Molecular anatomical investigation of the 2-AG signaling pathway at glutamatergic synapses

Doctoral thesis

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Introduction

In contrast to anterograde signaling, our knowledge about retrograde signaling operating as a feedback mechanism at chemical synapses is rather limited. It turned out during the last decade that endocannabinoid signaling plays a general physiological role in retrograde signaling (Kreitzer és Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson és Nicoll, 2001). This system has a fundamental role in controlling anterograde neurotransmitter release in an activity-dependent manner from the spinal cord to the neocortex (Castillo et al., 2012; Ohno-Shosaku és Kano, 2014). The prevailing view is that the synaptic endocannabinoid signaling pathway primarily operates as a negative feedback and mediates short-term, long-term or tonic depression of chemical synapses (Castillo et al., 2012). It is currently widely accepted that the physiological and pathophysiological significance of endocannabinoids are based on this modus operandi.

The molecular and anatomical organization of the endocannabinoid system
Three major groups of the molecular components of the endocannabinoid system are distinguished:

1. G protein-coupled receptors, such as the classical CB_1 and CB_2 receptors:

In both rodents and humans, the CB_1 cannabinoid receptors play the most important roles in mediating the effects of phytocannabinoids such as THC or the endocannabinoids (Ledent et al., 1999; Zimmer et al., 1999; Huestis et al., 2001; Monory et al., 2007, Kano et al., 2009). These $G_{i/o}$-coupled receptors are the most abundant GPCRs in the brain. On the other hand, other receptors are also emerging as potential candidate molecular targets of phytocannabinoids and endocannabinoids.

2. Endocannabinoids: Anandamide and 2-AG are the most intensively investigated classical endocannabinoid molecules. Both are lipid derivates of precursor phospholipids and are located in cell membranes. Research efforts in the last ten years revealed that 2-AG may be the canonical synaptic endocannabinoid. Pharmacological and genetic evidence also suggest that 2-AG mediates CB_1 cannabinoid receptor-mediated synaptic plasticity (Ohno-Shosaku és Kano, 2014). Therefore, a major objective of my doctoral thesis was to delineate the molecular organization of the retrograde 2-AG pathway.

3. Metabolic enzymes: Biochemical experiments identified numerous enzymes, which may potentially synthesize or degrade 2-AG or anandamide. The most important 2-AG synthesizing pathway involves phospholipase Cβ (PLCβ) (Stella et al., 1997; Hashimotodani et al., 2005), which makes 1,2-diacyl-glycerol (DAG), which is then further hydrolized by a plasma membrane-integrated diacylglycerol lipase enzyme. Two isoforms DGL-α and DGL-β have been described in the brain, but knockout animal studies revealed that it is the α isoform, which produces 2-AG (Gao et al., 2010; Tanimura et al. 2010, Yoshino et al., 2011). The vast majority (85%) of 2-AG is degraded by monoacylglycerol lipase (MGL) in the brain (Blankman et al. 2007).

The operation of the synaptic endocannabinoid pathway

When our team started research presented in my thesis, it was still unknown of which endocannabinoid plays the most important role in synaptic endocannabinoid signaling as a messenger and it was also unclear which enzymes may regulate synaptic endocannabinoid levels. Since then substantial progress led to a generally accepted operation mode, which integrates the findings presented in the Results section. To provide a general scheme, here I briefly summarize to most-up-to-date information, which does not fully follows the chronological sequence of discoveries.

The fact that 2-AG is a full agonist of CB_1 cannabinoid receptors makes it ideal as a synaptic messenger. Its synthesizing enzyme DGL-α is postsynaptic, whereas its molecular target CB_1 as well as its degrading enzyme MGL is presynaptic in axon terminals (Katona et al., 1999; Dinh et al., 2002; Gulyás et al., 2004). This synaptic molecular architecture is in agreement with the model of being a retrograde messenger, which is also supported by pharmacological experimemnts. Therefore, I will briefly outline synaptic endocannabinoid signaling focusing on the 2-AG pathway. Electrophysiological studies have revealed that two principal mechanisms, depolarization and activation of plasma membran $G_{q/11}$ protein-coupled receptors, such as $mGluR_1$ and $mGluR_5$ glutamate receptors, or the combination of these two mechanisms can trigger 2-AG release (Ohno-Shosaku és Kano, 2014). Quantitative neuroanatomical investigations have uncovered that $mGluR_1/mGluR_5$ receptors, a $G_{q/11}$ proteins and the PLCβ enzyme subsynaptic localization exhbits a characteristic pattern. These proteins are absent from the postsynaptic density (PSD) of excitatory synapses, but are accumulated at the edge of

the PSD within the so-called perisynaptic zone (Baude et al., 1993; Lujan et al., 1996; Tanaka et al., 2000; Uchigashima et al., 2007; Fukaya et al. 2008). In parallel, our research has shown that DGL-α is also concentrated in a perisynaptic annulus (see in details the Results section), which was confirmed by others (Yoshida et al., 2006). The Homer scaffold proteins are important to provide a platform for the macromolecular complex involved in 2-AG synthesis (Brakeman et al., 1997; Jung et al., 2007; Tang és Alger, 2015). This complex is termed as the perisynaptic machinery (PSM) to distinguish from the functionally different PSD complex (Katona és Freund, 2008). The PSM translates the extent of anterograde transmission into a retrograde feedback signal. Excess presynaptic activity activates perisynaptic metabotropic glutamate receptors, which then leads to 2-AG production. The retrograde signal attenuates futher presynaptic transmitter release as a synaptic circuit-breaker. The postsynaptically released 2-AG binds presynaptic CB_1 receptors and triggers molecular cascades leading to the depression of synaptic transmission. Synaptic depression can be short-term or long-term and can occur at both excitatory and inhibitory synapses (for review see Castillo et al., 2012). Short-term and long-term depression requires different second messenger pathways. Transient receptor activation triggers Gprotein $\beta\gamma$ subunit-mediated inhibition of voltage-gated calcium channels into the axon terminals (Mackie és Hille, 1992; Herlitze et al., 1996). Persisent receptor activation the G subunit $\alpha_{i/o}$ subunit results in the inhibition of adenylyl cyclase activity and reduced cAMP levels, which then culminates in long-term depression of neurotransmitter release (Chevaleyre et al., 2007).

In the present study, I have first investigated the 2-AG pathway in the hippocampus, which is well known for its role in learning and memory and also for the detailed knowledge on synaptic plasticity. In addition, we have also studied the 2-AG pathway in the dopaminergic neurons of the ventral tegmental area (VTA), which plays a pivotal role in reward signaling and in motivational behavior.

Aims

To understand the operational principles of the synaptic endocannabinoid system, it is important to elucidate the precise cellular and subcellular distribution of its molecular components. Therefore, we aimed to determine:

I. The cellular expression and subcellular position of DGL-α, the synthesizing enzyme of 2-AG, and the CB_1 cannabinoid receptors in hippocampal principal cells.

We aimed to address the following specific aims:

1. Is the mRNA and protein of DGL-α present in hippocampal excitatory neurons?
2. What is the subcellular distribution pattern of DGL-α, is it present at excitatory synapses?
3. Which type of excitatory cells express CB_1 mRNA and what is its subcellular location?
4. Is DGL-α and the CB_1 receptor co-occur in the same excitatory synapse?

It has been well known for long time that endocannabinoids play an important roles in many forms of synaptic plasticity such as long-term synaptic depression (LTD). Interestingly, some reports suggested that hippocampal interneurons do not express endocannabinoid-LTD. Therefore, our second major objective was to determine:

II. Are the molecular components of endocannabinoid signaling present at afferent excitatory synapses of hippocampal interneurons, which could provide a molecular platform for endocannabinoid-LTD?

We aimed to address the following specific aims:

5. Is the gene encoding DGL-α is turned on in hippocampal interneurons, in other words, do these cells express DGL-α mRNA?
6. Is the DGL-α enzyme protein is present in parvalbumin- and somatostatin-containing interneurons?
7. What is the subcellular distribution pattern of the DGL-α enzyme in hippocampal interneurons?

The ventral tegmental area (VTA) is a central region of the reward and motivational systems of the brain. It is well established that enhanced activity of VTA dopaminergic neurons plays an important role in addiction. Cannabis causes addiction and the endocannabinoid system is necessary for other drugs to evoked addiction. Therefore, we aimed to determine:

III. The precise molecular and anatomical organization of the endocannabinoid signaling pathway in the VTA and to test the hypothesis that the molecular blueprint of the synaptic 2-AG pathway, which we described in the hippocampus can also be extented to other brain regions.

We aimed to address the following specific aims:

8. Do neurons in the VTA produce the mRNA of DGL-α?
9. Is the DGL-α protein is present in dopaminergic neurons?
10. What is the precise subcellular distribution of DGL-α in dopaminergic neurons?
11. Do CB_1 cannabinoid receptors occur in the VTA?
12. What is the subcellular localization of CB_1 receptors in the GABAergic and excitatory synapses of the VTA?
13. Do DGL-α enzymes and CB_1 receptors colocalize in synapses?

Methods

All animal experiments presented in the doctoral thesis were approved by the Committee of the Scientific Ethics of Animal Research of IEM HAS (22.1/4027/003/2009) and were performed according to institutional guidelines of ethical code and the Hungarian Act of Animal Care and Experimentation (1998. XXVIII. Section 243/1998). The experiments were carried out in agreement with the 3R principles (replacement, reduction, refinement).

1. Tissue preparation for anatomical experiments

We have performed the experiment in wild-type C57BL/6H, C57BL/6J, and CD1 mice és CB_1 knockout mice (Ledent et al. 1999). The animals were anesthesized by pentobarbital-containing Equithesin intraperitoneal injection, perfused and the 40-50 μm thick sections of the fixed brains were used for in situ hybridization and immunohistochemistry experiments. The former case involbed the treatment of section with diethyl-pyrocarbonate (DEPC)-treated buffer and sectioning was performed under RNAse-free conditions.

2. Tissue preparation for molecular biology experiments

Riboprobes for in situ hybridization were obtained from C56BL/6H mice hippocampal total cDNA samples. The mice were anesthesized with isoflurane, the brains removed and freezed over dry ice. Ultrasonic homogenization of the samples on ice was used to obtain total RNA samples and then reverse transcription was carried out to obtain the cDNA samples.

3. In situ hybridization

The following approach was exploited to obtain riboprobes for in situ hybridization: blunt-end PCR products of two non-overlapping segments of the coding region of DGL-α were generated by Pfu-mediated polymerase reaction from the total cDNA samples. PCR primers were designed by the Primer 3 software (Rozen and Skaletsky, 2000). The amplicons were inserted into the SmaI site of the pBluescript II SK⁻ vector. The plasmids were transformed into bacteria and after selection, amplification and sequencing steps, we obtained the final plasmids at large yield. Restriction endonuclease were used to

linearize the plasmids and the riboprobes were prepared by T3 and T7 RNA polymerase-mediated in vitro transcription. This reaction tagged the riboprobes recognizing the coding sequence of DGL-α by digoxigenin. Sections used for in situ hybridization were 40 μm thick. The free-floating sections were first washed with DEPC-treated PB (pH=7.4). To monitor low expression levels of DGL-α in interneurons, we post-fixed the section for 4 hours in 4% paraformaldehyde solution. Section were washed 3 times for 20 minutes to facilitate penetration in 0.1% Tween-20-containing PB (pH=7.4). Hybridization step was performed with continous rinsing in a hybridization chamber overnight at 65°C for expression studies in hippocampal principal cells and for 24 hours at 60°C for hippocampal interneurons. The non-specifically bound riboprobes were washed afterwards and the sections were incubated with goat anti-digoxigenin Fab fragment conjugated with alkaline phosphatase dissolved in TBSTN overnight at 4°C. Next day, the sections were washed in TBST and the reaction was developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) és nitroblue-tetrasoniumchloride (NBT) chromogene mixture. The development of the reaction took 4-6 hours for principal cells and 12 hours for interneurons. Afterwards, the reaction was stopped by thorough 0.1 M PB (pH=7.4) washing. The section were covered with Vecatshield or Vectamount in case of the combined in situ hybridization-immunostaining experiments. The experiments were analyzed by a ZEISS Axioplan 2 microscope and the light micrographs of the hippocamus and VTA were taken with an Olympus DP70 digital camera.

4. Immunohistochemistry

The forebrain sections used for immunohistochemistry experiements were 50 μm thick. To facilitate the penetration of the antibodies, we placed the sections into 30% succrose solution then freeze-thawed 4 times over liquid nitrogen, which generates ruptures in the membranes without major disruption of the ultrastructure, but which are large enough to allow access of the IgG molecules. Two major immunohistochemistry approaches were used: immunoperoxidase reaction and immunogold labeling and we also combined these approaches in certain experiments. In case of the peroxidase enzyme-based immunostaining, we first blocked the endogenous peroxidase activity with 1%-os H_2O_2 for 10 minutes. Afterwards, the sections were treated with 5% normal goat serum (NGS)

for one hour and then incubated with the respective primary antibodies at 4°C for 48 hours. The specificity of the antibodies was validated by the lack of immunostaining in sections derived from CB_1 KO mice. In case of DGL-α, two independent antibodies raised against non-overlapping epitopes were used and resulted in an identical staining pattern. Incubation with the primary antibody was followed by extensive washing and the sections were incubated with secondary antibodies. Regarding the goal of the experiment and the mode of development either biotinylated secondary antibodies (Vector Laboratories, Burlingame) or gold-conjugated secondary antibodies (Aurion, Wageningen) were used. In case of the combined immunogold-immunoperoxidase reaction, the sections were incubated with the coctail of the two primary antibodies raised in different species. Afterwards the immunogold labeling was developed first followed by the immunoperoxidase reaction.

5. Electron microscopy

Hippocampal and VTA sections were dehydrated with an ascending ethanol series and embedded into epoxy resin (Durcupan, ACM, Fluka). The region-of-interest was selected with light microscope and reembedded into blocks for ultramicrotome sectioning by a Leica Reichert ultramicrotome into 60 nm thin serial sections. The sections were placed onto Formvar membrane-coated copper grids, contrasted with lead citrate and investigatd with a Hitachi 7100 electron microscope (Tokyo, Japan).

6. Quantitative electron microscopic analysis of DGL-α distribution

To establish the subcellular localization of DGL-α in relation to synapses within the dendritic spine heads of pyramidal cells, we carried out a quantitative analysis on 300 immunogold labeled excitatory synapses located in the CA1 stratum radiatum (n=3 animals). First we measured the distance of immunogold particles representing the position of DGL-α enzim from the edge of the postsynaptic density of the afferent excitatory synapse. Because silver-intensified gold particles have a variable size, we used the centrum of the particle and the measurement was performed along the plasma membrane. The obtained distance values were segregated into 60 nm bins. The data were compared with Kruskal-Wallis non-parametric statistics and presented as mean and standard deviation. Because the samples obtained from the 3 animals were not different,

we pooled the data and presented as the ratio of gold particles in a given bin compared to the number of all gold particles. A similar approach was followed to determine the subcellular position of DGL-α in relation to afferent excitatory synapses of aspiny dendritic branches and long filopodial spines of hippocampal interneurons. Immunogold particles representing the position of DGL-α in parvalbumin-positive interneurons were analyzed in the CA1 stratum radiatum, whereas mGluR1a-positive dendrites were analyzed in the CA1 stratum oriens. Electron micrographs were taken at 50,000 X magnification and the quantitative data were collected and evaluated by the Analysis és Statistica softwares.

Results

1. Endocannabinoid system at the afferent excitatory synapses of hippocampal principal cells

The lipid derivative 2-AG is the most abundant endocannabinoid in the central nervous system. The serine hydrolase DGL-α was one of its candidate synthesizing enzymes among many potential other enzymes around the time when our research programme has been started (Bisogno et al., 2003). Therefore, our first step was to test the hypothesis if the gene *dagla* encoding the DGL-α enzyme is expressed in hippocampal cells. To establish DGL-α mRNA expression pattern, we performed in situ hybridization on free-floating forebrain sections. The highest DGL-α expression levels were observed in the hippocampus. The cell bodies of CA1 and CA3 pyramidal neurons showed stronger chromogene reactions than the granule cells of the dentate gyrus. Some weakly expressing cells were also found in the hilus, which are likely to be the glutamatergic mossy cells, because the expression levels of DGL-α in GABAergic interneurons and glial cells did not reach the detection threshold in other subfields of the hippocampal formation. One must note however that the strength of the in situ hybridization reaction is strongly influenced by the temperature and length of the hybridization step. In the present experiments we selected more stringent conditions to avoid non-specific background labeling, which may have reduced the sensitivity of the reaction.

Our next aim was to determine the precise subcellular localization of DGL-α in hippocampal principal cells. Because DGL-α knockout mice as controls have not yet been available, we have used two independent antibodies raised against non-overlapping epitopes (ab-INT és ab-L26) to validate the immunoperoxidase reactions, which were visualized with the DAB precipitate. The immunostaining pattern obtained with these antibodies were highly similar and gave a characteristic laminar distribution at low magnification. This pattern reflects the segregated distribution of excitatory pathways and it is in agreement with the findings of the in situ hybridization experiment. DGL-α-immunoreactivity was granular in the neuropil at high magnification. This compartmentalized immunostaining pattern suggested that this enzyme may be concentrated around excitatory synapses.

It has been debated whether 2-AG is a true retrograde messenger, or it plays an indirect autocrine role in the regulation of neurotransmitter release. Therefore, our next aim was to determine the precise subcellular localization of DGL-α by using electron microscopy. The electron dense DAB precipitate representing the position of DGL-α was exclusively localized in the dendritic spine heads of principal cells in the samples obtained from CA1 stratum oriens. These spine heads were innervated by DGL-α-immunonegative axon terminals forming asymmetric synapses. This observation indicates that DGL-α may be the start point of synaptic endocannabinoid signaling and the 2-AG messenger produced by this enzymes reaches presynaptic CB_1 receptors via a retrograde route.

Despite their tiny sizes, dendritic spine heads have a surprisingly complex structure. Several molecularly and functionally distinct microdomains have been distinguished within spine heads with specific roles in the regulation of synaptic transmission and plasticity (for review see Rácz és Weinberg, 2013). To determine where exactly DGL-α is positioned with the spine heads we employed pre-embedding immunogold staining. The gold particles were always attached to the inner surface of the plasma membrane, which is in agreement with the facts that DGL-α is a transmembrane protein and both epitopes are positioned on its intracellular C-terminus. To quantitatively characterize the subcellular distribution of DGL-α, we measured the distance of gold particles from the edge of the postsynaptic density in randomly selected samples obtained from the CA1 stratum radiatum. The highest density of gold particles were found in the first 60 nm bin and the density decreased gradually with the distance from the synapse. This perisynaptic distribution pattern is similar to the localization of $mGluR_5$ receptors (Lujan et al. 1996, 1997), and suggests that the molecular components involved in 2-AG synthesis and mobilization are concentrated around the synapses in a perisynaptic annulus as part of a macromolecular complex.

Because DGL-α is the predominant synthesizing enzyme of 2-AG, it was conceivable to hypothesize that CB_1 receptors, the primary molecular targets are also localized at the same excitatory synapses. To test this hypothesis, we used a novel polyclonal antibody, which is sensitive to visualize even low copy numbers of CB_1 receptors (Fukudome et al., 2004). As a first step, we validated the specificity of this antibody in CB_1 knockout mice. Immunostaining for CB_1 revealed a characteristic

laminar distribution pattern following the trajectory of excitatory pathways in wild-type, but not in knockout mice. The strongest immunostaining was found in the inner molecular layer of the dentate gyrus. High density of CB_1-immunoreactivity was also found in the strata radiatum and oriens of the hippocampal CA1 and CA3 subfields, where dense granular staining in the neuropil was observed together with large GABAergic fibers. This light microscopic distribution pattern raised the possibility that CB_1 receptors are indeed present on excitatory axon terminals. To test this hypothesis next we performed an electron microscopic analysis. In samples obtained from the inner third of stratum moleculare, high levels of DAB- based immunolabeling was found in axon terminals forming asymmetric synapses. In addition, similar presynaptic CB_1-immunoreactivity was also observed in glutamatergic excitatory and GABAergic inhibitory boutons throughout the CA1 and CA3 subfields.

2. Endocannabinoid system at the afferent excitatory synapses of hippocampal interneurons

The in situ hybridization experiments presented in the previous section did not reveal the presence of DGL-α in hippocampal interneurons. On the other hand, one must emphasize that the absence of DGL-α mRNA in inhibitory cells may simply be due to the limitation of detection sensitivity and DGL-α may still be expressed by these cells albeit at lower levels. This is an important question for two reasons. First of all, it was unknown whether the 2-AG signaling pathway plays a role in synaptic plasticity exclusively in certain cellular components of hippocampal networks, or the molecular architecture we uncovered may be a more general property of glutamatergic synapses? Secondly, a lower DGL-α mRNA level may indicate that there is a cell-type-specific optimalized induction threshold and efficiency of the 2-AG signaling pathway. Several experimental parameters, such as the reduction of the temperature of the hybridization step, increasing its length, higher concentration of riboprobes used in the incubation, shortened washing steps, longer chromogene development can all facilitate the sensitivity of in situ hybridization. By systematically changing these parameters, we could provide direct evidence that DGL-α mRNA is expressed by hippocampal GABAergic interneurons at

very low levels. These experiments were validated by the lack of staining when using the negative control riboprobe.

In the next experiments in situ hybridization was combined with immunostaining to establish, which interneuron types express DGL-α mRNA. Hippocampal GABAergic cells consist of numerous types, we selected two of these for further studies. One interneuron type, which contains parvalbumin as a neurochemical marker has aspiny dendrites, but still this type receives the highest density of excitatory innervation (Gulyás et al., 1999). The other type is also innervated by high numbers of glutamatergic synapses, which target the filopodial spines on the dendrites of these cells. This spine interneuron type expresses the neurochemical marker somatostatin (Maccaferri et al. 2000; Klausberger és Somogyi, 2008). Notably, numerous DGL-α mRNA-containing interneurons were scattered throughout all hippocampal subfields. In the CA1 region in two animals, we investigated 58 parvalbumin-positive cell bodies and all were DGL-α-positive. In addition, DGL-α mRNA expression level reached the detection threshold in 58 cell bodies out of 59 somatostatin-immunoreactive interneurons.

In order to determine the presence of the DGL-α enzyme protein in interneurons and its precise subcellular position, we performed combined immunogold-immunoperoxidase staining. Because somatostatin labels only the cell body and the axon arbor of an interneuron, we used $mGluR_{1a}$-immunostaining, which visualizes the somatodendritic domain of the same interneuron type. It was notable that immunogold particles representing the position of DGL-α were always found perisynaptically in interneuron dendrites. The electron dense DAB precipitate provided direct evidence that the gold particles were present on parvalbumin-positive dendrites in the CA1 stratum radiatum and on $mGluR_{1a}$-immunoreactive dendrites in the CA1 stratum oriens. Importantly, the gold particles were most concentrated in the 60 nm bins at the edge of the afferent excitatory synapses and their density gradually decreased away from the synapse along the plasma membrane.

3. Endocannabinoid system at the afferent synapses of ventral tegmental area dopaminergic neurons

In the next experiments, we aimed to reveal the distribution pattern of DGL-α in the VTA, where the dopaminergic neurons involved in reward signaling are located. We were also interested in determining if the molecular organizational principles observed in the hippocampus can also be extended to another brain regions. In situ hybridization revealed weak DGL-α expression in neurons scattered throughout the VTA and also in the substantia nigra pars compacta. To establish the precise cellular and subcellular distribution of the DGL-α protein we then performed immunohistochemistry experiments. Immunoreaction was visualized by using the DAB precipitate. In agreement with our previous observations in the hippocampus, immunostaining for DGL-α revealed a dense granular staining pattern throughout the VTA suggesting that the enzyme has a compartmentalized subcellular localization. Interestingly, even the DAB precipitate, which is a diffusible labeling material, was attached to the plasma membrane next to excitatory and inhibitory synapses. Immunogold staining was always found at the inner surface of the plasma membrane, which is consistent with the fact that DGL-α is a transmembrane protein and the epitopes are located on its intracellular C-terminus. Based on morphological criteria, it is likely that those synapses which contained postsynaptic DGL-α are formed by either cortical and subcortical glutamatergic afferents of the VTA (Sesack and Pickel, 1992; Carr and Sesack, 2000; Omelchenko and Sesack, 2007); or by the axons of encephaline and dynorphin-containing GABAergic medium spiny neurons located in the nucleus accumbens (Pickel et al., 1993, Sesack and Pickl, 1992, 1995). Some nerve terminals may derive from local GABAergic interneurons as well (Bayer and Pickel, 1991).

To determine whether the VTA dopaminergic neurons also produce DGL-α protein, we exploited double immunostaining. DGL-α was visualized by immunogold labeling, whereas the dopaminergic nature of the respective profiles were shown by tyrosine-hydroxylase (TH)- immunoperoxidase staining (DAB). The enzyme was presenr at both afferent excitatory and inhibitory synapses, although the diffusible DAB precipitate rendered often difficult to establish the type of the synapse. We also found DGL-α in TH-immunonegative profiles in our samples.

Given the postsynaptic presence of DGL-α, we asked the question whether CB_1 cannabinoid receptors are located presynaptically in the VTA GABAergic and glutamatergic synapses. Earlier anatomical studies showed very low levels of CB_1 in the VTA (Herkenham et al. 1991b; Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998). Therefore, we used the novel high sensitivity CB_1 antibody to address this issue (Fukudome et al., 2004). Immunostaining resulted in high density neuropil labeling in wild-type, but not in knockout mice in the midbrain. To uncover the subcellular localization of CB_1 receptors we carried out electron microscopic analysis on the VTA samples. CB_1 receptor-immunostaining was restricted onto axon terminals and immunogold particles were always attached to the plasma membrane extrasynaptically. To provide direct evidence for the presence of the molecular architecture of endocannabinoid signaling necessary for homosynaptic depression, we finally performed double immunostaining for DGL-α és CB_1 in the VTA. These experiments confirmed that these two molecular players occur together at the opposite sides of the same symmetric and asymmetric synapses. This molecular organization thus demonstrate the presence of the retrograde 2-AG signaling pathway in the VTA and, in agreement with our findings in the hippocampus, raise the possibility that endocannabinoid signaling may be a general feature of chemical synapses throughout the central nervous system.

Conclusions

In the present doctoral thesis, I examined the distribution of two molecular components of 2-AG-mediated endocannabinoid signaling in the hippocampus and in the midbrain VTA. DGL-α, the synthesizing enzyme of 2-AG was always found postsynaptically at the edge of the synapses. In contrast, CB_1 cannabinoid receptors, the molecular targets of 2-AG is always presynaptic on axon terminals. Because we found a similar molecular organization in both brain regions, we propose that the subcellular distribution of these molecular components may follow a general principle. In addition, these findings support prior physiological findings that 2-AG may be a bona fide retrograde synaptic messenger.

Endocannabinoid signaling plays an important role in several forms of synaptic plasticity. Our studies in the hippocampus shed light onto the fact that afferent excitatory synapses of both principal cells and interneurons carry the necessary molecular architecture for endocannabinoid-mediated LTD. To evoke LTD, one may need to use cell-type-specific induction protocols, for instance the threshold for endocannabinoid-LTD is lower in principal cells. This may be important in those physiological or pathophysiological processes at the network level, when excess excitation triggers endocannabinoid release, which then attenuates excitatory input of excitatory cells, but the excitatory input of inhibitory cells still remain functional. This phenomenon may have a protective function against pathological network activity.

The VTA is a central component of the reward system of the brain, and most of those dopaminergic neurons involved in reward signaling are located in this region. Addictive drugs generally facilitate dopamine release, and by blocking the dopaminergic system, the rewarding effect of most drugs can be prevented. Addiction is a pathological form of learning based on pathological synaptic plasticity. Our data show that both glutamatergic and GABAergic afferent synapses of the dopaminerg neurons contain the molecular components of the endocannabinoid signaling pathway hence endocannabinoid signaling may play an important role to weight the different afferent inputs of dopaminergic neurons via endocannabinoid-mediated synaptic plasticity.

List of publications

Publications included in the doctoral thesis:

1. Péterfi Z*, Urbán GM*, Papp OI, Németh B, Monyer H, Szabó G, Erdélyi F, Mackie K, Freund TF, Hájos N, Katona I. (2012) Endocannabinoid-mediated long-term depression of afferent excitatory synapses in hippocampal pyramidal cells and GABAergic interneurons. **Journal of Neuroscience**, 32(41):14448-63. *=equal contributions as first-authors

2. Mátyás F, Urbán GM, Watanabe M, Mackie K, Zimmer A, Freund TF, Katona I. (2008) Identification of the sites of 2-arachidonoylglycerol synthesis and action imply retrograde endocannabinoid signaling at both GABAergic and glutamatergic synapses in the ventral tegmental area. **Neuropharmacology**, 54(1):95-107.

3. Katona I, Urbán GM, Wallace M, Ledent C, Jung KM, Piomelli D, Mackie K, Freund TF. (2006) Molecular composition of the endocannabinoid system at glutamatergic synapses. **Journal of Neuroscience**, 26(21):5628-37.

Other publications:

4. Nyilas R, Dudok B, Urbán GM, Mackie K, Watanabe M, Cravatt BF, Freund TF, Katona I. (2008) Enzymatic machinery for endocannabinoid biosynthesis associated with calcium stores in glutamatergic axon terminals. **Journal of Neuroscience**, 28(5):1058-63.

5. Berghuis P, Rajnicek AM, Morozov YM, Ross RA, Mulder J, Urbán GM, Monory K, Marsicano G, Matteoli M, Canty A, Irving AJ, Katona I, Yanagawa Y, Rakic P, Lutz B, Mackie K, Harkany T. (2007) Hardwiring the brain: endocannabinoids shape neuronal connectivity. **Science**, 316(5828):1212-6.

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