**Final Report on NKFIH Frontline KKP 129961 grant**

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Our Frontline research grant aimed to define the cellular and domain-specific nano-organization of the classical synaptic endocannabinoid signalling pathway. We also aspired to identify and characterize new forms of cannabinoid-mediated signalling mechanisms.

In summary, the experimental results addressing these broad questions have been presented in eight publications in prestigious life science journals (2 in Nature Communications, 2 in Science Advances (Barti et al also appears on cover page), PNAS, Cerebral Cortex, Cell Reports, Methods in Molecular Biology). Two additional manuscripts are under consideration at PLOS Biology and PNAS.

The PI submitted the Advanced ERC grant in 2022 to the ERC LS5 neuroscience panel and received an evaluation score „A": „fully meets the ERC's excellence criterion and is recommended for funding if sufficient funds are available". The reviewers' opinions were overall very positive which encourages us to resubmit the revised Advanced ERC proposal in the summer of 2025. We are performing further preliminary experiments along the suggestions of reviewers to strengthen the application. Moreover, our team received another NKFIH grant, Excellence starting on January 1$^{st}$, 2025, that ensures the successful continuation of our research program and facilitates our chances to obtain the Advanced ERC grant.

As an important mentoring aspect of the grant, talented PhD students have also been involved in the Frontline Research program. Four students have successfully defended their doctoral thesis (Zsófia László, Vivien Miczán, Susanne Prokop and Benjámin Barti), whereas Miklós Zöldi will defend in 2025. These young researchers all continued in academia as postdoctoral researchers. Zsófia László and Susanne Prokop won the prestigious Junior Prima Award in Hungarian Science category. Several undergraduate researchers have also participated in the Frontline research program (Márton Vámosi, Dárius Leszkó, Dániel Nagy, Sámuel Szabó). They also received awards and recognition at Student Scientific Conferences (OTDK conference) and continued/will continue their research as PhD students.

The Covid-19 pandemic caused a delay in the experimental work during the second year of the project. This delay together with the long process of publication in high profile journals prompted us to request an extension of the Frontline Research grant until the end of 2024 that was granted.

Summary of major findings of the Frontline KKP 129961 research program:

1) A major objective of the current research was to decipher how the cell-type- and subcellular compartment-specific nanoscale $CB_1$ receptor distribution defines the physiological properties of synapses. To achieve this, we first correlated physiological parameters such as presynaptic neurotransmitter release probability in individual axon terminals with nanoscale molecular abundance data. A major accomplishment was the development of a workflow that required several innovations in experimental tools, labelling processes and in experimental data analysis. To correlate electrophysiological parameters with quantitative molecular and anatomical data within a single synapse, we performed paired patch-clamp electrophysiological recordings and post hoc STORM nanoscale molecular imaging at synapses between $CB_1$ interneurons and postsynaptic CA1 pyramidal cell pairs. We found that the receptor-effector nanoscale stoichiometry within the intra/perisynaptic domain is an important determinant of neurotransmitter release probability at GABAergic synapses. The underlying reason is the substantial population of tonically active $CB_1$ cannabinoid receptors located in the nanoscale vicinity of the release machinery. Unexpectedly, we also discovered that this form of cannabinoid signalling remains intact in the absence of the synthesizing enzymes of the two main endocannabinoid molecules, anandamide and 2-AG. Ongoing research aims to elucidate the more precise molecular mechanisms of this new non-canonical form of cannabinoid signalling. The functional consequences of altering presynaptic $CB_1$ numbers was investigated by using $CB_1$ receptor heterozygous (HET) mice. We observed 40-50% reduction in receptor number on the entire bouton surface measured by correlated confocal and STORM microscopy. In contrast, super-resolution imaging showed that the intra/perisynaptic pool of $CB_1$ receptors remains unaltered indicating that the bouton first fill up the nanodomain around synaptic release sites. Accordingly, the nanoscale receptor/effector ratio and synaptic cannabinoid tone were similar between the HET and WT genotypes. In contrast, in vivo treatment by the psychoactive phytocannabinoid $\Delta^9$-tetrahydrocannabinol (THC) disrupted the intrasynaptic nanoscale stoichiometry and eliminated tonic cannabinoid signalling further highlighting the functional significance of the nanoscale organization of the receptors. In addition, this experiment also gain insight into the molecular and synaptic effects of cannabis exposure and suggests that impaired GABAergic control of pyramidal neurons may be an important consequence. Our results in Science Advances and the study were featured on the cover page of the journal *(Barti et al., Science Advances, 2024)*.

2) Interestingly, we found that the impaired nanoscale receptor-effector ratio selectively affected the synaptic cannabinoid tone, whereas phasic cannabinoid signalling such as depolarization-induced suppression of inhibition (DSI) remained unaffected. This finding indicates that different mechanisms underlie phasic and tonic forms of synaptic cannabinoid signalling. In a follow-up study in collaboration with Mario van der Stelt's laboratory at Leiden University, we aimed at further characterizing the classical synaptic endocannabinoid signalling pathway important in DSI. Since the precise mechanisms governing the release and transport of key endocannabinoid lipid molecules, such as 2-AG, remain elusive and controversial, we set out to investigate the potential roles of microvesicle release in this process. Our experiments led us to propose a new model that emphasizes the role of extracellular microvesicles in 2-AG signalling. Specifically, our lab conducted paired patch-clamp electrophysiological experiments on acute brain slices, introducing either vehicle or pharmacological inhibitors of microvesicle release into the postsynaptic cell. These experiments revealed altered properties of depolarization-induced suppression of inhibition (DSI) between synaptically coupled cell pairs. These results are in press in PNAS and will be published in February 2025 *(Straub, Barti et al., PNAS, 2025)*.

3) We also established calcium imaging from $CB_1$-positive hippocampal interneuron axon terminals (see Barti et al 2024). In terms of these experiments, we aimed to determine which calcium-binding proteins may regulate presynaptic calcium transients in this specific interneuron population, because these cells do not express the traditional calcium-binding proteins. Therefore, we characterized the molecular properties of these interneurons by using *in silico* database screening together with sophisticated experimental and imaging methods. We revealed that NECAB1 and NECAB2 are the characteristic calcium-binding proteins of $CB_1$-interneurons in the hippocampus, somatosensory cortex and basolateral amygdala. Combination of patch-clamp electrophysiology, confocal, and STORM super-resolution microscopy uncovered subcellular nanoscale differences between the two calcium-binding proteins indicating functional differences. We published these findings in Cerebral Cortex and the study was also highlighted on the cover page of the journal *(Miczán et al. Cerebral Cortex, 2021)*.

4) To further exploit the single-molecule sensitivity of STORM imaging, and to circumvent potential pitfalls of antibody labelling (such as penetration problems at dense molecular structures, lack of specific antibodies of certain targets, batch-to-batch variance), we developed the PharmacoSTORM method that uses fluorescent small molecules (ligands) for super-

resolution imaging of target proteins. In collaboration with the group of Prof. György Keserű from the Research Centre for Natural Sciences, we have successfully developed a fluorescent ligand for the $CB_1$ cannabinoid receptor. The novel cannabinol analogue showed high-affinity binding to $CB_1$ receptor (Ki = 3.5 nM) and potent $CB_1$ agonism (EC50=68.8 nM in cAMP assay). The selectivity of the pharmacoprobe was tested on $CB_1$ receptors expressed in HEK293 cells, and the visualization of fluo-cannabinoid binding could be readily combined with the detection of receptor-immunostaining. The novel labelling tool was suitable for epifluorescence, confocal as well as for STORM microscopy. In the latter case, fluo-cannabinoid could visualize $CB_1$ receptors with nanometer localization accuracy (~5 nm). Competitive ligand binding measurements provided a straightforward way to test the specificity of labelling without the need of genetic modifications of the receptor. Rimonabant (unlabelled $CB_1$ antagonist) pretreatment prevented fluo-cannabinoid binding in vitro and eliminated completely the STORM signal from the plasma membrane. The exceptional detection sensitivity of STORM has the capacity to visualize single fluorescent ligands bound to their target in the cell membrane. Our methodological developments can help to reveal the dynamic nanoscale distribution of receptors with unique localization accuracy and improved quantitative precision, and address long-debated questions of the endocannabinoid field. We have also successfully designed, synthesized and characterized a fluorescent pharmacoprobe for monoacylglycerol lipase (MAGL) enzyme, another key molecular player of the endocannabinoid system in collaboration with the Keserű and van der Stelt labs. We have also used a fluorescent ligand for an ion channel and a fluorescent medicine to demonstrate the broad applicability of our approach that we termed “PharmacoSTORM”. Our work published in Nature Communications also describes the nanoscale binding pattern of cariprazine in the brain. Cariprazine was developed in Hungary, and as a third-generation antipsychotic it is used in the treatment of schizophrenia and bipolar disorders reaching blockbuster status with $2.3 billion in sales in 2024. However, the underlying neurobiological mechanisms of its therapeutic effects remained largely unknown. Unexpectedly, we found the highest intensity fluorescent cariprazine binding on the axons of the granule cells located in the so-called Islands of Calleja brain region. Taken together, we provided the first nanoscale visualization of the binding sites of a clinically applied drug on an identified neuronal compartment within native brain tissue. Importantly, Susanne Prokop, a key person in our Frontline research program received the prestigious Junior Prima Award in 2023 for developing the PharmacoSTORM approach. Moreover, the National Institute on Drug Abuse highlighted our study as one of the 5 most important research findings in 2021 *(Prokop et al., Nature Communications, 2021)*.

5) Astrocytes are essential components of the physiological and pathophysiological mechanisms in the brain. However, they represent a long-time challenge for molecular imaging due to the diffraction-limited diameter of most of their processes. Therefore, we also developed a new approach to visualize the fine astrocytic processes and the nanoscale organization of the endocannabinoid system in astrocytes by using STORM super-resolution imaging. To achieve the sparse labelling of astrocytes that enables the unequivocal localization of low-copy number proteins, we used postnatal electroporation. We also applied Voronoi tessellation to our dual-color imaging and demonstrated that this approach can discriminate astrocytic ultra-fine processes and closely attached presynaptic contacts at the nanoscale level. Our quantitative analysis showed that the major endocannabinoid-degrading enzyme MAGL has two major populations. A highly abundant and clustered pool in presynaptic terminals and a homogeneously distributed pool in astrocytic processes. This cell-type-specific nanoscale organization is important and likely to subserve specific physiological needs, because presynaptic MAGL controls synaptic 2-AG-signalling, whereas astrocytic MAGL produces arachidonic acid for prostaglandin synthesis. This study was presented as an invited lecture at the Cannabinoid Gordon Conference by a PhD student of the team, Miklós Zöldi and the manuscript summarizing these results is under consideration at PLOS Biology *(Zöldi et al)*.

6) In a review published in the Endocannabinoid Signalling edition of the Methods in Molecular Biology book series, Miklós Zöldi and István Katona also presented the detailed step-by-step protocol for the cutting-edge technique of STORM super-resolution microscopy used for imaging of $CB_1$ receptors in tissue preparations *(Zöldi and Katona, Endocannabinoid Signalling, 2023).*

7) To exploit his expertise in cell-type- and compartment-specific STORM imaging, Miklós Zöldi also investigated the cortico-striatal glutamatergic afferents of D1 direct and D2 indirect pathway medium spiny neurons. He discovered together with our French guest researcher Vincent Page-Blanc that behavioural flexibility is associated with increased abundance of the postsynaptic 2-AG-synthesizing enzyme DAGL-a and presynaptic $CB_1$ cannabinoid receptors specifically in D2 indirect pathway medium spiny neuron afferent synapses. Importantly, behavioural rigidity in aging animals is associated with the lack of increased endocannabinoid signalling at the same synapses indicating a hitherto undiscovered molecular plasticity process involving synaptic endocannabinoid signalling. The manuscript is under consideration in PNAS (*Paget-Blanc and Zöldi et al).*

8) We also aimed at characterizing another player of the endocannabinoid system, ABHD4, a putative synthesizing enzyme of the endocannabinoid anandamide. We have shown that ABHD4 is expressed in radial glia progenitor cells, immature cells of the embryonic brain that are still undergoing cell divisions. By using an arsenal of state-of-the-art methods such as in utero electroporation and correlated confocal and super-resolution imaging we discovered that ABHD4 functions as a pro-apoptotic molecule triggering cell-death upon abnormal delamination of the radial glia progenitor cells thereby preventing pathological developmental processes. Importantly, the level of ABHD4 protein is rapidly downregulated following neuronal cell fate commitment under normal developmental conditions indicating that the delaminated daughter neuroblasts escape from apoptosis by silencing ABHD4-expression. This new safeguarding mechanism has been termed developmental anoikis, as this process is conceptually analogous to anoikis, a detachment-induced form of apoptosis that is the central protective mechanism against pathological forms of epithelial-mesenchymal transition. We have shown the potential clinical importance of ABHD4-mediated developmental anoikis in a mouse model of fetal alcohol syndrome, since maternal alcohol exposure often results in microcephaly due to the loss of radial glia progenitor cells. These findings have been published in Nature Communications *(László et al., Nature Communications, 2020)*, and the first author, Zsófia László received Junior Prima Award.

9) In a previous study from our lab, we showed nanoscale molecular changes of synaptic endocannabinoid signalling in the ventral tegmental area of offspring whose mother has been exposed to THC (*Frau and Miczán et al, 2019, Nature Neuroscience*). In collaboration with Joe Cheer (University of Maryland) and Miriam Melis (University of Cagliari), we contributed to a study showing that maternal cannabis exposure induced hyperdopaminergia also affects the dopaminergic and the behavioural response to natural rewards and synthetic opioids (*Lujan et al 2024 Science Advances*).

10) Last, but not least, in collaboration with the group of Ádám Dénes from HUN-REN KOKI, using our in-utero electroporation approach, we studied microglial somatic contacts in the developing embryonic brain. Our work elucidated the pivotal role played by P2Y12 receptors on microglial processes in neurodevelopment by establishing junctions with cell bodies of developing neurons throughout embryonic, early postnatal and adult neurogenesis (*Cserép et al. Cell Reports, 2022*).