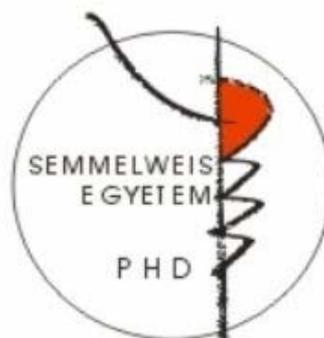


Pre- or postsynaptic distribution of distinct endocannabinoid-synthesizing enzymes at chemical synapses

Doctoral (Ph.D.) Theses

Rita Nyilas

Semmelweis University
János Szentágothai School of Ph.D. Studies



Supervisor: István Katona, Ph.D.

Scientific Referees for the Ph.D. Dissertation: Katalin Halasy, Ph.D., D.Sc.
Zita Puskár, Ph.D.

Committee Members at the Comprehensive Exam: Béla Halász, Ph.D., D.Sc.
Katalin Schlett, Ph.D.
József Takács, Ph.D.

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INTRODUCTION

Molecular and anatomical architecture of the endocannabinoid system

The endocannabinoid molecules are endogenous bioactive lipid-derivatives acting on type 1 and 2 cannabinoid receptors (CB₁ and CB₂), the molecular targets of Δ^9 -tetrahydrocannabinol (THC; Gaoni & Mechoulam, 1964), the main active compound of marijuana (*Cannabis sativa*). They are generated from neuronal cell membrane component polyunsaturated fatty acids with long carbon chains through several enzymatic steps (Piomelli, 2003). Besides the first discovered and most well-known anandamide (N-arachidonylethanolamine; Devane et al, 1992), acting as a full CB₁ agonist, the second identified endocannabinoid, the 2-arachidonoylglycerol (2-AG; Mechoulam et al, 1995; Sugiura et al, 1995) seems to have a more significant physiological role.

Cannabinoid receptors in the brain

The effects of endocannabinoids in the nervous system is predominantly mediated by the G_{i/o} protein-coupled, the 7 transmembrane domain-containing CB₁ cannabinoid receptor (Matsuda et al, 1990). Its significance is supported by the fact that mainly this receptor is responsible for the behavioral effects of THC (Ledent et al, 1999; Zimmer et al, 1999, Monory et al, 2007; Huestis et al, 2001). The highest expression level of CB₁ is in the central nervous system in the body (Howlett et al, 2002; Pacher et al, 2006), and it is one of the most abundantly expressed G-coupled receptor proteins in the brain (Herkenham et al, 1990).

2-AG mediated signaling in the central nervous system

2-AG that is synthesized in a 170 times larger amount than anandamide (Stella et al, 1997), plays an outstanding role in the processes of the nervous system. It is considered to be the real endogenous ligand of the CB₁ receptor, although it also activates CB₂ (Sugiura et al, 1999, 2000, 2006), and presumably functions as a synaptic endocannabinoid signaling molecule in the central nervous system (Freund et al, 2003; Kim & Alger, 2004; Makara et al, 2005).

Molecular elements of 2-AG synthesis and degradation

2-AG, being a molecule, which can easily diffuse through the plasma membrane of cells, is not stored in synaptic vesicles, but synthesized 'on demand' (Piomelli, 2003). The biosynthesis of 2-AG in the nervous system occurs predominantly in two enzymatic steps, mediated by phospholipase C β (PLC β) and diacylglycerol lipase- α (DGL; Sugiura et al, 2006), and it is degraded by monoacylglycerol lipase (MGL; Dinh et al, 2002; Blankman et al, 2007).

2-AG as retrograde synaptic endocannabinoid signaling molecule

A basic molecular organization scheme of 2-AG signaling in several brain areas supports the retrograde action of 2-AG in chemical synapses (Lafourcade et al, 2007; Uchigashima et al, 2007; Mátyás et al, 2008) with presynaptic positioning of CB₁ receptor and MGL (Katona et al, 1999, 2000, 2001, 2006; Gulyás et al, 2004) in axon terminals, and postsynaptic localization of DGL- α (Katona et al, 2006; Yoshida et al, 2006), the primary biosynthetic enzyme of 2-AG (Bisogno *et al.*, 2003), and PLC β (Fukaya et al, 2008). Electrophysiological studies also imply a retrograde role of 2-AG as a synaptic messenger (Melis et al, 2004; Makara et al, 2005; Hashimotodani et al, 2007, 2008).

2-AG has been proposed to function as a synaptic circuit breaker at brain glutamatergic synapses (Katona & Freund, 2008). Upon excess presynaptic activity, it is released from the postsynaptic neuron, passes the synaptic cleft and activates presynaptic CB₁ receptors, leading to the reduction of further neurotransmitter release from the axon terminals (Wilson & Nicoll, 2002). Variations of this basic functional logic are responsible for various forms of short-term and long-term synaptic plasticity mediated by 2-AG (Chevaleyre et al, 2006), which can be the mechanisms underlying activity-dependent or pathological modulations of specific neuronal functions.

Anandamide and other N-acyletanolamines (NAEs) in the brain

Among endogenous cannabinoids, the first discovered anandamide (Devane et al, 1992) is the most intensively studied signaling molecule. Based on its chemical structure, it belongs to the a sizeable family of bioactive lipids, the N-acyletanolamines (NAEs), with whom it shares common metabolic pathways for generation and inactivation in neurons (Di Marzo et al, 1994; Cravatt et al).

Metabolic pathways of NAEs

Anandamide and related NAEs are synthesized from their membrane precursors N-acyl-phosphatidyl-ethanolamines (NAPEs; Di Marzo et al, 1994; Okamoto et al, 2007). NAPEs are generated from phospholipids by a not-yet identified, calcium-dependent N-acyl-transferase (NAT) enzyme in the brain (Cadas et al, 1997). In the most well-known, direct metabolic cascade, NAPE molecules are hidrolized to phosphatidic acid and NAEs by an enzyme, called N-acylphosphatidyl-ethanolamine-hydrolizing phospholipase D (NAPE-PLD; Okamoto et al., 2004). Distinct NAE molecules have different acyl-groups bound to ethanolamine, which is arachidonic acid in anandamide (AEA), palmitoic acid in N-palmitoylethanolamine (PEA), stearic acid in N-stearoylethanolamine (SEA), and oleic acid in N-oleoylethanolamine (OEA).

Effects of NAEs in the nervous system

Besides its well-known psychoactive role, a broad physiological and behavioral activity profile, including anxiolysis (Gaetani et al, 2009), significance in neurodegeneration (Hansen et al,

2001; van der Stelt et al, 2002; Basavarajappa et al, 2009), antidepressant effect (Mangieri & Piomelli, 2007; Bambico et al, 2008) has been documented for anandamide. PEA exerts antiepileptic, antinociceptive (Calignano et al, 1998; Cravatt és Lichtman, 2004), while OEA and SEA has anorexic (Rodriguez de Fonseca, 2001; Oveisi et al, 2004) effects, but OEA also has antinociceptive activity (Suardiaz et al, 2007). However, despite their compelling functional importance *in vivo*, the underlying molecular architecture of anandamide and NAE synthesis and their cell-physiological roles in the brain have so far been elusive.

The role of the endocannabinoid system in the processes of CNS with excess neuronal activity

The endocannabinoid system, through activity-dependent modulation of short-term and long-term synaptic efficacy, plays an important role in hippocampal functions, such as learning and memory (Chevalere et al, 2006). In pathological states, characterised by augmented neuronal excitability, the same endocannabinoid system, crucial for synaptic plasticity in learning and memory processes, acts as a synaptic circuit breaker and exerts neuroprotective and anticonvulsive effects (Nagayama et al, 1999; Panikashvili et al, 2001; van der Stelt et al, 2001; Wallace et al, 2001, 2002, 2003; Shafaroodi et al, 2004).

Another example for a neuronal network function with synaptic plasticity and pathological excess neuronal activity, which is modulated by endocannabinoids, is nociception. Cannabinoids, that have been used to alleviate pain since antiquity, produce antinociception both in animal models of acute and reduce hyperalgesia and allodynia in persistent pain and in clinical studies (Pertwee, 2001; Walker és Huang, 2002; Walker és Hohmann, 2005; Pacher et al, 2006; Kogan és Mechoulam, 2007). The antinociceptive role of endogenously produced cannabinoids has also been shown both in physiological (Hohmann et al, 2005; Suplita et al, 2006; Kinsey et al, 2009), both in pathological (Mittrirattanakul et al, 2006; Petrosino et al, 2006) pain states. However, in contrast to the proposed critical role of endocannabinoid 2-AG in the regulation of nociception and synaptic plasticity, the molecular machinery responsible for its action and its involvement in pain sensation has not been identified before in the spinal cord.

1) Molecular architecture of the 2-AG signaling pathway in inhibitory and excitatory synapses of the dorsal horn of the spinal cord and its role in the modulation of nociception

In our studies, we aimed to identify the molecular and anatomical basis of 2-AG synthesis and action in the dorsal spinal cord, underlying the role of 2-AG signaling in pain regulation by answering the following questions:

- Where is 2-AG synthesized in the spinal cord, do dorsal horn neurons express diacylglycerol lipase- α (DGL- α), the biosynthetic enzyme of 2-AG?
- In which dorsal horn layers are the DGL- α protein and the CB₁ cannabinoid receptor, the molecular target of 2-AG, present?
- Where are DGL- α , mGluR₅, the upstream activator of 2-AG synthesis and CB₁ receptor localized subcellularly in the spinal dorsal horn?
- In what types of synapses is CB₁ receptor situated in the superficial dorsal horn?
- What is the role of the spinal retrograde 2-AG signaling a behavioral phenomenon, called stress-induced analgesia? (in collaboration with Laura Gregg and Andrea Hohmann)
- What is the role of presynaptic CB₁ receptors located on inhibitory dorsal horn interneurons in activity-dependent central hyperalgesia? (in collaboration with Hanns Ulrich Zeilhofer's laboratory)

2) Localization of NAPE-PLD in hippocampal excitatory axon terminals

In our further experiments, we aimed to uncover the regional, cellular and subcellular distribution of N-acylphosphatidyl-ethanolamine-hydrolyzing phospholipase D (NAPE-PLD), a biosynthetic enzyme of anandamide and its related bioactive congeners (Okamoto et al., 2007), in the central nervous system. Our questions were the following:

- Which cell types of the forebrain and the hippocampus express NAPE-PLD mRNA?
- In which hippocampal region is the protein of NAPE-PLD enzyme present?
- At electron microscopic level, does NAPE-PLD have a pre- or a postsynaptic localization in hippocampal glutamatergic synapses?
- In what subcellular compartment does NAPE-PLD accumulate in hippocampal mossy fiber and Schaffer collateral terminals?

METHODS

Preparation of tissue sections for anatomy and molecular biology

Experiments were carried out using C57BL/6, CB₁ knockout (KO; Zimmer et al, 1999) and NAPE-PLD KO (Leung et al, 2006) mice and Wistar rats. For immunocytochemistry and *in situ* hybridization, 50 µm-thick sections were made from fixed tissue of perfused animals, deeply anaesthetized with a mixture of ketamine-xylazine. For sections subjected to *in situ* hybridization, diethylpyrocarbonate (DEPC)-treated buffer was used and sectioning was performed under RNase-free conditions.

Riboprobes for *in situ* hybridization and cDNA for *real-time* PCR reactions derived from total mouse hippocampal or frontal cortex mRNA. Animals under isoflurane anaesthesia were decapitated, the brains were immediately removed from the skull, and the specific brain regions were isolated on dry ice. Afterwards, the unfixed tissue was homogenized by an ultrasonic homogenizer on ice, and total RNA samples were isolated and purified from the lysates. cDNA samples were prepared by reverse transcribing 1 µg of total RNA.

In situ hybridization

In our experiments, digoxigenin-labeled RNA-probes prepared against the mouse DGL- α and NAPE-PLD coding sequence were used. The whole hybridization procedure was carried out in a free-floating manner. Slices were incubated with sheep anti-digoxigenin Fab fragment conjugated to alkaline phosphatase in a blocking solution containing normal goat serum. The reaction was developed with the chromogen solution containing 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium chloride.

Immunocytochemistry

Free-floating spinal cord and forebrain sections were blocked in normal goat or donkey serum, then were incubated with the appropriate primary antibody. Then, depending on the developing method of the immunostaining, biotinylated secondary antibodies (immunoperoxidase-based immunocytochemistry) or secondary antibodies conjugated with fluorescent molecules or biotinylated secondary antibodies followed by fluorescently labeled tertiary antibodies (immunofluorescence staining) or 0.8 nm gold-conjugated goat anti-guinea pig or goat anti-rabbit secondary antibodies (pre-embedding immunogold labeling) were used.

After incubation with the biotinylated secondary antibody, localization of the protein of interest was visualized using avidin biotinylated-horseradish peroxidase complex followed

by H₂O₂ and 3,3'-diaminobenzidine (DAB) as chromogen. NAPE-PLD was developed with DAB-Ni method. Pre-embedding immunogold labeling was silver intensified.

Co-localization of two antigens was studied by double fluorescence immunostaining or, on electron microscopic level, by immunogold labeling combined with immunoperoxidase method. Sections for electron microscopy were treated with OsO₄ and uranyl acetate, dehydrated in an ascending series of ethanol and acetonitrile, and embedded in resin.

Electron microscopy and statistical analysis

For electron microscopic analysis, series of ultrathin sections (60 nm thickness) were collected on Formvar-coated single-slot grids and stained with lead citrate.

For the identification of primary afferent terminals in the spinal dorsal horn, we used the following morphological criteria defining synaptic glomeruli described previously (Ribeiro-da-Silva & Coimbra, 1982; Ribeiro-da-Silva, 1995). To determine whether the terminals fulfill these criteria, they were followed through consecutive serial sections.

Localization and density of NAPE-PLD immunoreactivity in hippocampal excitatory axon terminals was examined as a double blind experiment on immunogold labeled corresponding sections of wild-type and NAPE-PLD knockout littermate mice. For the comparison of immunogold labeling in NAPE-PLD wild-type and knock-out mice, χ^2 test was used. In the case of the analysis of the subcellular distribution of gold particles within hilar excitatory boutons, two groups were compared using two-tailed nonparametric Mann-Whitney *U* test.

Confocal microscopic analysis of immunofluorescence stainings

Immunofluorescence staining of spinal cord sections were analysed using a confocal laser-scanning microscope in a sequential acquisition mode. Colocalization of CB₁ with VGAT (VIAAT) or CGRP in axon terminals were counted in laminae I and II outer of the superficial dorsal horn, their ratios were calculated for each animal. Data were presented as mean \pm SD.

Quantitative real-time PCR measurements

For quantitative real-time PCR measurements, we used cDNA samples prepared from wild type and NAPE-PLD KO mice. Primers for the examined genes were designed to generate amplicons of 100–150 bp. In our negative control measurements, distilled water and samples without reverse transcriptase were used. To ensure reaction specificity and accurate

quantification, melting curve analysis was performed after each reaction, the length of the amplified fragments was further verified by gel electrophoresis.

Each reaction was repeated minimum three times in parallel, and the values included in statistical analysis were the means of these measurements. Expression level of the target genes was normalized to the expression level of β -actin. To compare the expression level of target genes between the experimental groups of wild type and NAPE-PLD KO animals, the efficiency calibrated model of Pfaffl was applied (Ludányi et al, 2008; Pfaffl, 2001, 2002). Statistical analysis of data were performed by two-sided nonparametric Mann–Whitney *U* test.

Behavioral experiments

The existence of the 2-AG signaling pathway in the spinal cord was confirmed in an *in vivo* animal model of stress-induced analgesia in collaboration with Andrea Hohmann's laboratory, using Sprague-Dawley rats. Analgesia was evoked by a brief electric footshock, different drugs or vehicle were administered intrathecally. Stress antinociception was quantified behaviorally by measuring the tail-flick latency.

The role of CB₁ receptors located on dorsal horn inhibitory axon terminals in secondary hyperalgesia was studied *in vivo* by Hanns Ullrich Zeilhofer's research group in Switzerland. Behavioral testing was done in adult wild-type (C57BL/6), CB₁ KO (Marsicano et al, 2002), *sns*-CB₁ KO (Agarwal et al, 2007) and *ptf1a*-CB₁ KO (Kawaguchi et al, 2002) mice and their floxed littermates. Intrathecal injections of drugs were made at the lower lumbar level under light isoflurane anesthesia. Subcutaneous injections of 10 μ l capsaicin were done in the hindpaw to evoke secondary hyperalgesia. Mechanical sensitivity (paw withdrawal thresholds) was assessed with dynamic von Frey filaments.

***In vitro* electrophysiology**

The inhibitory action of CB₁ receptors, situated on dorsal horn GABA/glycinergic axon terminals, on inhibitory synaptic inputs of excitatory neurons conveying nociceptive information, was examined in collaboration with Hanns Ullrich Zeilhofer and his colleagues. *In vitro* whole-cell patch-clamp recordings of postsynaptic current responses were made from superficial dorsal horn neurons in transverse lumbar spinal cord slices (250 – 300 μ m thick) prepared from young C57BL/6J mice.

RESULTS

Molecular bases of endocannabinoid modulation of nociception in the dorsal horn of the spinal cord

Expression and distribution of the DGL- α enzyme and the CB₁ receptor protein in the spinal cord

To determine the cellular source of 2-AG in the spinal cord, we first examined the expression pattern of DGL- α , its main synthesizing enzyme (Bisogno et al, 2003). Non-radioactive free-floating *in situ* hybridization on mouse spinal cord sections with an antisense riboprobe revealed widespread DGL- α expression throughout the spinal cord, whereas no significant labeling was found with the control sense probe. Most, if not all neurons expressed DGL- α mRNA at variable levels in the dorsal horn, but DGL- α positive cells were also scattered in the intermediate spinal cord. Remarkably, the highest DGL- α mRNA expression in the spinal cord was observed in large cells presumably corresponding to the spinal motoneurons in the ventral horn.

To study the distribution of the DGL- α protein in the spinal cord, immunostainings for DGL- α were carried out on wild-type mouse and rat spinal cord sections. A widespread punctate immunolabeling pattern was found with both antibodies directed against independent epitopes across the entire grey matter, with the highest density located in the dorsal horn of the spinal cord, the central termination zone of primary afferents. Besides the characteristic punctate labeling, scattered cell bodies immunoreactive for DGL- α were also visualized by the staining in the ventral spinal cord. When the peptide fragment used as epitope for antibody generation was added to the primary antibody incubation mixture no labeling was found on spinal cord sections.

After elucidating the characteristic staining pattern of DGL- α , the biosynthetic enzyme of 2-AG, we wanted to determine the localization of CB₁ cannabinoid receptor, the molecular target of 2-AG, in the dorsal horn of the spinal cord, a key area in pain information processing. Light microscopic analysis of peroxidase-based (DAB) CB₁ immunostaining performed by a highly sensitive antibody revealed a very similar distribution of the CB₁ receptor to that of DGL- α in the mouse and rat dorsal spinal cord. The most profound CB₁-immunoreactivity was seen in the superficial layers, where strong accumulation of the receptor protein was

represented by very dense puncta at higher magnification. Strong staining was also observed in the dorsolateral funiculus, where longitudinal fibers of intrasegmental connections, ascending projections and descending modulatory systems are situated, and also in lamina X, around the central canal, in a site of termination of visceral afferents. The specificity of the antibody was confirmed by the lack of immunolabeling in sections derived from CB₁ knockout mice.

Taken together, immunostaining for DGL- α and CB₁ provide evidence that the molecular components responsible for mediating 2-AG synthesis and action are present in the spinal cord with the highest density in the termination zone of primary nociceptive axons.

DGL- α enzyme is located postsynaptically, while CB₁ receptor is present in a presynaptic position at primary nociceptive synapses of the spinal dorsal horn

To determine which subcellular compartments underlie the characteristic punctate staining pattern observed at the light microscopic level, we performed a detailed electron microscopic analysis of DGL- α localization in mouse and rat spinal cord sections. Samples for electron microscopy were selected from layers I-III of the dorsal spinal cord to ensure complete primary afferent distribution in the superficial layers. High-magnification electron microscopic analysis demonstrated that the diffusible DAB end product of the immunoperoxidase staining, which indicates the subcellular localization of DGL- α , was concentrated in a large number of dendritic spine heads and also in small diameter dendrites. The DGL- α -immunopositive spines and dendrites received asymmetric synapses from DGL- α -negative excitatory axon terminals, including boutons belonging to either type I or type II synaptic glomeruli based on their morphological features (Ribeiro-da-Silva & Coimbra, 1982) and corresponding to nerve endings of unmyelinated C- or myelinated A δ -fibers, respectively.

Silver-intensified immunogold staining also confirmed the subcellular accumulation of DGL- α on spine heads and dendrites situated postsynaptically to small excitatory axon terminals and both types of synaptic glomeruli in the superficial dorsal horn.

To determine the precise subcellular distribution of 2-AG's molecular target, we used a highly sensitive second generation CB₁ antibody whose specificity has been confirmed in CB₁ knockout animals. Electron microscopic analysis in the superficial dorsal horn of mouse and rat spinal cords revealed that the diffusible reaction product DAB, indicating the presence of CB₁ receptor, was concentrated predominantly in excitatory axon terminals, forming asymmetrical synapses. Moreover, presynaptic CB₁ immunolabeling was also found in axon

endings that can be identified by morphological criteria as forming type I or type II synaptic glomeruli.

Immunogold staining also confirmed presynaptic CB₁ receptors on small boutons and on primary nociceptive axon terminals both forming asymmetrical synapses on their postsynaptic targets. Gold particles, indicating the precise subcellular position of CB₁, were always attached to the presynaptic plasma membrane, often adjacent to the active zone.

CB₁ receptor is present in a significant subset of central peptidergic primary nociceptive axon terminals

To further confirm that CB₁ cannabinoid receptors do occur presynaptically on central synapses of primary nociceptive afferents and, to quantify its distribution in boutons of peptidergic primary afferents, we performed confocal microscopic analysis on double immunofluorescence staining for CB₁ and calcitonin gene-related peptide (CGRP), a neurochemical marker of peptidergic sensory afferent fibers. Altogether 384 CGRP-positive axon terminals were analyzed in the superficial dorsal horn (n = 3 mice) and 42.9 ± 6.8% of these boutons were positive for CB₁. On the other hand, in the same spinal cord area, 41.56 ± 23.74% of 391 CB₁-immunoreactive terminals (n = 3 mice) contained CGRP.

mGluR₅ glutamate receptor, the upstream activator of 2-AG biosynthesis, co-localizes with the DGL- α enzyme at the postsynaptic side of primary nociceptive synapses in the mouse spinal cord

Our results, discussed above, provided anatomical evidence that mGluR₅ and DGL- α , the first and last molecular components in the biosynthetic pathway of the endocannabinoid 2-AG, are concentrated on the postsynaptic side of primary nociceptive synapses, while its effector, the CB₁ cannabinoid receptor is enriched presynaptically on primary afferent fibers and other excitatory axon terminals.

In the next set of experiments, we aimed to unravel whether there is an anatomical basis of group I metabotropic glutamate receptor (mGluR)-induced 2-AG synthesis in the dorsal spinal cord, as previously described in several brain areas (Robbe et al, 2002; Jung et al, 2005, 2007; Lafourcade et al, 2007; Uchigashima et al, 2007; Drew et al, 2008). In co-localization experiments, we performed double immunogold-immunoperoxidase (DAB) immunostainings using anti-DGL- α and anti-mGluR₅ antibodies. Extensive electron

microscopic analysis confirmed that DGL- α was indeed localized on mGluR₅-positive dendritic spines receiving asymmetrical synapses from central axon terminals of C- and A δ -fibers (type I or II synaptic glomeruli, respectively). These anatomical findings demonstrate that the enzymatic machinery of 2-AG synthesis and its upstream triggering receptor are located around the postsynaptic side of primary nociceptive synapses.

Retrograde 2-AG signaling pathway mediates mGluR₅-dependent stress-induced analgesia *in vivo*

The above anatomical findings indicate that the molecular machinery initiating retrograde 2-AG signaling is present at nociceptive synapses. Thus, in subsequent experiments, in collaboration with Andrea Hohmann's laboratory, we examined functional evidence for the involvement of this molecular machinery in endocannabinoid-mediated stress-induced analgesia (SIA), a behavioral phenomenon in which animals are less responsive to noxious stimulation following exposure to an environmental stressor. This animal model of stress-induced analgesia (Hohmann et al, 2005) was previously demonstrated to mobilize 2-AG in the lumbar spinal cord (Suplita et al, 2006).

Intrathecal administration of the group I mGluR agonist DHPG induced a time-dependent enhancement of post-shock tail-flick latencies relative to vehicle controls. On the contrary, the mGluR₅ antagonist MPEP strongly suppressed the antinociceptive effect produced by footshock and altered the time course of stress antinociception. The CB₁ antagonist rimonabant and THL, the most potent pharmacological inhibitor of DGL (Bisogno et al, 2003; 2006) presently available also blocked the time-dependent enhancement of stress antinociception induced by DHPG, supporting the central role of the mGluR₅-DGL- α -CB₁ pathway in mediating stress-induced analgesia in the spinal cord.

CB₁ cannabinoid receptors are also present presynaptically on inhibitory axon terminals of the superficial spinal dorsal horn

To study the presence of CB₁ cannabinoid receptor on inhibitory axon terminals, first we performed extensive electron microscopic analysis on serial spinal cord sections stained by peroxidase-based immunocytochemistry. We used an antibody against CB₁, of which specificity has been confirmed in CB₁ KO animals. Based on our experiments, a subset of putative inhibitory axon terminals forming characteristic symmetric synapses also

accumulated the diffusible reaction end product DAB, indicating the presence of CB₁ receptors in inhibitory axon terminals in the superficial dorsal horn (laminae I and II).

To determine the precise subcellular localization of CB₁ cannabinoid receptor on these boutons, we used the silver-enhanced immunogold technique with the same antibody. Immunogold labeling revealed the same result as immunoperoxidase staining, several axon terminals forming symmetric synapses contained gold particles indicating the precise localization of CB₁. These gold particles were always attached to the inner surface of the presynaptic plasma membrane, which is in accordance with the topological position of the epitope of the antibody, providing further support for the specificity of the antibody.

Although the morphological characteristics suggested that these symmetrical synapses are inhibitory axon terminals to confirm that this is indeed the case, we carried out colocalization experiments in the dorsal horn of the spinal cord. Two different anti-CB₁ antibodies and an antibody against vesicular GABA transporter (VGAT) were used in sequential double stainings combining the immunogold and the immunoperoxidase (DAB) techniques for each antibody in both directions. Extensive electron microscopic analysis confirmed that CB₁ is indeed localized (by both antibodies) on VGAT-positive GABAergic/glycinergic axon terminals giving a the characteristic symmetric synapses on dendritic shafts.

A smaller subpopulation of CB₁ receptors is found on the axon terminals of inhibitory interneurons

To quantify the ratio of inhibitory axon terminals among the CB₁ cannabinoid receptor-positive axon terminals, we performed confocal microscopic analysis on double immunofluorescence staining for CB₁ and VGAT. Altogether 641 CB₁-positive axon terminals were analyzed in the superficial dorsal horn (n=3 mice) and $7.86 \pm 2.57\%$ of these boutons were positive for VGAT. These findings using highly sensitive antibodies provide evidence that, besides on primary afferents and local excitatory interneurons (Farquhar-Smith et al, 2000), 5-10 % of CB₁ cannabinoid receptors do occur presynaptically on spinal GABAergic/glycinergic synapses.

CB₁ mediated reduction of neurotransmission of GABA-/glycinergic inhibitory interneurons is a key process leading to central hyperalgesia

The physiological significance of CB₁ receptors, located on inhibitory terminals has been revealed by our collaborators, Hanns Ullrich Zeilhofer and his research group. To define the role of CB₁ receptors in dorsal horn neuronal circuits, in their experiments, the effects of CB₁ receptor activation on neurotransmission was characterized in mouse transverse spinal cord slices. The mixed CB₁/CB₂ receptor agonist WIN 55,212-2 reversibly reduced the amplitudes of inhibitory postsynaptic current responses (IPSCs) mediated by GABA_A and strychnine-sensitive glycine receptors, evoked by extracellular electrical field stimulation and recorded from visually identified neurons located in the superficial spinal dorsal horn (laminae I and II). Inhibition of IPSCs by WIN was reversed by the CB₁ receptor antagonist/inverse agonist AM 251, providing evidence on the functioning CB₁ receptors on inhibitory axon terminals of the dorsal horn of the spinal cord, detected by high-resolution anatomical methods.

In the next set of experiments, it was tested whether stimulation of endogenous endocannabinoid production through activation of group I metabotropic glutamate receptors (mGluR_{1/5}) would have a similar effect on inhibitory synaptic transmission. These experiments were carried out in GlyT2-EGFP transgenic mice (Zeilhofer et al, 2005), which allowed targeted recordings from glycinergic and non-glycinergic, presumed excitatory, interneurons of the superficial layers. DHPG, an agonist at mGluR_{1/5}, reduced IPSC amplitudes via CB₁ activation (it was reversed by AM 251) in non-glycinergic, presumed excitatory superficial dorsal horn neurons, while glycinergic input to glycinergic neurons was less sensitive to DHPG. Moreover, depolarization-induced suppression of inhibition (DSI), which is known to be mediated by endocannabinoids, could be induced in the putative excitatory neurons, but was not seen in glycinergic neurons, indicating that mGluR_{1/5} activation disinhibited preferentially excitatory neurons in the superficial dorsal horn. These *in vitro* electrophysiological data suggest that upon activation of mGluR_{1/5}, endocannabinoids are released in the spinal dorsal horn and, through the activation of CB₁ receptors, reduce the release of GABA / glycine from inhibitory nerve terminals and disinhibit excitatory interneurons located in the superficial layers.

Next, the effects of pharmacological and genetic manipulation of the endocannabinoid system on subcutaneously injected capsaicin- (C-fiber-) induced secondary hyperalgesia were tested in wild-type mice. Consistent with the role of CB₁ receptors in synaptic disinhibition,

AM 251 reversed mechanical sensitization and, inhibition of endocannabinoid degradation with URB 597 (Piomelli, 2003) or of endocannabinoid re-uptake with UCM 707 (Piomelli, 2003) prolonged secondary hyperalgesia.

In line with these pharmacological experiments, global CB₁ KO mice (Marsicano et al, 2002) and mice lacking CB₁ receptors specifically in dorsal horn inhibitory interneurons (*ptf1a*-CB₁ KO mice; Glasgow et al, 2005) were protected from capsaicin-induced mechanical sensitization. By contrast, mice devoid of CB₁ receptors only in primary afferent nociceptors (*sns*-CB₁ KO mice; Agarwal et al, 2007) developed normal secondary hyperalgesia indicating that the CB₁ receptors on inhibitory dorsal horn neurons and not those on primary nociceptors mediated capsaicin-induced secondary hyperalgesia.

Taken together, these results imply critical role for CB₁ receptors situated on dorsal horn inhibitory axon terminals in central hyperalgesia, mediated by endocannabinoids that are released upon activation of mGluRs by nociceptive C-fibers.

Localization of a new lipid signaling pathway in the excitatory axon terminals of the hippocampus

N-acylethanolamine synthesizing NAPE-PLD is expressed in several brain regions of the mouse forebrain, with the highest level in the granule cells of the dentate gyrus

Despite that of 2-AG, little is known about the exact sites of synthesis and cellular role(s) of the first discovered endocannabinoid anandamide and its bioactive congeners in the nervous system. Therefore, in our further experiments, we studied the regional, cellular and subcellular localization of N-acylphosphatidyl-ethanolamine-hydrolyzing phospholipase D (NAPE-PLD), the biosynthetic enzyme of N-acylethanolamines (NAEs).

Nonradioactive free-floating *in situ* hybridization on mouse forebrain sections revealed that granule cells in the dentate gyrus may have the highest expression of NAPE-PLD in the forebrain. In contrast, no labeling was observed on sections derived from NAPE-PLD knockout animals, proving the specificity of the reaction. A somewhat weaker but still high expression level was found in CA3 pyramidal neurons, whereas NAPE-PLD mRNA was not observed in CA1 pyramidal neurons, in hippocampal interneurons, and in glial cells. Modest levels of NAPE-PLD mRNA were also observed in several other regions, e.g., in layers II–III of the neocortex, in layer II of the piriform cortex, in higher-order thalamic nuclei, and in the

ventromedial hypothalamic nuclei.

Localization of NAPE-PLD protein shows a characteristic laminar pattern in the hippocampus and refers to the presence of the enzyme in glutamatergic axon terminals

The highest amount of NAPE-PLD mRNA was found in the hippocampus thus, we focused our further analysis to this region. To determine the localization of NAPE-PLD protein, we used two independent antibodies, which stained identically in wild-type mice, but did not stain in knockout animals. In accordance with the high mRNA levels in dentate gyrus, immunoperoxidase staining for NAPE-PLD visualized a dense meshwork of labeling in the hilus and stratum lucidum, which are the termination zones of mossy fibers, the axons of granule cells. A weaker but still characteristic NAPE-PLD staining followed the laminar pattern of other glutamatergic fibers. Importantly, the stratum (str.) radiatum of CA1 subfield, which is the termination zone of Schaffer collaterals, the axons of CA3 pyramidal cells, was also visualized, in accordance with the moderate NAPE-PLD mRNA expression in these cells. At high magnification, immunostaining consisted of tiny granules homogeneously distributed in the neuropil.

NAPE-PLD is located presynaptically, in intracellular membrane cisternae of glutamatergic mossy fiber and Schaffer collateral terminals

The immunostaining pattern described previously, and the *in situ* hybridization results suggest that NAPE-PLD is a presynaptic enzyme confined to glutamatergic axon terminals. To test this hypothesis, we performed high-resolution immunogold staining for NAPE-PLD at the electron microscopic level with the anti-NAPE-PLD antibody that gave the stronger immunostaining in our preceding experiments. Quantitative analysis confirmed the presence of this enzyme in large mossy terminals in the hilus of wild-type but not of knockout animals ($n = 300$ – 300 axon terminals; $p < 0.001$). On the other hand, specific immunoreactivity was absent in the thorny excrescences, the postsynaptic targets of mossy fibers ($n = 300$ – 300 postsynaptic profiles; $p = 0.243$). To estimate the density of NAPE-PLD labeling in boutons, mossy terminals were followed through serial sections for a distance of 300 nm. Within this distance, 86.67% of the mossy terminals contained immunogold particles representing at least one NAPE-PLD molecule (median value; range, 73.33–93.33%; $n = 45$ terminals, 3 animals) thus, we can conclude that most if not all large mossy terminals contain NAPE-PLD.

To extend our observation to other glutamatergic synapses, we performed a similar analysis in stratum radiatum of the CA1 subfield, which revealed that Schaffer collaterals, but not their postsynaptic targets, also contain NAPE-PLD ($n = 300$ – 300 profiles; $p = 0.00148$ or $p = 0.257$ for Schaffer collaterals or postsynaptic spines, respectively).

Previous immunogold electron microscopic studies have shown that the biosynthetic enzyme of 2-AG is selectively localized to the postsynaptic plasma membrane (Katona et al, 2006). In contrast, the present quantitative analysis of immunogold particle distribution at high resolution found that NAPE-PLD was not associated with the plasma membrane, but rather accumulated on the external surface of intracellular membrane compartments. Furthermore, the majority of gold particles were attached to cisternae of smooth endoplasmic reticulum (SER; median, 58.08%; range, 52.63–62%; $n = 352$ gold particles; $n = 4$ animals).

The expression of TRPV4 channel is robustly down-regulated in NAPE-PLD knockout animals compared to wild type mice, which suggest a potential functional relationship between these two proteins

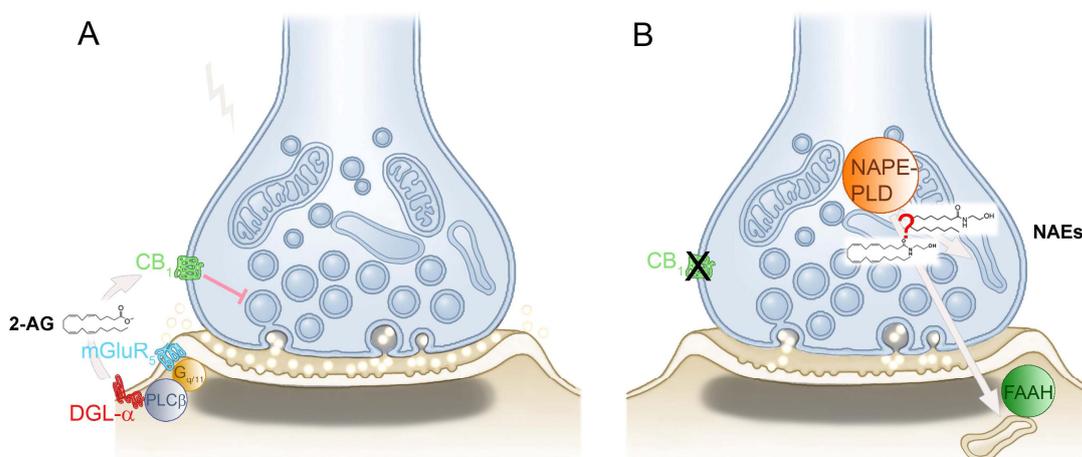
Anandamide, the first described endovanilloid, in certain circumstances, can act as a full agonist of the transient receptor potential vanilloid type-1 (TRPV1) channel (Ross, 2003) and is an indirect ligand of TRPV4 (Watanabe et al, 2003). Therefore, we wanted to know whether the presence or absence of NAPE-PLD, the synthesizing enzyme of anandamide and other NAEs, has an influence on the hippocampal expression of brain TRPV channels. In our experiments, quantitative *real-time* PCR measurements were done using cDNA samples derived from wild type mice and their NAPE-PLD knockout littermates to detect mRNA expression levels of TRPV1-4. Based on our data, gene expression level of the TRPV4 is robustly down-regulated (to approximately half of its normal level) in NAPE-PLD KO hippocampi as compared to wild type control tissue, suggesting a potential functional relationship between NAPE-PLD and TRPV4. In contrast to TRPV4, real-time PCR measurement did not reveal significant changes in the mRNA level of either of the other three TRPV channels (TRPV1, TRPV2 and TRPV3) in NAPE-PLD KO versus wild type hippocampi.

CONCLUSIONS

1) Our anatomical data provided evidence that mGluR₅ and DGL- α , the first and last molecular components in the biosynthetic pathway of the endocannabinoid 2-AG, were concentrated on the postsynaptic side of primary nociceptive synapses, while its effector, the CB₁ cannabinoid receptor was enriched presynaptically (summary figure, A). Furthermore, it was also demonstrated that these molecular players were functionally linked together to mediate endocannabinoid-mediated stress-induced analgesia *in vivo*. These findings suggest that nociceptive synapses are equipped with an intrinsic feedback system, the retrograde 2-AG signaling pathway, which regulates nociception in an activity-dependent manner.

2) On the other hand, spinal endocannabinoids, produced upon intense nociceptive stimulation, were demonstrated *in vitro* to activate also and reduce synaptic inhibition of spinal output neurons. On a behavioural level, this leads to pain sensitization called hyperalgesia, a key feature of pathological pain, revealing a novel, unexpected role of spinal 2-AG signaling in nociception.

3) Finally, our data demonstrated that, in contrast to its predicted role in retrograde signaling, NAPE-PLD was concentrated presynaptically, on intracellular membrane cisternae of hippocampal excitatory axon terminals (summary figure, B). Furthermore, the highest density of NAPE-PLD was found in mossy terminals of granule cells, which do not express CB₁ receptors. The remarkably different sites of NAE synthesis and action from that of 2-AG at glutamatergic synapses imply heterogeneous physiological roles for distinct endocannabinoids in the regulation of synaptic functions.



Summary figure. (A) Molecular architecture of 2-AG signaling regulating nociception in spinal primary nociceptive synapses. (B) Presynaptic localization of NAPE-PLD in hippocampal excitatory axon terminals and potential signaling pathways used by NAEs. (The schematic diagram of the chemical synapse was adapted and modified from www.universe-review.ca.)

PUBLICATIONS

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

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Nyilas R, Dudok B, Urban 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. *J Neurosci*, 28:1058-1063.

Other publications:

Papp AM, Nyilas R, Szepesi Z, Lorincz ML, Takacs E, Abraham I, Szilagyi N, Toth J, Medveczky P, Szilagyi L, Juhasz G, Juhasz G. (2007) Visible light induces matrix metalloproteinase-9 expression in rat eye. *J Neurochem*, 103:(6) 2224-2233.

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