneuron type. Similarly to PVBCs, ChCs can also fire at high frequency but are differently modulated by rhythmic network activities compared to PVBCs.

Although these three cell types can efficiently influence postsynaptic activity, it has been described in the hippocampus and the amygdala that PTIs show distinct characteristics regarding their intrinsic membrane properties and their input and output synapses. As the main site of information flow between neurons, chemical synapses introduce large variability into the postsynaptic effect of a presynaptic action potential, and characterization of synaptic properties advances our understanding of the contribution of PTIs to neuronal operations. Despite the importance of PTIs in shaping local activity, their

properties have not yet been compared in the prefrontal cortex (PFC), the region indispensable in functions like working memory, decision making and reward processing.

Besides general principles, however, synaptic properties are influenced by experiences that can cause long-term changes within a network. Such long-term change in the flow of information can take place following memory formation. To effectively induce memory formation and to study its neuronal correlates, classical fear conditioning has been used as a powerful paradigm. Classical fear conditioning involves a noxious stimulus (like a footshock) as an unconditioned stimulus (US) and a previously neutral stimulus (like a tone), which will be associated with the noxious stimulus during fear conditioning. As a result, the previously neutral stimulus will signal the threat and become the conditioned stimulus (CS) that also elicits fear response. The acquisition and expression of fear memories rely on processes taking place in a network of brain regions including the lateral and basal amygdala nuclei (LA and BA, respectively), but how different PTI types participate in or how they are affected by fear learning within these nuclei remains elusive. A method that provides insight into long-term alterations in excitatory input of a cell type following fear learning is quantifying the properties of miniature excitatory postsynaptic currents (mEPSCs), these action potential-independent events that could highlight differences in information flow concerning PTIs following memory formation.

2. Objectives

In order to uncover how the perisomatic region-targeting interneurons are embedded into the microcircuits of the PrL, and to investigate in the BA how excitatory inputs of PTIs are affected in a higher order cortical operation like memory formation, our aims were to:

1) Investigate how prelimbic PTIs integrate their input and translate it to output activity;
   - What are the active and resting membrane properties of PTIs?

2) Reveal the electrophysiological properties of excitatory and inhibitory connections between PNs and PTIs in the PrL;
   - What are the synaptic features of the uEPSCs evoked by PNs on PTIs?
   - How do uIPSCs evoked by PTIs on PNs differ?

3) Identify the connectivity of BCs in in the PrL;
   - Are the two BC types synaptically connected in the mPFC or do they form separate circuits?

4) Determine the changes in the excitatory inputs of PTIs in the BA following fear memory formation;
   - What are the effects of a tone, a footshock and complete fear conditioning on the features of mEPSCs recorded from the three PTIs?

3. Methods

Animals.

All procedures involving animals were performed according to methods approved by the Hungarian legislation (1998. XXVIII. section 243/1998, renewed in 40/2013), institutional guidelines, and in compliance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Directive 2010/63/EU). For the PFC study, both male and female adult mice were used in the electrophysiological experiments from the following transgenic mouse strains: BAC-CCK-DsRed (n=35) (Mate et al., 2013), BAC-PV-eGFP (n=48) (Meyer et al., 2002) and Pvalb-IRES-Cre crossed with BAC-CCK-DsRed (n=3). For anatomical quantification, C57Bl6 (n=3) and VGAT-IRES-Cre::BAC-CCK-GFP-coIN (n=4) (Vereczki et al., 2021) adult (P49-150) mice were used. For the BA study, adult male mice (P40-100) were used from the following strains: BAC-CCK-DsRed (n =16), BAC-PV-eGFP (n =19), or their offspring (*n*=4) expressing both eGFP and DsRed controlled by Pvalb and Cck promoters, respectively.

Behavioral tests

For the BA study, cue-dependent fear conditioning took place in a chamber with black dotted white background and metal rod floor (context A). Mice were allowed to habituate to this context for 5 min, then were returned to their home cage. After 1 h, mice were transferred

back to context A, where either of the following three protocols were used: (1) only CS group: CS was presented 7 times without US; (2) unsigned US group: 7 CS and 7 US were presented randomly; (3) signed US group: CS presentations co-terminated with the US. On the next day during testing cued fear expression, mice were subjected to a CS in a novel context. Freezing as an index of fear was *post hoc* measured manually on video recordings by trained observers blind to the animal treatment.

Electrophysiological recordings

Slice preparation

Following deep anesthesia induced by isoflurane, the brain was quickly removed from the skull and was placed into an ice-cold cutting solution bubbled with carbogen gas. 200 μm thick coronal slices containing the mPFC, or 200 μm thick horizontal slices containing the BA were prepared with a vibratome and were incubated in an interface-type holding chamber for at least 1 hour in artificial cerebrospinal fluid (ACSF) bubbled with carbogen gas, that was let to cool down gradually from 36 °C to room temperature.

Electrophysiological recordings

During recordings, slices were placed into a submerged-type of chamber and were perfused with ACSF kept at 32 °C. Patch-clamp recordings were performed under visual guidance of a differential interference contrast microscope using a 40x water dipping objective. Neurons were visualized with an sCMOS camera and fluorescent

protein expression was tested with the aid of a mercury arc lamp. Patch pipettes (4-7 MΩ) were pulled from borosilicate capillaries with an inner filament. Pipettes used for patching interneurons for paired recordings with PNs were filled with a K-gluconate based intrapipette solution containing 0.2% biocytin. A K-gluconate based intrapipette solution with high Cl^- concentration was used for patching PNs and for homotypic BC paired recordings. For optogenetic and pharmacological experiments, the intrapipette solution was Cs-gluconate based with 5 mM QX-314-Cl and 0.2% biocytin.

Whole-cell patch clamp recordings were performed with a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA, USA), low-pass filtered at 3 kHz, digitized at 10-50 kHz and not corrected for junction potential. Data were recorded with Clampex 10.4 (Molecular Devices) or an in-house acquisition and stimulus software (Stimulog, courtesy of Prof. Zoltán Nusser, Institute of Experimental Medicine, Budapest, Hungary).

The protocol used for recording firing patterns in current-clamp mode consisted of alternating 800 ms long depolarizing and hyperpolarizing current steps. A holding potential of -65 mV was applied during the recordings.

Paired recordings between interneurons and PNs were recorded with 5 action potentials elicited at 33 Hz with an inter-stimulus interval of 20 seconds. During paired recordings between homotypic BCs, 10 action potentials were elicited in the presence of AM251 (1 μM). Postsynaptic cells were clamped at -65 mV. Reported features of the postsynaptic currents are based on the analysis of the

postsynaptic response evoked by the 1st action potential in each train of stimuli.

For mEPSC recordings, neurons were held at a holding potential of -65 mV in voltage clamp mode in the presence of tetrodotoxin (TTX, 1 μM) and gabazine (5 μM) in the PFC or TTX (0.5 μM) and picrotoxin (100 μM) in the BA.

The presence of inhibitory inputs on PVBCs originating from CCKBCs was tested by evoking postsynaptic currents in PVBCs by using extracellular theta electrode stimulation in brain slices prepared from BAC-PV-eGFP mice. Ionotropic glutamate receptor-mediated postsynaptic currents were blocked by adding kynurenic acid in the recording solution. Neurons were held at -65 mV in voltage clamp mode. For testing PVBC input on CCKBCs, we performed simultaneous whole-cell recordings from CCKBCs and PNs in brain slices prepared from mice obtained by crossing the Pvalb-IRES-Cre with the BAC-CCK-DsRed mice. Offspring were injected with AAV5-EF1a-DIO-hChR2-eYFP in the mPFC to enable optogenetic control of local PVBC activity.

Post-hoc identification of cell types

After the recordings, slices were placed into a fixative solution to enable *posthoc* visualization of biocytin filled interneurons by applying fluorophore conjugated streptavidin. Biocytin filled PVBCs in the PFC were distinguished from fast-spiking ChCs based on the morphology of their axons, while CCKBCs showed strong DsRed expression and accommodating firing pattern. To distinguish between

PVBCs and ChCs in the BA, immunostaining against calbindin was performed and fast spiking interneurons with calbindin expression in their somata and/or axon terminals were considered BCs (Vereczki et al., 2016). CB1 content of CCKBCs was tested by immunolabeling and only those cells with CB1 immunopositive axonal boutons were included in the study. PNs in the mPFC were identified based on their slower membrane kinetics and their distinctive firing pattern characterized by regular spiking and characteristic afterhyperpolarization.

Immunohistochemistry

For labeling putative contacts in the mPFC to visualize CCKBC input on PVBCs, coronal sections of 80 µm thickness containing the mPFC were prepared from C57Bl6 (wild type) mice. Immunostainings were performed to label CB1, gephyrin and PV content. For labeling putative contacts on CCKBCs from PVBCs, mPFC slices were prepared from VGAT-IRES-Cre::BAC-CCK-GFP-coIN mice. Immunostainings were performed to label CB1, gephyrin and PV content along with the amplification of GFP signal in CCK cells. Using 3D confocal images, interneurons within ~30 µm depth from the surface of the sections were selected for analysis irrespective of the number of synaptic contacts on them. The surface of the somata and the putative contacts on it were manually labeled and quantified.

Statistical tests

Data from only the two BC types were compared with Mann-Whitney U-test. Recordings of the three PTI types in the BA and the mPFC were compared with Kruskal-Wallis ANOVA and *post hoc* Dunn's test. The level of significance was set to 0.05. Statistics were performed using Origin 2018 or Origin 2021.

4. Results

4.1. Part I: The microcircuit of pyramidal neurons and perisomatic region-targeting inhibitory cells in the mouse prefrontal cortex

4.1.1. Contrasting active and resting membrane properties of basket cells in the PrL

One of the key factors that determine the function of a given cell type is how its inputs are integrated and converted into output activity. Therefore, we first studied the active and resting membrane properties of PTIs. Acute slices containing the PrL were prepared from BAC-CCK-DsRed or BAC-PV-eGFP mice, where CCKBCs or PVBCs and ChCs express fluorescent proteins, respectively. Analysis of voltage responses upon the injection of hyperpolarizing and depolarizing current steps revealed that PVBCs had the smallest input resistance, the fastest membrane time constant and more hyperpolarized spike threshold than CCKBCs and ChCs. Among the three PTI types, ChCs had the smallest capacitance. PVBCs and ChCs exhibited a fast spiking phenotype and showed significantly less accommodation than

CCKBCs, a notable difference between the PTI types in other cortical areas. These diverse membrane properties suggest that signals in the three PTI types are translated into distinct activity patterns and have different integration properties in the PrL as it has been first proposed about basket cells in the hippocampus.

4.1.2. Local PNs evoke larger uEPSCs on PVBCs with short latency and fast kinetic properties

To determine how PTIs are embedded into the local excitatory network, we performed paired recordings between PNs and PTIs and analyzed the properties of the unitary excitatory postsynaptic currents (uEPSCs). Due to the low connectivity rate between PNs and ChCs sampled in our experiments (12.2%, 6 pairs from 49 tests), only results with BCs are included in the analysis. Our recordings revealed that uEPSCs evoked in PVBCs have significantly larger amplitude and shorter latency, but release from excitatory synapses to CCKBCs or PVBCs were found to be similarly successful. Kinetic properties of uEPSCs recorded from CCKBCs, however, were significantly slower both in terms of their 10-90% rise time and half-width (T50). These differences were recapitulated in mEPSC recordings as well, suggesting that the differential kinetic properties of uEPSCs are not due to sampling biases but reflect the distinct features of local excitatory inputs on the two BC types.

By quantifying the short-term plasticity in these connections, we found that the amplitude of uEPSCs in PVBCs did not decrease considerably in response to 5 action potentials. Conversely, CCKBCs

were innervated by depressing excitatory synapses. Taken together, our data suggest that PVBCs receive larger and faster synaptic excitation that is maintained if repetitive firing in PNs is induced, whereas the local PN population gives rise to smaller and slower postsynaptic events in CCKBCs that show prominent depression when trains of action potentials are evoked in PNs.

4.1.4. Fast spiking INs innervate local PNs with reliable but depressing synapses

The influence of PTIs on prelimbic processes is largely determined by their impact on postsynaptic partners. Action potentials evoked in PVBCs during paired recordings elicited unitary inhibitory postsynaptic currents (uIPSCs) in PNs with significantly larger amplitude than those evoked by CCKBC spikes. While PVBC and ChC synapses were highly reliable, almost every third action potential in CCKBCs failed to evoke a uIPSC in PNs. Notably, postsynaptic events upon PVBC and ChC discharges showed significantly shorter latency than those following CCKBC activation. Nonetheless, the three PTI types evoke postsynaptic events in PNs with similar kinetics both in terms of their 10-90% rise time and T50.

Investigation of the short-term dynamics in these inhibitory synapses revealed that uIPSCs evoked by CCKBCs became neither potentiated nor depressed. On the other hand, synapses arriving from PVBCs and ChCs showed significant short-term depression, a phenomenon previously observed in other cortical regions as well. Taken together, our results indicate that fast spiker INs generate fast

and reliable inhibition with PVBC spikes evoking significantly larger uIPSCs than CCKBCs, but in case of prolonged PTI activation, PNs can receive a steadier level of inhibition from CCKBCs.

4.1.5. Homotypic basket cell connections

Our paired recordings with the same type of BCs revealed that uIPSCs tended to have larger potency in synapses between PVBCs and displayed shorter latency compared to synaptic contacts between CCKBCs, although these differences did not reach the level of significance. On the other hand, failure rate was significantly higher for CCKBC than for PVBC synapses. Sustained presynaptic activity, however, revealed that homotypic CCKBC synapses display strong facilitation, while PVBCs receive uIPSCs of decreasing amplitude. Taken together, homotypic BC pairs display different synaptic characteristics and connectivity patterns in the PrL.

4.1.6. Heterotypic basket cell connections

To determine the presence or absence of functional synaptic connections on PVBCs established by CCKBCs in the PrL, we used a pharmacological approach and took advantage of CB1 expression on CCKBC axon terminals that is selective for this cell type among cortical inhibitory neurons (Wilson et al., 2001). Extracellular stimulation was used to evoke IPSCs in PVBCs while glutamatergic synaptic transmission was blocked by 2 mM kynurenic acid. Bath application of the CB1 agonist WIN55,212-2 (1 μM) reduced the amplitude of evoked postsynaptic currents below 50% of the initial

amplitude values demonstrating the presence of CB1-sensitve inputs to PVBCs. These electrophysiological data were further supported by our anatomical results showing CB1+ axonal boutons apposed to the majority of sampled PV+ somata (29 out of 33 cells, 87.9%).

To study the potential synaptic connections established by PVBCs on CCKBCs, we crossed BAC-CCK-DsRed mice with PV-Cre mice. Therefore, in offspring, we could target fluorescently labeled CCKBCs and activate PVBCs via Cre-dependently expressed channelrhodopsin (ChR2). During the optical stimulation of the PV population, we recorded from CCKBCs and PNs simultaneously in whole-cell configuration. The mean amplitude of IPSCs recorded from CCKBCs reached 11.1% of the IPSCs evoked simultaneously in PNs and the latency of the responses was significantly larger in CCKBCs. In addition, we also identified PV+ inhibitory synaptic contacts on the somata of CCKBCs in slices containing the PrL prepared from the offspring generated by crossing VGAT-IRES-Cre and BAC-CCK-GFP-coIN mice. Immunostaining against PV and gephyrin revealed that the majority of the CCK/GFP+ somata received PV+ input (27 out of 32 cells, 84.4%). In summary, these results demonstrate the reciprocal innervation between the two BC types in the PrL.

4.2. Part II: Fear learning and unsigned noxious stimuli change excitatory inputs on perisomatic region-targeting inhibitory cells in the basal amygdala

4.2.1. Separation of the effects of fear memory formation and sensory inputs

To distinguish between the effects of fear memory formation and the CS/US presentations on the excitatory synaptic inputs of BA PTIs, either of the following three behavioral paradigms were used. To test the behavioral consequences of the CS presentation, mice were repeatedly subjected to the CS (tone) without the US (shock, only CS group). To test the effects of CS and US without association, tones and shocks were presented randomly during conditioning (unsigned US group), therefore, the association between CS and US did not form. In contrast, when tones co-terminated with mild electrical shocks (signed US group), the fear memory was formed and CS presentation the next day in a different context induced significant freezing. Our experimental design, therefore, allows the separation of the consequences of fear memory formation from those caused by sensory signals.

4.2.2. Excitatory synaptic inputs in the 3 PTI types

To test whether excitatory synaptic inputs of different PTI types in the BA are capable of plastic changes, acute brain slices containing the amygdala were prepared immediately after cued fear testing. mEPSCs were recorded in whole-cell patch-clamp mode in the presence of 0.5 μM tetrodotoxin (TTX, voltage-gated Na^+ channel

blocker) and 100 μM picrotoxin ($GABA_A$ receptor antagonist). Amplitudes of mEPSCs in PVBCs showed an 11% decrease in the unsigned US group and a 5% decrease in the signed US group compared to the only CS group, and there was a significant increase (7%) in this mEPSC feature if we compared the unsigned and signed US groups. This observation implies that the US itself can elicit changes in mEPSC peak amplitudes in PVBCs, but the associated learning decreases those changes. Regarding the inter-event intervals (IEI) of mEPSCs, the unsigned US presentation led to a 26% increase in IEIs (i.e. reduced rate) compared to the only CS group, and the signed US group also showed a 23% increase. Rise time and decay kinetics of mEPSCs were not different in the three paradigms. Taken together, these results suggest that the US presentation decreases mEPSC amplitudes and their occurrence in PVBCs, but fear memory formation may cause a slighter reduction in the amplitude of their excitatory synaptic inputs in comparison to CS presentation only.

In CCKBCs, mEPSC peak amplitudes showed a slight (3%) but significant increase in the signed US groups compared to the only CS controls. However, there was no significant difference in case of the other two group comparisons. Interestingly, there were no changes in the IEI and the rise time of mEPSCs either, however, there was a significant 17% decrease in the decay time constant when we compared the only CS group to the unsigned US group. Taken together, these results show that fear learning increases the amplitude of mEPSCs and the unsigned US accelerates the mEPSC decaying phase in the CCKBC population.

In contrast to BCs, excitatory synaptic inputs in ChCs did not show change in terms of their amplitude, however, the rate of mEPSCs were 7% higher in the signed US group than in the only CS group. Rise time and decay kinetics of mEPSCs were not different in the three paradigms. Taken together, our data indicate that there is a unique increase in mEPSC rates in ChCs upon signed US presentation following fear memory formation that was not present in any other PTI type.

5. Conclusions

By performing *in vitro* whole-cell recordings from PTIs of the PrL, we found that PVBCs significantly differ from the other two PTI types with their low input resistance, short membrane time constant or large AHP amplitude, while CCKBCs fired the widest action potentials with the lowest maximum firing rate. After obtaining paired recording, analysis of the uEPSCs recorded from PTIs showed that PVBCs receive synaptic excitation from local PNs with short latency and large amplitude from synapses that are less depressing than earlier works described. Unitary EPSCs from CCKBCs had the slowest kinetic properties, which seemed as a universal feature of the synaptic excitation targeting the two basket cells, as contrasting kinetics of mEPSCs resembled that of uEPSC. We found excitatory connections on ChCs in only a handful of cases, but ChCs innervated neighboring PNs with high probability and evoked uIPSCs very reliably that showed similar short-term depression as connections established by PVBCs. Activation of PVBCs and ChCs gave rise to uIPSCs with

significantly shorter latency compared to events evoked by CCKBC spiking which could contribute to their role in selecting active members of the neuronal population.

Homotypic synaptic connections between basket cells are frequent and show cell type specific features in the PrL that resemble uIPSCs evoked in PNs. Our pharmacological and optogenetical tools supported by anatomical data allowed us to provide evidence for the functional connectivity between the two types of basket cell populations.

To study how the inputs of PTIs in the BA are affected by noxious stimulation and fear memory formation itself, we investigated how the mEPSCs recorded in PTIs are altered by the unsigned and signed footshock compared to when only the tone is presented. Using these 3 different behavioral conditions we found that ChCs were the only cells that received heightened rate of excitation following fear conditioning. Kinetics of the recorded events were affected only in CCKBCs after unsigned US presentation, while events recorded from PVBCs decreased both in their rate and amplitude. Therefore, these cell types might be differentially involved in processing aversive stimuli and fear memories.

6. Bibliography of the candidate's publications

Publications related to the thesis:

Fekete, Z., Weisz, Veres, J. M., F., Karlócai, M. K., Andrási, T., Hájos, N. (2024). Synaptic communication within the microcircuits of

pyramidal neurons and basket cells in the mouse prefrontal cortex. The Journal of Physiology. https://doi.org/10.1113/JP286284

Veres, J. M., Fekete, Z., Müller, K., Andrási, T., Rovira-Esteban, L., Barabás, B., Papp, O. I., Hájos, N. (2023). Fear learning and aversive stimuli differentially change excitatory synaptic transmission in perisomatic inhibitory cells of the basal amygdala. Front. Cell. Neurosci 17:1102338. https://doi.org/10.3389/fncel.2023.1120338

Publications not related to the thesis:

Nagy-Pál, P., Veres, J. M., Fekete, Z., Karlócai, M. K., Weisz, F., Barabás, B., Reéb, Z., Hájos, N. (2023). Structural organization of perisomatic inhibition in the mouse medial prefrontal cortex. The Journal of Neuroscience 43(42) 6972-6987. https://doi.org/10.1523/JNEUROSCI.0432-23.2023

Vereczki, V. K., Müller, K., Krizsán, É., Máté, Z., Fekete, Z., Rovira-Esteban, L., Veres, J. M., Erdélyi, F., Hájos, N. (2021). Total number and ratio of GABAergic neuron types in the mouse lateral and basal amygdala. The Journal of Neuroscience 41(21) 4575-4595. https://doi.org/10.1523/JNEUROSCI.2700-20.2021