Scientific Final Report for OTKA/NKFI – 124972 Grant

The role of DGL-alpha enzyme in the regulation of hippocampal GABAerg neurotransmission

(A DGL-alfa enzim szerepe hippokampális GABAerg neurotranszmisszió szabályozásában)

Detailed report of scientific results

The 3 specific aims of the project proposal were the following in brief: 1) to study the properties of basic synaptic transmission and presynaptic Ca^{2+} transients in mice lacking DGLα enzyme, 2) conducting electrophysiological and lipidomic experiments to find out whether DGLα enzyme mediated 2-AG synthesis is indeed responsible for persistent cannabinoid signaling and to examine whether there are any alternative routes on which tonic eCB signaling can be performed in a DGLα independent manner, 3) using combined confocal and super-resolution STORM imaging in cell-type specific manner to address if the lack of DGLα enzyme results in morphological alterations in interneurons or any kind of changes in CB_1 receptor numbers on the surface of the axon terminals.

By the end of the financed period all planned experiments have been successfully performed by the researchers listed in the proposal. Since the latest report we have efficiently increased the sample numbers, finished all necessary experiments and finalized the data for publication. These results will be discussed below.

Previously, several studies have shown that tonic or persistently ongoing endocannabinoid signaling can be unmasked via pharmacologically blocking CB_1 receptors with the specific antagonist/inverse-agonist AM251. However, there is also evidence supporting the notion that AM251 can activate other receptors or signaling machineries beside CB_1 receptors. Moreover, functional CB_1 receptors can also be expressed postsynaptically. In light of these findings, our laboratory found it important to address the question whether direct involvement of CB_1 receptors mediate tonic signaling or there are other considerable CB_1 independent, off-target effects of the drug AM251.

Thus, in the first set of experiments we performed paired whole-cell patch-clamp measurements on *in vitro* slices in the CA1 region of the hippocampus between CB_1 expressing GABAergic perisomatic interneurons and postsynaptic pyramidal cell partners in wild type (WT) and CB_1 receptor knockout (KO) mice (Figure 1). Cells were filled with biocytin to allow post-hoc morphological reconstruction and further molecular investigation. In agreement with

previous studies performed on wild type animals, bath application of 10 µM AM251 increased inhibitory postsynaptic current (IPSC) amplitudes (IPSC: 39 ± 7 pA; +AM251: 52 ± 10 pA) as well as probability of successful synaptic events (success rate: 62 ± 7 %; +AM251: 71 ± 7 %) between CB_1 expressing GABAergic interneurons and pyramidal cells (Figure 1/B). However, in experiments carried out on CB_1 KO animals AM251 was unable to alter synaptic transmission (IPSC: 47 ± 7 pA; +AM251: 46 ± 6 pA, success rate: 68 ± 4; +AM251: 70 ± 5, Figure 1/D) indicating the direct involvement of CB_1 receptors in effects exerted by the drug action.

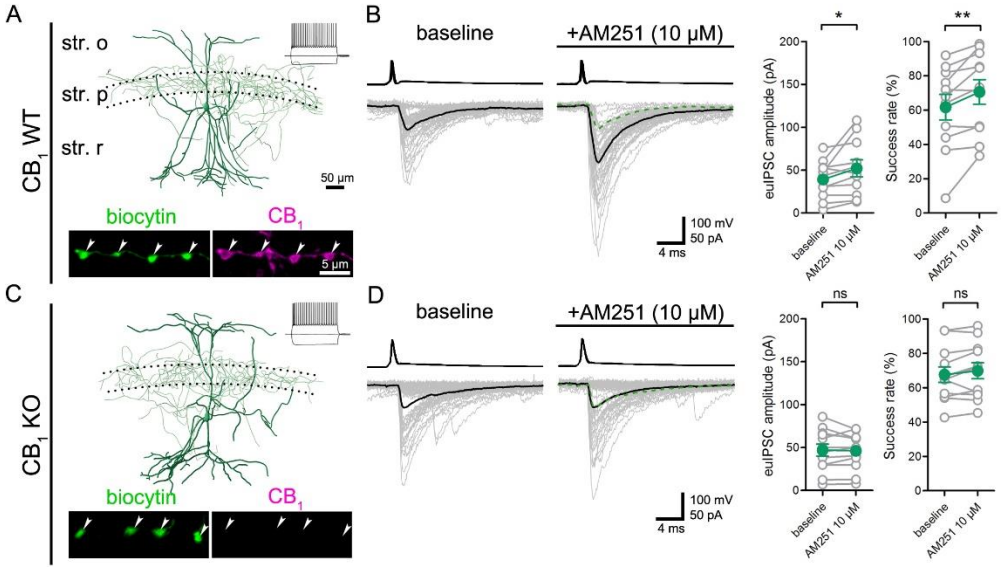

Figure 1: CB_1 receptors are indispensable for persistent cannabinoid modulation of synaptic transmission. **A, C)** Neurolucida reconstruction of perisomatically-targeting interneurons derived from CB_1 wild-type and CB_1 knockout tissue in the CA1 region of the hippocampus. Basket cells showed similar morphology and firing pattern between genotypes. Inset shows example voltage traces of response to -200 pA, 0 pA and +200 pA hyperpolarizing and depolarizing current steps from resting membrane potentials recorded in whole-cell current-clamp configuration. Axon terminals were mainly distributed across the pyramidal cell layer, showed immunopositivity for CB_1 in case of wild-type cells. **B)** Example traces of presynaptic action potentials evoked on perisomatic interneuron (top black traces) and respective postsynaptic responses (bottom traces) in CA1 pyramidal cell. Fifty consecutive euIPSCs (gray) and their averages (black) are presented before and during the application of the CB_1 receptor antagonist/inverse agonist AM251 from the same cell pair. AM251 (10 µM, 10 min) substantially increased GABAergic synaptic transmission between CB_1-positive perisomatic interneurons and CA1 pyramidal cells (*p = 0.0441; **p= 0.0065; n = 11 pairs, Paired t test). **D)** AM251 was unable to alter synaptic transmission in CB_1 KO mice (p_{ns} = 0.7551; p_{ns} = 0.0949; n = 12 pairs, Paired t test). str. o: stratum oriens, str. p: stratum pyramidale, str. r: stratum radiatum.

However, with the genetic deletion of CB_1 receptors from the whole brain of the animal we were unable to address whether the phenomena happen through pre- or postsynaptic receptors. To overcome this obstacle, we performed two-photon calcium imaging experiments, that allowed us to monitor the changes of intracellular Ca^{2+} levels in the axon terminals of perisomatically targeting interneurons before and after applying AM251 (Figure 2). After

application of the drug, intracellular Ca^{2+} levels greatly increased in boutons (normalized fluorescence (ΔF/F peak): 1 ± 0.01, +AM251: 1.08 ± 0.02) meaning AM251 has a unique action on presynaptic CB_1 receptors in the regulation of tonic signaling.

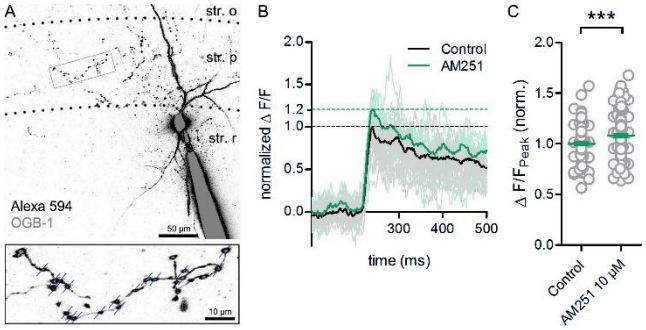

Figure 2: AM251 acts presynaptically on hippocampal inhibitory synapses. **A)** Perisomatically targeting interneuron filled with fluorescent and with Ca^{2+} sensitive dye (Alexa 594 and OGB-1 respectively) to allow two-photon imaging of presynaptic Ca^{2+} levels. **B)** Representative traces and their averages (bold lines) of ΔF/F fluorescence values following action potential initiation obtained from confocal line-scans performed on 18 boutons (blue lines on boxed region in A) before and after AM251 application. **C)** AM251 further elevated ΔF/F fluorescence values, indicating increased Ca^{2+} influx in presynaptic axon terminals compared to control measurements (n = 146 and 147 boutons from 12 interneurons, ***p = 0.0004, Unpaired t test).

After unmasking the precise receptor population which were involved in the studied mechanism, with the next set of experiments we moved toward understanding the role and the possible contribution of DGLα. As we concluded from our preliminary experiments using liquid chromatography tandem mass spectrometry, genetic deletion of DGLα dramatically reduced 2-AG levels in the brain (Figure 3/A). In such conditions, measured phasic eCB signaling was completely absent in these mice in line with the literature. Lipidomic experiments in combination with the inhibition of 2-AG degradation route via preincubation of the slices with monoacylglycerol lipase (MGL) inhibitor JZL184 (100 nM), showed a tonic production of synaptic 2-AG in slice preparations. This continuous ligand production was impaired in DGLα KO mice (Figure 3/A), meaning that DGLα enzyme is responsible for the tonic production of 2-AG which can be one underlying mechanism of tonic eCB regulation of synaptic transmission. Surprisingly, when we tested the presence of tonic eCB signaling via electrophysiological paired recording experiments we saw that despite the reduced 2-AG levels, deletion of DGLα did not alter the synaptic communication between GABAergic interneurons and pyramidal cells. Baseline synaptic transmission, characterized by the average amplitudes of IPSCs (WT: 116 ± 20 pA; KO: 106 ± 43 pA) and successful events (WT: 80 ± 3 %; KO: 76 ± 4 %) were unchanged after DGLα deletion (Figure 3/B, C).

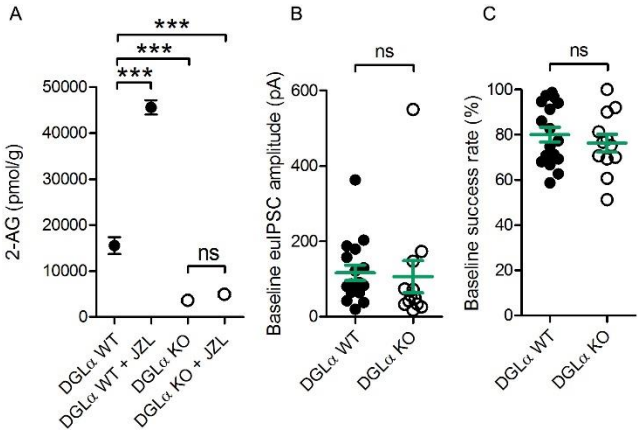

Figure 3: DGLα KO mice retain baseline synaptic transmission properties, however persistent 2-AG synthesis requires DGLα. **A)** 2-AG levels measured by liquid chromatography-tandem/mass spectrometry. Pretreatment of acute hippocampal slices with the irreversible MGL inhibitor JZL184 (100 nM) for 40 minutes evoked a robust increase in hippocampal 2-AG levels in WT animals. DGLα KO animals on the other hand did not show increased 2-AG levels after JZL184 treatment (WT: 15540 ± 1835 pmol/g; WT,+JZL184: 45621 ± 1552 pmol/g; KO: 3635 ± 356 pmol/g; KO,+JZL184: 4945 ± 496 pmol/g, n = 6 animals/group, ***$p < 0.0001$, $p_{ns} = 0.0576$, Unpaired t test). **B, C)** Baseline synaptic transmission properties remain unaltered after DGLα deletion, both euIPSC amplitudes (B) and success rates (C) obtained in paired recordings from DGLα KO mice are indistinguishable from WT animals (euIPSC: $p_{ns} = 0.1060$; success rate: $p_{ns} = 0.6105$, n = 12 pairs, Mann-Whitney U test).

One reason which can cause unaltered physiological synaptic parameters is a presence of a possible compensatory mechanism, which can imply changes in cell morphology, CB_1 receptor expression levels or altered CB_1 receptor numbers in DGLα KO compared to WT. To address this possibility, we exploited STORM super-resolution imaging microscopy to investigate nanoscale localization and the number of CB_1 receptor at the axon terminals of hippocampal CB_1 expressing interneurons in a cell type-specific manner. After the electrophysiological characterization, interneurons were filled with biocytin to allow correlative confocal and STORM imaging (Figure 4). We found that gross morphological appearance of perisomatic interneurons was similar in WT and DGLα KO mice, furthermore, the presynaptic CB_1 receptor numbers and density of receptors remained unaltered at axon terminals of DGLα KO mice compared to WT littermates (Figure 4/C, D). Additionally, interneuron bouton size was also unaffected (Figure 4/E). Despite the significant role of DGLα in neural pathfinding and synaptogenesis, interestingly no morphological alterations were observed at perisomatic interneurons in mice lacking DGLα.

Given the unchanged baseline synaptic transmission levels and the unaltered anatomical parameters of interneurons in the absence of DGLα, we hypothesized the existence of another eCB regulatory mechanism, which can set the magnitude of synaptic transmission. As a first step, the involvement of the non-canonical eCB system was studied.

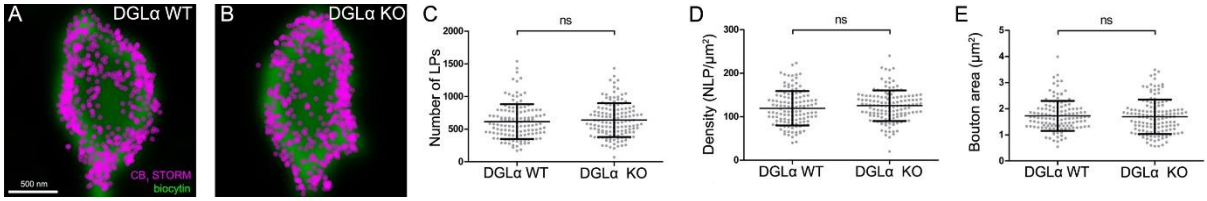

Figure 4: Deletion of DGLα does not cause adaptive change in the CB_1 receptor numbers, density or bouton size of GABAergic perisomatic interneurons. **A, B)** STORM super-resolution imaging of CB_1-immunofluorescence staining in the axon terminals of biocytin-filled perisomatic interneurons from DGLα WT and KO animals respectively. Magenta dots represent the nanoscale localization of CB_1 receptors overlaid on the confocal image of the corresponding interneuron terminal visualized by biocytin in green. **C, D)** Quantification of CB_1 receptor numbers (C; WT: 615.2 ± 23.68, n = 127 boutons; KO: 640.6 ± 22.15, n = 137 boutons, p_{ns} = 0.4341, Unpaired t test) and CB_1 receptor density (D; WT: 119.4 ± 3.52 NLP/µm^2, n = 126 boutons; KO: 125.3 ± 3.018 NLP/µm^2, n = 136 boutons, p_{ns} = 0.2037, Unpaired t test) by STORM super-resolution localization points (NLPs). **E)** Measurement of bouton area of biocytin-filled perisomatically-targeting GABAergic interneuron terminals (WT: 1.727 ± 0.05 µm^2, n = 127 boutons; KO: 1.694 ± 0.06 µm^2, n = 137 boutons, p_{ns} = 0.6650, Unpaired t test). str. o: stratum oriens, str. p: stratum pyramidale, str. r: stratum radiatum.

We repeated the paired electrophysiological experiments with AM251 treatment to test other possibly CB_1-mediated mechanisms in WT and DGLα KO slices (Figure 5). Wild type mice showed marked increase in IPSC amplitude (116 ± 20 pA; +AM251: 143 ± 23 pA), success rates (80 ± 3 %; +AM251: 85 ± 4 %) and elevated intracellular Ca^{2+} levels (normalized ΔF/F: 1 ± 0.02, +AM251: 1.084 ± 0.02) in axon terminals of presynaptic interneurons after application of AM251 (Figure 5/A-C). When recordings were carried out in slices from DGLα KO tissue, surprisingly tonic endocannabinoid-mediated suppression of GABA transmission was still present. Blockade of CB1 receptors via AM251 application significantly increased the magnitude of neurotransmission: the amplitude of IPSCs were robustly elevated (106 ± 43 pA; +AM251: 129 ± 46 pA) and success rates also increased (76 ± 4 %; +AM251: 82 ± 4 %) (Figure 4/D, E). Additionally, we again observed increased calcium influx into presynaptic boutons of perisomatic interneurons (normalized fluorescence (ΔF/F): 1 ± 0.02, +AM251: 1.07 ± 0.02, Figure 5/F), suggesting a similar involvement of a presynaptic phenomenon. The observed changes in physiological parameters and intracellular Ca^{2+} levels after AM251 application was identical between the two genotypes. These results support that beside the continuously released 2-AG ligand, another route of tonic endocannabinoid signaling is present at the hippocampal GABAergic synapses.

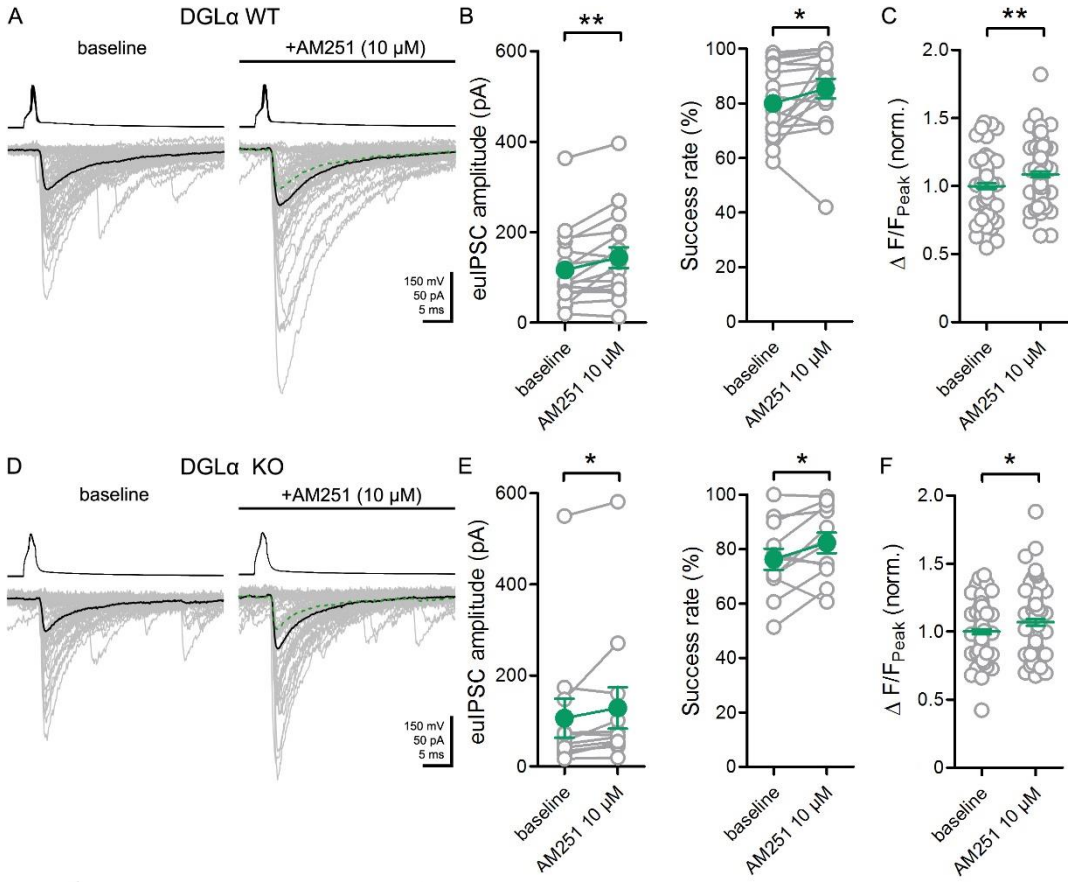

Figure 5: DGLα independent tonic cannabinoid signaling is unmasked by CB_1 receptor antagonist. **A)** Example traces of presynaptic action potentials evoked in a perisomatic interneuron (top) and respective postsynaptic responses (bottom) in CA1 pyramidal cells of WT mice. Fifty consecutive euIPSCs (gray) and their averages (black) are presented before and during the application of AM251 from the same cell pair. **B)** Summary graphs showing AM251 substantially increased GABAergic synaptic transmission including euIPSC amplitude and success rate (n = 17 pairs; **p = 0.0080, *p= 0,0416, Wilcoxon signed rank test). **C)** Presynaptic Ca^{2+} levels of boutons located in the pyramidal layer measured by two photon calcium imaging are elevated after AM251 application (n = 87 boutons from 6 cells, **p = 0.0090, Wilcoxon signed-rank test). **D)** Example traces as in A) from pairs derived from DGLα KO mice. **E, F)** AM251 elevated euIPSC amplitudes, success rates, (n = 12 pairs, euIPSC: *p = 0.0161; success rate: *p = 0.0312, Paired t test) and raised presynaptic Ca^{2+} levels (n = 86 boutons from 7 cells, *p = 0.0476, Wilcoxon signed-rank test) in the absence of DGLα.

Based on our results, that synaptic 2-AG independent tonic eCB signaling is still present in DGLα KO slices, an obvious explanation would be that this process is mediated by anandamide (AEA). AEA is the second most commonly studied cannabinoid lipid messenger in the brain and can also be a key player in the regulation of tonic eCB signaling in various brain regions including the hippocampus. N-acylphosphatidyl-ethanolamine-specific phospholipase D (NAPE-PLD) is described as the primary enzyme responsible for the synthesis of AEA. Thus, we extended our experiments to unfold whether continuous AEA synthesis is the underlying pathway which regulates tonic DGLα independent eCB signaling. We used genetically modified mice from which NAPE-PLD enzyme was missing, thus AEA synthesis was partially disrupted. This was verified by using liquid chromatography/tandem mass spectrometry, which

resolved a 42% drop of AEA levels in mice lacking NAPE-PLD. After lipidomic experiments we performed similar physiological paired recording experiment, to address the intactness of tonic eCB signaling. CB_1 receptor inhibition again elevated the amplitude (39 ± 11 pA; +AM251: 65 ± 14 pA) and success rates (58 ± 9 %; +AM251: 79 ± 7 %) of inhibitory currents. These experiments revealed that persistently active eCB signaling was unaltered in NAPE-PLD KO mice, indicating that the ligands generated by NAPE-PLD are also not responsible for this phenomenon. Further experiments are required however, to uncover the precise molecular mechanism through which this pathway is regulated.

Taken together, our major results depict a previously undescribed novel form of tonic endocannabinoid signaling which requires neither DGLα generated 2-AG nor NAPE-PLD generated AEA, thus suggests a ligand-independent form of persistent synaptic modulation by the presynaptic CB_1 receptors.

List of related scientific publications

The abovementioned results achieved by the primary support of the OTKA/NKFI – 124972 Grant will shortly be submitted for **scientific publication** to an international research journal as: Barti B., Kenesei K., Ledri M., Kisfali M., Tóth B., Miczán V., Glavinics RJ., Horvai G., Vizi ES. and Katona I. (2020) Differential Contribution of Diacylglycerol Lipase-alpha to Phasic and Tonic Endocannabinoid Signaling at Hippocampal GABAergic Synapses. The manuscript has been written and will be submitted in early 2020 with the NKFIH support highlighted in the Acknowledgment.

Major part of the results has been presented in some of the most prestigious conferences of the field in form of poster and lecture, in which the Grant was also highlighted:

Invited Lecture: Katona I. (2018) Correlation of physiological, morphological and molecular parameters by combined patch-clamp and STORM imaging at hippocampal synapses. Society for Neuroscience (SfN) Minisymposium, 003.05.

Poster: Kenesei K., Barti B., Ledri M., Kisfali M., Tóth B., Miczán V., Glavinics RJ., Kelemen K., Horvai G. and Katona I. (2019) Differential Contribution of Diacylglycerol Lipase-alpha to Phasic and Tonic Endocannabinoid Signaling. Cannabinoid Function in the CNS, Gordon Research Conference, #29.