Neurobiological characterization of the endocannabinoid signaling in postmortem and epileptic human hippocampus

Doctoral (Ph.D.) Theses

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INTRODUCTION

Molecular and anatomical architecture of endocannabinoid signaling, and its functional significance in the nervous system

Behavioural and therapeutic effects of chemical compounds (mainly Δ^9-tetrahydrocannabinol) of Cannabis plant have been known since antiquity. These effects are mediated by CB_1 cannabinoid receptors located on cells of the nervous system. Endocannabinoids are endogenously generated cannabinoid ligands that are structurally similar to Δ^9-tetrahydrocannabinol: the best-known endocannabinoids are N-arachidonoyl-etanolamine (anandamide) and 2-arachidonoylglycerol (2-AG).

CB_1 receptor belongs to the $G_{i/o}$ subgroup of seven-transmembrane domain containing G-protein coupled receptors. CB_1 is one of the most abundant G-protein coupled receptors in the central nervous system, and is present on both glutamatergic and GABAergic terminals in numerous rodent brain areas. Its presynaptic localization suggests that the endocannabinoid system mediates retrograde synaptic signaling.

Given that endocannabinoids are not stored in synaptic vesicles, but are generated from structural lipid components of the cellular membrane in an activity-dependent manner, the rate of their enzymatic generation and degradation is of critical importance. 2-AG is generated from the second messenger molecule diacylglycerol by the sn-1 specific diacyl-glycerol lipase (DGL). DGL enzyme has two isoforms that differ in their C-terminal tail: DGL-β is expressed mostly in the developing nervous system and in the liver, while DGL-α is present in the adult brain and is responsible for the stimulation-induced synthesis of 2-AG. Degradation of 2-AG into arachidonic acid and glycerol is primarily performed by monoacyl-glycerol lipase (MGL) in the brain. Presynaptic localization of CB_1 receptor imply that its endocannabinoid ligands may function as retrograde signaling molecules, which is confirmed by the subcellular localization of the sythesizing and degrading enzymes of 2-AG. DGL-α, the enzyme responsible for the production of 2-AG, is found in postsynaptic spines of the excitatory cells, whereas MGL, the degradation enzyme of 2-AG, is present in presynaptic axon terminals, similarly to CB_1 cannabinoid receptors. The other well-known endocannabinoid, anandamide, is synthesized by presynaptic N-acyl-phosphatidyletanolamine

phospholipase D (NAPE-PLD), and degraded by postsynaptic fatty acid amine hydrolase (FAAH): localization of these enzymes suggests that anandamide is not a retrograde, but presumably an anterograde or intracellular signaling molecule.

One of the most important CB_1-mediated physiological phenomenon, considering the activity of neurons, is that βγ subunits from activated G-proteins are able to inhibit the permeability of calcium channels resulting in a CB_1-receptor-dependent decrease of neurotransmitter release. This suggests that endocannabinoid system has particular importance in controlling excess neuronal activity. Indeed, synaptic neurotransmission can be modulated by interventing the level of 2-AG, supporting the role of 2-AG as an endogenous ligand of CB_1 receptor and mediator of retrograde signaling.

Epilepsy

Approximately 1% of the human population is affected by epilepsy which is one of the most common chronic neurological diseases recognized since antiquity. The disease is a chronic pathophysiological condition characterised by spontaneous recurrent seizures with various etiology. Epilepsy can often be controlled by medication, however, it is common that the prolonged use of antiepileptic drug results in a resistance to the medication, and currently 30% of the patients do not have adequate seizure control despite the wide rage of antiepileptic drugs available. In patients whose seizures remain resistant to treatment with anticonvulsants, the surgical removement of epileptogenic focus area can be beneficial.

Temporal lobe epilepsy, one of the most common form of epilepsy in adults, is a simple or complex symptomatic type of epilepsy syndrome, and in most cases the epileptogenic area is found in the hippocampus. Patients with temporal lobe epilepsy usually do not respond sufficiently to medication, for these patients surgical removal of the epileptic focus can result in seizure control.

The role of endocannabinoid system in epilepsy

The use of cannabis for therapeutic purposes dates back thousands of years: treatment of epileptic seizures with the use of hashish was first described in the 15th century.

Anticonvulsant effects of cannabinoids were first shown experimentally in the 1970s. Since then, it has been demonstrated in several experimental animal models of epilepsy that the activation of the endocannabinoid system affects the formation of seizures by blocking neuronal excitability and protecting against excitotoxic damage. Thus, activation of retrograde endocannabinoid signaling may be a key function in attenuating seizures associated with excess presynaptic activity. Generally, the phytocannabinoids and synthetic CB_1 agonists are anticonvulsants, while CB_1 antagonists evoke epileptic seizures. In addition, endocannabinoid synthesis occurs in the brain as a consequence of seizures, and these substances also affect the outcome of the brain trauma in animals. Expression of the cannabinoid receptor may also change due to the convulsions, however, results are controversial partly because changes in excitatory and inhibitory terminals are not considered separately.

AIMS

Although the anatomical localization and functional significance of the endocannabinoid system is relatively well-known in the nevous system of rodents, despite its therapeutic significance, almost no data are available about the anatomical arrangement and physiological function of molecular elements of the endocannabinoid system in the human brain. Therefore we aimed to investigate the anatomical localization of the metabolic enzymes and receptor of synaptic endocannabinoid 2-AG in the excitatory synapses of human hippocampus. In our study, we aimed to investigate the following questions:

1. In which layers of the human hippocampus is the 2-AG synthesizing enzyme DGL-α expressed, and in which subcellular domain is it situated?
2. What is the distribution of 2-AG's degrading enzyme MGL in the human hippocampus?
3. Is CB_1 cannabinoid receptor present in the excitatory axon terminals of the human hippocampus?

The role of the endocannabinoid system has been demonstrated in several pathophysiological conditions using animal models. Activation of the endocannabinoid system exerts anticonvulsant and neuroprotective effects, which suggests a novel therapeutic potential in human patients. However, the progession of epilepsy may impair the function of this endogenous safeguard system protecting against excessive stimulation, for example in multidrug resistant patients who are regularly exposed to epileptic seizures. Therefore, we examined whether the operation of the endocannabinoid system is impaired due to chronic epilepsy, more precisely, if the quantity of the elements of the endocannabinoid signaling is changed in the hippocampus of temporal lobe epileptic patients. We aimed to answer the following questions in our study:

4. Is the gene expression level of CB_1 receptor or cannabinoid receptor interacting proteins changed in the hippocampus of temporal lobe epileptic patients?
5. Is the gene expression level of enzymes responsible for metabolism of 2-AG and anandamide altered in the hippocampus of temporal lobe epileptic patients?
6. Is the quantity of CB_1 receptor protein changed in the hippocampus of temporal lobe epileptic patients?
7. Which type of axon terminals are affected by the changes of CB_1 cannabinoid receptor in the epileptic patients?

MATERIALS AND METHODS

Human tissue samples

We have examined control and epileptic human hippocampi in our experiments. Control subjects died suddenly from causes unrealted to any brain disease, and surgically removed epileptic hippocampal samples were obtained from patients with therapy-resistant temporal lobe epilepsy. Two major types of epileptic hippocampal samples were selected for our study: non-sclerotic samples showed only minimal cell loss in the CA1 region, however in sclerotic samples the CA1 region was robustly shrunken and atrophic due to the loss of 90% of principal cells.

Mouse tissue samples

We used adult CD1 mice in qPCR experiments: control animals were killed immediately after anesthesia and were compared with animals submitted to 4 hour long postmortem period or 4 hour long anesthesia, respectively. DGL-α knock-out and wild type mice kindly provided by Prof. Masanobu Kano were used to investigate the specificity of DGL-α antibody. Flourescent immunocytochemical reactions were performed in mice on C57BL/6H background that were perfused transcardially with 4% paraformaldehyde.

Quantitative real time polimerase chain reaction

For qPCR reactions total RNA samples were isolated from hippocampal tissue samples and cDNA samples were prepared by reverse transcription. Expression level of target genes was determined by quantitative real time PCR. To ensure reaction specificity melting curve analysis, sequencing and gel electrophoresis verifying the amplified fragments, were performed. Expression level of the target genes was normalized to the expression level of two different reference genes. To compare the expression level of target genes between different experimental groups model of Pfaffl was applied, and statistical analysis of data was performed by REST software.

Immunocytochemistry

60μm thick slices were cut from the tissue blocks and were processed for immunstaining. Sections were first blocked then incubated in the antibody of interest, then treated with a biotinylated secondary antibody generated against the animal producing the primary antibody. Sections were then incubated with avidin biotinylated-horseradish peroxidase complex, and reaction showing the localization of the protein of interest was developed using DAB as chromogen. Sections were treated with osmium and uranyl then dehydrated in an ascending series of ethanol, and embedded in resin. In fluorescence immunostainings sections were incubated in secondary antibodies labeled with fluorescent dyes, and fluorescent signal was detected by a Nikon Eclipse Ti-E inverse microscope equipped with an A1R confocal system.

Quantitative electronmicroscopical analysis

To investigate the specificity of the antibody recognizing DGL-α, we performed an electronmicroscopic analysis in the stained CA1 radiatum of the hippocampi of wild type and knock-out mice. Tissue samples of interest we reembedded, resectioned and series of ultrathin sections were collected on Formvar-coated grids, then counterstained with lead citrate. Electron micrographs were taken at 20000x magnification with a Hitachi 7100 electron microscope. DGL-α immunopositive and immunonegative dendritic spine heads receiving asymmetric synapses were counted in a counting frame of identical area in wild type and knock-out mice.

Series of ultrathin human sections from the inner molecular layer of the dorsal dentate gyrus were prepared and photographed similarly, and were analysed with the physical disector stereological method by counting CB_1 immunopositive and immunonegative axon terminals forming asymmetric or symmetric synapses.

RESULTS

Light- and electronmicroscopic distribution of the molecular elements of 2-AG mediated signaling in the human hippocampus

Specificity of the antibody recognizing DGL-α enzyme

We first aimed to indentify a DGL-α specific antibody with high specificity using hippocampi of wild type and DGL-α knock-out mice. In the wild type animals a strong DGL-α-immunreactivity is present according to the topographic arrangement of glutamatergic pathways of the hippocampus. At higher magnification, an intense punctate staining was observed in the CA1 region of the hippocampus, however, immunoreactivity was almost fully absent in DGL-α knock-out hippocampus. Further electron microscopic analysis revealed that in the wild type mice DGL-α immunoreactivity was present in 24% of dendritic spine heads receiving asymmetric synapses, in contrast, sections taken from the DGL-α knock-out mouse contained only a negible amount (1%) of DGL-α-immunoreactivity in spine heads.

Regional distribution of the enzymes responsible for 2-AG metabolism in the mouse and human hippocampus

At low magnification, immunofluorescent reaction for DGL-α resulted in a layer-specific fluorescent signal in the CA1-CA3 regions of the hippocampus and dentate gyrus, in mice and human. With the lack of MGL knock-out mice we could not prove the specificity of antibodies raised against MGL, so instead we performed immunocytochemical investigations with two independent antibodies (MGL-mid and MGL-NT) raised against different epitopes of MGL. In the hippocampus of mice the two independent antibodies revealed an almost identical immunofluorescent signal. Similarly to mice, MGL staining highlighted the layers of the human hippocampus corresponding to excitatory pathways.

Localization of 2-AG synthesizing DGL-α enzyme in the human hippocampus

Immunostaining in the human hippocampus showed striking similarities with the pattern observed in wild type mice at a high magnification: a widespread granular pattern of DGL-α immunoreactivity was visible in the human hippocampus. Immunopositive granules were distributed along the immunonegative main trunk of apical dendrites of pyramidal cells in the CA1 stratum radiatum of hippocampus and oblique dendrites of granule cells in the molecular layer of dentate gyrus, respectively.

To determine which subcellular compartment corresponds to the punctate DGL-α staining pattern, stratum radiatum of CA1 region and molecular layer of the dentate gyrus from human hippocampal sections were processed for electron microscopy. DAB end product of the immunoperoxidase staining procedure, representing the subcellular position of DGL-α, was concentrated in dendritic spine heads, in accordance with the localization of DGL-α in rodent hippocampi.

Distribution of 2-AG degrading enzyme MGL in the human hippocampus

Similarly to DGL-α immunostaining, the distribution pattern of MGL mirrored the laminar structure of the human hippocampal formation. At higher magnification, dendrites of pyramidal and granule cells were largely devoid of immunolabeling for MGL, however, neuropil among these dendrites contained a dense, punctate MGL-positive staining.

To understand the ultrastructural distribution of MGL, we performed electron microscopic analysis of MGL-immunostaining in the human hippocampus. At asymmetric, presumably glutamatergic synapses, MGL-immunoreactivity was restricted to presynaptic axon terminals both in the stratum radiatum of CA1 region of the hippocampus or molecular layer of the dentate gyrus. DAB end product indicating the presence of MGL protein was predominantly found close to synaptic vesicles and to active zone release site. Our results are in accordance with MGL-immunoreactivity in rodent hippocampi suggesting MGL to be located in boutons terminating on excitatory synapses.

Localization of CB_1 cannabinoid receptor in the human hippocampus

To investigate the CB_1-immunoreactivity in the human hippocampal formation we took advantage of a novel, highly sensitive antibody for CB_1 that visualizes both glutamatergic and GABAergic axon terminals in the rodent. Immunostaining for CB_1 receptor revealed a similar distribution to that previously reported in mouse hippocampi in previous reports. CB_1-immunoreactive fibers covered the hippocampus according to the laminar structure of the hippocampus. Beside the axonal immunostaining, scattered interneuron bodies immunoreactive for CB_1 were also observed.

To determine the ultrastructural localization of CB_1-immunolabeling we examined the molecular layer of dentate gyrus by electron microscopy in the human hippocampal formation. CB_1 immunoreactivity was detected in two types of axon terminals at the electron microscopic level: besides the previously described GABAergic boutons, the glutamatergic axon terminals also contained CB_1 immunolabeling. GABAergic terminals forming symmetric synapses on dendrites usually showed a stronger immunoreactivity than glutamatergic terminals that innervate dendritic spine heads.

Expressional changes in the elements of the endocannabinoid system in the human temporal lobe epileptic hippocampus

CB_1 cannabinoid receptor mRNA level is downregulated in the hippocampus of temporal lobe epileptic patients

Quantitative real time PCR measurements revealed that CB_1 receptor mRNA level was robustly downregulated in epileptic samples compared to expression level in control subjects. In non-sclerotic epileptic hippocampi, mRNA level of CB_1 was decreased to 37,1% or 46,5% normalized to β-actin ($p < 0,01$) or GAPDH ($p = 0,032$), respectively. Likewise, mRNA level of CB_1 in sclerotic epileptic hippocampi was reduced to 28,9% or 30,1% normalized to β-actin ($p < 0,001$) or GAPDH ($p < 0,01$), respectively.

The effect of postmortem delay or prolonged anesthesia on the mRNA level of CB_1 receptor

Epileptic and control postmortem hippocampal samples are processed in a different way prior to their removal, which may influence the interpretation of the above

measurements. To investigate the possibility that either postmortem delay or sustained anesthesia caused gene expression change for CB_1 receptor we studied two animal models, however neither a postmortem period of 4 hours nor sustained anesthesia for 4 hours affected the mRNA level of CB_1. These experiments indicate that these effects are unlikely to account for the downregulation of CB_1 receptors in the human epileptic hippocampus.

Expression level of cannabinoid receptor-interacting protein CRIP1 in epileptic human hippocampi

qPCR measurements uncovered that in the sclerotic epileptic hippocampus mRNA level of CRIP1a was significantly decreased to 69,6% or 68,2% normalized to β-actin ($p = 0,026$) or GAPDH ($p = 0,016$), respectively, compared to control hippocampi. In non-sclerotic epileptic hippocampi, mRNA level of CRIP1a did not change, and similarly, CRIP1b splice variant did not show any expression change in non-sclerotic or sclerotic epileptic hippocampi.

Expression level of enzymes responsible for endocannabinoid metabolism in the epileptic samples

First, we examined the mRNA level of enzymes synthesizing and degrading endocannabinoid 2-AG. In the sclerotic epileptic hippocampi, mRNA level of DGL-α synthesizing enzyme was significantly diminished to 44,6% or 37,9% compared to control samples normalized to β-actin ($p = 0,028$) or GAPDH ($p = 0,037$), respectively. In contrast, we found no difference in the expression level of DGL-α between non-sclerotic epileptic and control hippocampi. Expression level of synthesizing enzyme DGL-β and degrading enzyme MGL did not change in any of the examined epileptic tissue types compared to control.

Then we measured the expression of enzymes responsible for the level of endocannabinoid anandamide: we found no significant change in the gene expression of synthetic enzyme NAPE-PLD or degrading enzyme FAAH in the human epileptic hippocampal tissues, compared to control samples.

Density of CB_1 receptor immunoreactivity is reduced in the epileptic human hippocampus

Immunostaing for CB_1 receptor gave us the opportunity to extent the results obtained at the mRNA level also to the protein level. Compared with control hippocampi, general CB_1 immunostaining was much weaker in the hippocampus of non-sclerotic and sclerotic epileptic patients. The most striking difference in CB_1 immunoreactivity was in the inner molecular layer of the dentate gyrus: the dense CB_1-labeled neuropil present in control samples disappeared nearly completely in sclerotic samples, but labeling was also diminished in the hippocampi of non-sclerotic epileptic patients. Based on light microscopic investigation at high magnification, decrease in CB_1 immunoreactivity presumably affects smaller glutamatergic terminals, and not bigger GABAergic boutons.

Ratio of CB_1-positive excitatory axon terminals decreased in the inner molecular layer of dentate gyrus of epileptic hippocampi

To investigate the above observation we photographed serial ultrathin sections by electron microscopy in the inner molecular layer, and analysed them with a stereological method to estimate the numerical density of CB_1-positive and negative glutamatergic axon terminals. In control samples, the majority of glutamatergic boutons were positive for CB_1 (72,8 ± 2,1%), this ratio was significantly reduced in non-sclerotic (50,0 ± 2,8%) and sclerotic (21,0 ± 3,8%) epileptic human hippocampi ($p < 0,001$). The estimated numerical density of CB_1-positive axon terminals was 0,648 ± 0,075 /μm^3 in control samples, which decreased to 0,300 ± 0,051 /μm^3 and 0,112 ± 0,021 /μm^3 ($p < 0,001$) in non-sclerotic and sclerotic epileptic tissues, respectively. In contrast, the density of CB_1-negative boutons showed an increasing trend in the epileptic (non-sclerotic: 0,326 ± 0,062 /μm^3, sclerotic: 0,454 ± 0,070 /μm^3) samples, compared to control (0,248 ± 0,038 /μm^3), and a significant difference was found between control and sclerotic samples ($p = 0,037$).

However, ratio of CB_1-positive inhibitory axon terminals and estimated numerical density of CB_1-positive or negative symmetric inhibitory axon terminals did not change significantly between experimental groups.

SUMMARY

We explored the precise anatomical localization of molecular elements of the endocannabinoid system in the human hippocampus by light and electron microscopical investigations, and we found the following:

1. 2-AG's synthesizing enzyme DGL-α is localized postsynaptically in the dendritic spine heads of pyramidal and granule cells.
2. MGL, the enzyme responsible for 2-AG degradation, is present in the excitatory axon terminals presynaptically.
3. CB_1 cannabinoid receptor is also found on excitatory axon terminals, in addition to the previously described inhibitory terminals.

We examined if the quantity of different elements of the endocannabinoid system is altered in the hippocampus of temporal lobe epileptic patients. In our study we demonstrated the followings:

4. mRNA level of CB_1 cannabinoid receptor is decreased in non-sclerotic and sclerotic epileptic hippocampi compared to control values. Moreover, we found less of CRIP1a mRNA in the sclerotic epileptic hippocampi.
5. Gene expression level of DGL-α is decreased in the sclerotic epileptic hippocampi, however, we found no difference in the expression of enzymes responsible for anandamide synthesis or degradation.
6. Density of CB_1-immunoreactivity is reduced in the hippocampus of epileptic patients, mainly in the inner molecular layer of the dentate gyrus.
7. We found a descrease in the ratio of CB_1 receptor containing excitatory axon terminals in the inner molecular layer, but the ratio of CB_1-positive inhibitory terminals was not altered.

CONCLUSION

Immunocytochemical findings demonstrate the post- and presynaptic segregation of enzymes responsible for synthesis and elimination of 2-AG, respectively, and the presynaptic localization of CB_1 cannabinoid receptor in the human hippocampus. Thus, the molecular architecture of the 2-AG mediated endocannabinoid signaling supports the theory that 2-AG is a retrograde messenger molecule regulating synaptic activity by the modulation of neurotransmitter release. Its similar blueprint in rodents and humans further indicates that 2-AG's physiological role as a negative feed-back signal regulating neurotransmitter release is an evolutionarily conserved feature of excitatory synapses. Thus, understanding the functional role of the rodent endocannabinoid system may be of pivotal importance in the therapeutic exploitation of the endocannabinoid system in numerous pathological disorders of the nervous system.

We showed with comparative expression profiling and high-resolution electron microscopic analysis that CB_1 receptor expression and the fraction of glutamatergic axon terminals equipped with CB_1 are downregulated in the hippocampus of temporal lobe epileptic patients. These findings suggest that the protective endocannabinoid signaling pathway is disrupted in the hippocampus of drug resistant chronic epileptic patients. Along with the decrease in CB_1, the level of 2-AG synthesizing enzyme DGL-α was also negatively altered, implying that impairment of this synaptic circuit breaker in the excitatory synapses is diminished in the hippocampal formation of patients with intractable temporal lobe epilepsy, and may account for reduced seizure threshold and increased neuronal damage in epileptic patients.

LIST OF PUBLICATIONS

Publications that form the basis of the Ph.D. dissertation:

***Anikó Ludányi**, Loránd Erőss, Sándor Czirják, János Vajda, Péter Halász, Masahiko Watanabe, Miklós Palkovits, Zsófia Maglóczky, Tamás Freund, István Katona* (2008) Down-regulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus, The Journal of Neuroscience, 28:2976-90.

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Other publications:

*Viktor Varga, Balázs Hangya, Kinga Kránitz, **Anikó Ludányi**, Rita Zemankovics, István Katona, Ryuichi Shigemoto, Tamás Freund, Zsolt Borhegyi* (2008) The presence of pacemaker HCN-channels identifies theta rhythmic GABAergic neurons in the medial septum, The Journal of Physiology, 586:3893-915.

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