Studying the molecular plasticity of the nervous system using nanoscopy

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Theses of PhD dissertation

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1 Abstract

Understanding the diverse functions of the brain is hindered by its complex connectivity, the diversity and plasticity of its synapses and the thousands of proteins behind these actions. In our era neuroscientists are equipped with more and more effective tools to be able to observe this vast complexity. We have developed a method, which enables us to stain different cell types, individual cells and even subcellular compartments, and investigate the abundance and nanoscale distribution of proteins of interest within. Correlated confocal and super-resolution microscopy thus provide means of inferring from this molecular information the function of the investigated proteins within the cell or compartment. In my dissertation, I would like to present two different scientific research projects, where we have taken advantage of this correlated method.

In the first project we were looking for the characteristic calcium binding proteins of cannabinoid receptor (CB_1) positive GABAergic neurons of the hippocampus, basolateral amygdala and somatosensory cortex for a better understanding on the specific physiological properties of these cells. After thorough investigation of a single-cell *in silico* mRNA sequencing database we found two candidates: N-terminal EF-hand Calcium Binding Protein 1 and 2 (NECAB1 and NECAB2). Next, we performed different experiments to corroborate this finding on the mRNA level, then on the protein level with population and single-cell measurements, and lastly nanoscale investigation took place with correlated confocal

and super-resolution microscopy to uncover a subcellular compartment-specific difference of protein abundance and distribution between NECAB1 and NECAB2.

In the second research project we investigated the effect of Δ^9-tetrahydrocannabinol (THC) treatment of rat dams on the dopaminergic reward system of their offspring. We showed that prenatal cannabis exposure does not cause a difference in dopaminergic cell numbers or CB_1 receptor levels in the ventral tegmental area (VTA). Interestingly, a synapse-specific redistribution takes place in the inputs of dopaminergic cells: the number of excitatory syanpses as well as the strength of excitatory and inhibitory synapses have both changed upon treatment which was shown with correlated confocal and super-resolution microscopy. Then, with the help of our collaborators we uncovered the reasons and consequences of these changes and proposed a possible treatment for preventing the pathological signs.

In summary, we have successfully utilized the capabilities of the correlated method and have shown how it can answer important questions in neuroscience.

2 New scientific results

<u>**Thesis 1a:**</u> **I found that the CB_1+ interneurons of the mouse hippocampal CA1 and CA2/CA3 regions and the somatosensory cortex express the mRNAs of NECAB1 and NECAB2 calcium binding proteins based on *in silico* single-cell mRNA-sequencing databases. Then I corroborated these results with experiments on the mRNA and protein levels in the hippocampus, somatosensory cortex, and basolateral amygdala on the population level.**

Related publications of the author: [J4], [C8], [C9]

Interneuron researchers are in a constant search for the consensus calcium binding proteins of CB_1+ interneurons, which can serve as good population markers and may be responsible for the specific firing pattern and network role of these cells. Thanks to the advent of the single-cell mRNA sequencing technology we can now search for mRNAs of such proteins in cells of the hippocampus and somatosensoy cortex. NECAB1 and NECAB2 proteins were detected as tributes and we confirmed their presence in CB_1+ interneurons with experimental work. This project is a good example how the ever-growing data pools can be mined nowadays to answer specific scientific questions.

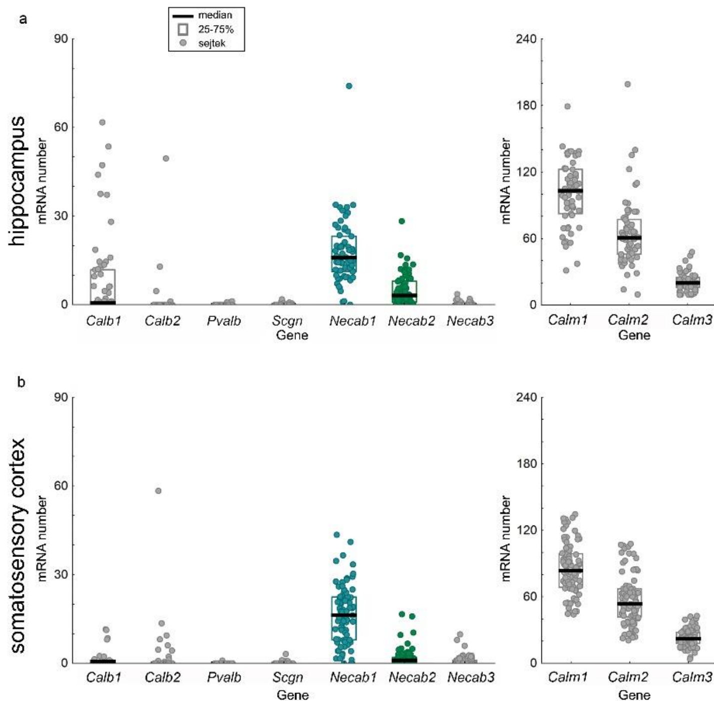

Figure 1. CB_1+ hippocampal interneurons express the EF-hand calcium-binding proteins NECAB1 and NECAB2 based on in silico data (figure: Miczán et al., modified)

a, b) In silico analysis of calcium-binding protein expression profiles of CB_1-positive interneurons based on a single-cell RNAseq database [1]. Classical calcium-binding proteins (calbindin – Calb1, calretinin – Calb2, parvalbumin – Pvalb and secretagogin – Scgn) are present in low levels and only in subgroups of CB_1+ cells, however the N-terminal EF-hand calcium-binding protein 1 and 2 (Necab1 and Necab2) are present in almost all CB_1+ hippocampal interneurons (n=61 cells) and interneurons of the somatosensory cortex (n=84 cells).

Calmododulin calcium binding proteins serve as positive controls for validation, since they are present in all eucaryotic cells.

Thesis 1b: I showed on single cells using correlated confocal and STORM super-resolution microscopy that the NECAB1 and NECAB2 proteins have different subcellular distribution in the CA1 region of the mouse hippocampus. NECAB1 protein is more abundant in the somatodendritic compartment, while NECAB2 is denser in the axon terminals.

Related publications of the author: [J4], [C8], [C9]

NECAB1 and NECAB2 proteins share great sequence homology and the question naturally arises why the same cell type needs both. We thus looked for localization differences among the two proteins. While we were not able to detect NECAB1 in the axon terminals with confocal micoscopy, we could utilize the higher sensitivity of STORM to detect it in boutons as well. In contrast, NECAB2 was more aboundant in axon terminals. This density difference can suggest slightly different functions of the two proteins.

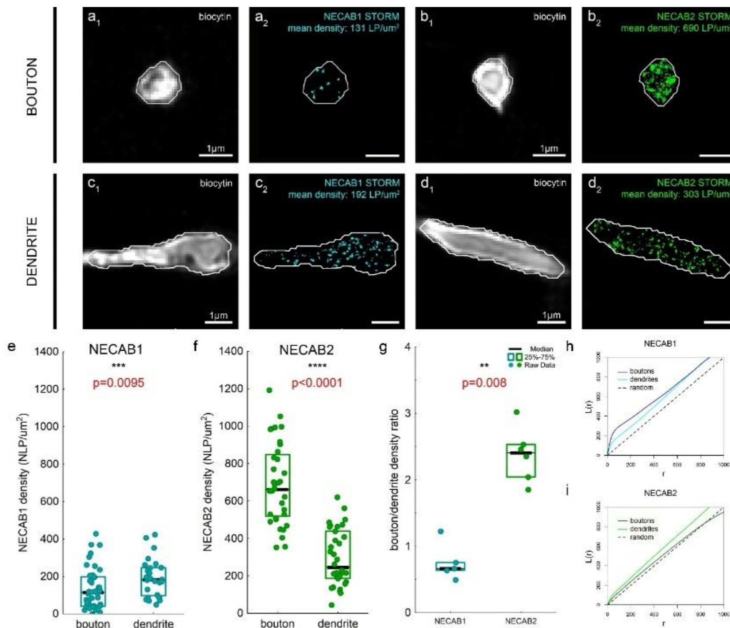

Figure 2 Subcellular density difference of NECAB1 and NECAB2 in perisomatic CB_1+ interneurons (figure: Miczán et al.)

a_1, b_1, c_1, d_1) Cell-specific biocytin-labeling of individual morphologically characterized CB_1-positive interneurons. (a_1, b_1, - axon terminal compartment, c_1, d_1 - dendritic compartment). A region of interest (ROI) is delineated with an Active Contour algorithm [2]

a_2, c_2) Axon terminal and dendrite showing NECAB1 positivity with STORM microscopy with different staining density. A ROI filter is applied on the STORM coordinates.

b_2, d_2) Axon terminal and dendrite showing NECAB2 positivity in STORM microscopy with different staining density. A ROI filter is applied on the STORM coordinates.

e) NECAB1 LP density is higher in the dendrites compared to axon terminals (N=6 and 5 animals, 43 and 28 ROIs, respectively, Mann-Whitney U-test, $p=0.0095$)

f) Higher density of NECAB2 was detected in axon terminals compared to dendritic compartments (N=6 and 6 animals, 36 and 34 ROIs, respectively, Mann-Whitney U-test, $p<0.0001$).

g) The ratio of the STORM localization point density residing in axonal and dendritic compartments is significantly different in the case of NECAB1 and

NECAB2 staining. NECAB1 is accumulated in the dendritic compartments, whereas NECAB2 is preferentially present in the axon terminals (N=5 and 6 animals, Mann-Whitney U-test, p=0.008).
h) Ripley's L-function visualization of LPs of NECAB1+ dendrites (cyan) and axon terminals (blue) showing that NECAB1 staining is more clustered in axon terminals than in dendrites (N=6 and 5 animals, n=51 and 40 ROIs, respectively). Black dashed line shows the function for Poisson distribution (random).
i) Ripley's L-function visualization of LPs of NECAB2+ dendrites (light green) and axon terminals (dark green) showing that NECAB2 staining is more clustered in dendrites than in axon terminals (N=6 and 6 animals, n=31 and 37 ROIs, respectively). Black dashed line shows the function for Poisson distribution (random).

Thesis 2a: I found using confocal and correlated confocal and STORM microscopy that there is no difference in the number of VTA dopaminergic neurons and the number of CB_1 receptors residing on vGluT1+ and VIAAT+ inputs of the cells following prenatal cannabis exposure in male rats.

Related publication of the author: [J3]

In parallel with the growing recreational and medical use of cannabis the number of pregnant women abusing the drug also rises worldwide. The women are often unaware of the fact that they can harm their fetuses. Thus, it is important to examine the changes in the children caused by the active components of cannabis, such as THC. We focused on the reward system, since most of the psychoactive drugs act on it. We used a rat model, where pregnant rats were administered THC during the gestational period. However, THC reportedly acts on CB_1 receptors, which actively shape brain development during the fetal period. We could not detect any differences among the treated and vehicle groups either in the

number of VTA dopaminergic cells or the number of CB_1 receptors on their excitatory (vGluT1+) or inhibitory (VIAAT+) afferents. During the experiements we took advantage of the sensitivity of correlated confocal and super-resolution microscopy, since conventional confocal microscopy could not detect the low number of CB_1 receptors on the afferents.

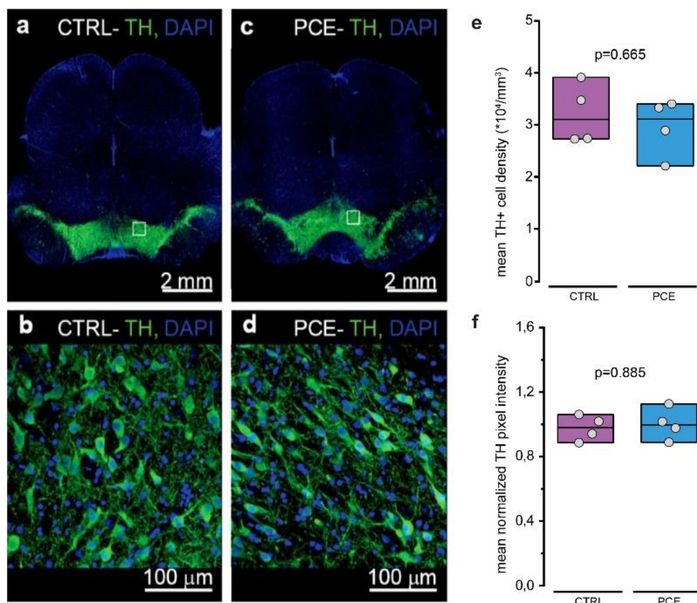

Figure 3. Prenatal cannabis exposure does not change the number of dopaminergic cells in the VTA (figure: Frau, Miczán et al., modified)

a) Anti-TH immunostaining (green) in control rat brain slice. Cell nuclei are stained with DAPI (blue).

b) Square area on panel a) magnified.

c) Anti-TH immunostaining in PCE-treated rat brain slice.

d) Square area on panel c) magnified.

n=12 images were taken per animal. Representative images are shown.

e) Mean TH+ cell density (N=4 és 4 animals, p=0.665, Mann–Whitney U-test).

f) Mean TH pixel intensity (N=4 és 4 animals, p=0.885, Mann–Whitney U-test)

<u>Thesis 2b:</u> I found using confocal and correlated confocal and super-resolution experiments, that prenatal exposure induces complex changes in the anatomy of the excitatory and inhibitory afferents of the TA dopaminergic cells in male rats. It causes a drop in vGluT1+ bouton numbers, which can more easily be excited, and in contrast the VIAAT+ synapses become weaker, but their number stays constant.

Related publication of the author: [J3]

We performed multi-channel fluorescent immunostaining against the excitatory vGluT1 and inhibitory VIAAT markers in slices containing filled VTA dopaminergic cells, then after high-resolution confocal imaging and image processing steps we found that the number of different input types of dopaminergic cells changes in different ways due to prenatal cannabis treatment. Molecular arrangement of synapses also changes. We could take advantage of correlated microscopy to study the synapse-specific density difference of the synaptic scaffolding protein bassoon. While in the PCE-treated animals the density of the scaffolding protein dropped, it escalated in inhibitory synapses. This suggests an increase in the strength of excitatory synapses and a decrease in the inhibitory ones since synaptic protein density affects the number of voltage-gated calcium channels in the active zone. Moreover, a constant amount of CB_1 receptors controls a different number of calcium channles which can contribute to the changes.

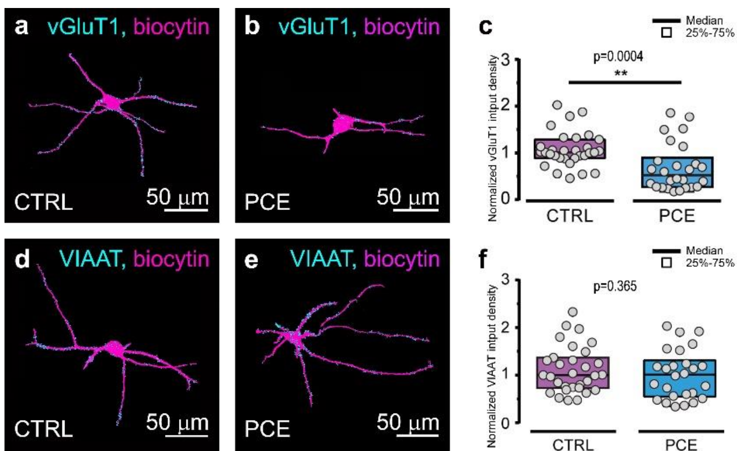

Figure 4. Number of excitatory inputs of dopaminergic cells decreases, while density of inhibitory inputs is constant (figure: Frau, Miczán et al., modified)

3D reconstruction of filled VTA dopaminergic cells (magenta).

a, d) control animals, b, e) PCE-treated animals.

a, b) vGluT1+ d, e) VIAAT+ synaptic inputs (cyan).

c) Quantification of vGluT1+ input densities normalized to the biocytin objects (p=0.0004, Mann–Whitney U-test n=31 és 26 images).

f) Quantification of VIAAT+ input densities normalized to the biocytin objects (p=0.365, Mann–Whitney U-test n=31 és 26 images).

3 Publications

[J1] L. Barna*, B. Dudok*, **V. Miczán**, A. Horváth, Z. I. László, and I. Katona, “Correlated confocal and super-resolution imaging by VividSTORM,” *Nat. Protoc.*, vol. 11, no. 1, pp. 163–183, 2016.

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[J3] R. Frau*, **V. Miczán***, F. Traccis, S. Aroni, C. I. Pongor, P. Saba, V. Serra, C. Sagheddu, S. Fanni, M. Congiu, P. Devoto, J. F. Cheer, I. Katona* and M. Melis*, „Prenatal THC exposure produces a hyperdopaminergic phenotype rescued by pregnenolone”, *Nat. Neurosci*., vol. 22 no. 12, pp. 1975-1985, 2019.

[J4] **V. Miczán**, K. Kelemen, J. R. Glavinics, Z. I. László, B. Barti, K. Kenesei, M. Kisfali, I. Katona, „NECAB1 and NECAB2 are consensus calcium-binding proteins of the CB_1 -positive interneuron population in the mouse forebrain”, *Cerebral Cortex*, 2020. In press

[J5] Z. I. László*, Z. Lele*, M. Zöldi, **V. Miczán**, F. Mógor, G. M. Simon, K. Mackie, I. Kacskovics, B. F. Cravatt, and I. Katona, “ABHD4-dependent developmental anoikis safeguards the embryonic brain,” *Nat. Commun*., vol. 11, no. 1, pp. 1–16, 2020.

[C1] **V. Miczán**, „Investigation of the subcellular distribution of CB_1 cannabinoid receptors with correlated confocal and superresolution STORM microscopy” *PhD Proceedings Annual Issues of The Doctoral School Faculty of Information Technology and Bionics* 10 pp. 85-88. (2015)

[C2] **V. Miczán**, „VividSTORM: a new software for the correlated visualization and analysis of pixel intensity-based and localization microscopy data”, *PhD Proceedings Annual Issues of The Doctoral School Faculty of Information Technology and Bionics* 11 pp. 75-78. (2016)

[C3] **V. Miczán**, „Novel analysis tools for correlated confocal and super-resolution microscopy”, *PhD Proceedings Annual Issues of The Doctoral School Faculty of Information Technology and Bionics 12: pp. 28-28., (2017)*

[C4] **V. Miczán,** B. Dudok, L. Barna, A. Horváth, I. Katona, „Probing identified synapses at the nanoscale: developing software tools for visualization and analysis of correlated confocal- and STORM super-resolution microscopy” *International Brain Research Organization Workshop (IBRO)*, 16-17 January, 2014, Debrecen (poster)

[C5] **V. Miczán**, B. Dudok, L. Barna, A. Horváth, I. Katona, „VividSTORM: a new software for the correlated visualization and analysis of pixel intensity-based and localization microscopy data” *Frontiers in Neurophotonics Symposium (FINS)*, 3-6 October, 2015, Quebec City (poster)

[C6] **V. Miczán**, L. Barna, B. Dudok, A. Horváth, J. R. Glavinics, Z. I. László, I. Katona, „VividSTORM: a novel open-source software for super-resolution and confocal microscopy images” *Society for Neuroscience (SFN) Meeting,* 12-16 November, 2016, San Diego (poster)

[C7] **V. Miczán**, J. R. Glavinics, A. Horváth, L. Barna, B. Dudok, Z. I. László, I. Katona, „New features in VividSTORM promote correlated super-resolution and confocal microscopy” *Federation of European Neuroscience Societies (FENS) Regional Meeting*, 20-23. September 2017, Pécs (poster)

[C8] **V. Miczán**, K. Kelemen, J. R. Glavinics, Z. I. László, I. Katona, „Calcium-binding protein profile of CB_1 cannabinoid receptor-positive interneurons in the hippocampus, neocortex and basolateral amygdala", *EMBO Workshop Cortical interneurons in health and disease*, 17 – 20 June 2018, Costa d´En Blanes (poster)

[C9] **V. Miczán**, K. Kelemen, J. R. Glavinics, Z. I. László, B. Barti, K. Kenesei, M. Kisfali, I. Katona, „NECAB1 and NECAB2 are consensus calcium binding proteins of the CB_1 -positive interneuron population in the mouse forebrain" *Cannabinoid Function in the CNS Gordon Research Conference (GRC)* 21-26 July 2019, Castelldefells (poster)

[C10] B. Barti, B. Dudok, K. Kenesei, **V. Miczán**, M. Ledri, I. Katona „Presynaptic nanoscale receptor/effector ratio controls neurotransmitter release probability at hippocampal GABAergic synapses" *Federation of European Neuroscience Societies (FENS) Regional Meeting*, 20-23 September 2017, Pécs (poster)

[C11] B. Barti, B. Dudok, K. Kenesei, **V. Miczán**, M. Ledri, G. Y. Balla, I. Soltész, I. Katona „The intra/perisynaptic CB1 cannabinoid receptor pool tonically controls GABA release at mouse hippocampal synapses" *Society for Neuroscience (SFN) Meeting,* 3-7 November, 2018, San Diego (poster)

[C12] B. Barti, B. Dudok, **V. Miczán**, K. Kenesei, G. Y. Balla, M. Ledri, D. Zala, Z. Lenkei, I. Soltész, I. Katona „Molecular stoichiometry of CB1 receptors and the release machinery predicts tonic cannabinoid control of GABA release" *Cannabinoid Function in the CNS Gordon Research Conference (GRC)* 21-26 July 2019, Castelldefells (poster)

[C13] J. R. Glavinics, **V. Miczán**, B. Dudok, Z. I. László, I. Katona "Nanoscale Bayesian clustering-based characterization of the presynaptic molecular architecture at GABAergic synapses" *Federation of European Neuroscience Societies (FENS) Regional Meeting,* 20-23. September 2017, Pécs (poster)

[C14] J. R. Glavinics, **V. Miczán**, K. Kelemen, Z. I. László, I. Katona “NECAB1 and NECAB2 are the two major calcium-binding proteins of the CB1 cannabinoid receptor-positive GABAergic interneuron population in the neocortex, hippocampus and the basolateral amygdala” *Society for Neuroscience (SFN) Meeting,* 3-7 November, 2018, San Diego (poster)

[C15] K. Kenesei, M. Ledri, B. Tóth, B. Dudok, B. Barti, **V. Miczán**, G. Horvai, I. Katona „Differential contribution of diacylglycerol lipase-alpha to phasic and tonic endocannabinoid signaling at hippocampal GABAergic synapses” *Society for Neuroscience (SFN) Meeting,* 12-16 November, 2016, San Diego (poster)

[C16] K. Kenesei, M. Ledri, B. Barti, B. Tóth, B. Dudok, **V. Miczán**, G. Horvai, J. Szabadics, I. Katona „Persistent cannabinoid control of GABA release does not require diacylglycerol lipase-alpha, the synaptic endocannabinoid-synthesizing enzyme” *Federation of European Neuroscience Societies (FENS) Regional Meeting,* 20-23. September 2017, Pécs (poster)

[C17] K. Kenesei, B. Barti, M. Ledri, M. Kisfali, B. Tóth, **V. Miczán**, J. R. Glavinics, K. Kelemen, G. Horvai, I. Katona „Differential contribution of diacylglycerol lipase-alpha to phasic and tonic endocannabinoid signaling” *Cannabinoid Function in the CNS Gordon Research Conference (GRC)* 21-26 July 2019, Castelldefells (poster)

4 References

[1] A. Zeisel, A. B. M͡oz-Manchado, S. Codeluppi, P. Lönnerberg, G. La Manno, A. Juréus, S. Marques, H. Munguba, L. He, C. Betsholtz, C. Rolny, G. Castelo-Branco, J. Hjerling-Leffler, and S. Linnarsson, “Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq,” *Science*, vol. 347, no. 6226, pp. 1138–1142, 2015.

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