Release and transport of endocannabinoids

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General introduction

Cannabinoid receptors

The plant *Cannabis sativa* has been used for medicinal and recreational purposes for centuries. It contains over 500 compounds, of which around 100 belong to the class of cannabinoids[1]. In the 1960s, the main psychoactive component, (−)-*trans*-Δ^9-tetrahydrocannabinol (THC), was isolated and characterized[2]. Three decades after this discovery, the cannabinoid (CB) receptors CB_1 and CB_2 were identified as the molecular targets of THC[3,4]. While they are activated by a common set of ligands, they share ~44% sequence homology and differ in their tissue expression profiles[5]. The CB_1 receptor is found throughout the body but is primarily expressed in the brain, where it is one of the most abundant G-protein-coupled receptors (GPCRs). The CB_2 receptor, on the other hand, is mainly expressed in cells of the immune system and is not expressed in the brain under physiological conditions[6]. As GPCRs, the CB receptors couple to intracellular heterotrimeric $G_{i/0}$ proteins (**Figure 1.1A**). Release of α and βγ subunits from the $G_{i/0}$ proteins upon receptor activation leads to adenylyl cyclase inhibition and stimulation of the MAPK and PI3K/Akt pathways, which can lead to changes in gene expression patterns. Additionally, activation of the CB_1 receptor also closes several types of calcium channels and opens inward rectifying potassium channels (**Figure 1.1A**)[7,8]. Coupling of CB receptors to β-arrestin through GPCR kinase activity results in internalization and desensitization[9]. Through these mechanisms, the CB receptors regulate a host of physiological functions including appetite, pain sensation, memory, and motor function.

The endocannabinoids

Shortly after the discovery of the first CB receptor, the endogenous ligands for the receptors (endocannabinoids), *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoyl glycerol (2-AG), were identified[10,11]. While other endogenous molecules, such as virodhamine, *N*-arachidonoyldopamine and noladin ether, are known to interact with the CB receptors, anandamide and 2-AG are the main endocannabinoids. Anandamide is a high-affinity partial agonist of the CB_1 receptor and has low-affinity binding for the CB_2 receptor[12,13]. 2-AG is a full agonist at both receptors with higher intrinsic activity than anandamide[14,15]. The endocannabinoids are derivatives of the polyunsaturated ω-6 fatty acid arachidonic acid and are each synthesized by their own set of enzymes from phospholipid precursors (**Figure 1.2**). Their destruction is also under tight enzymatic control.

Synthesis of anandamide starts with the formation of *N*-arachidonoyl phosphatidylethanolamine from phosphatidylethanolamine (PE) and a phospholipid containing arachidonic acid at its *sn*-2 position by an *N*-acyltransferase of the phospholipase A1/2 acyltransferase (PLAAT) family or the calcium-dependent *N*-acyltransferase (CaNAT) phospholipase A2 group IVE (PLA2G4E)[16]. Hydrolysis of the arachidonic acid containing *N*-acylphosphatidylethanolamine (NAPE) by NAPE-phospholipase D (NAPE-PLD) directly produces anandamide (**Figure 1.2**). Alternative pathways include hydrolysis of *O*-acyl chains of NAPEs prior to the release of

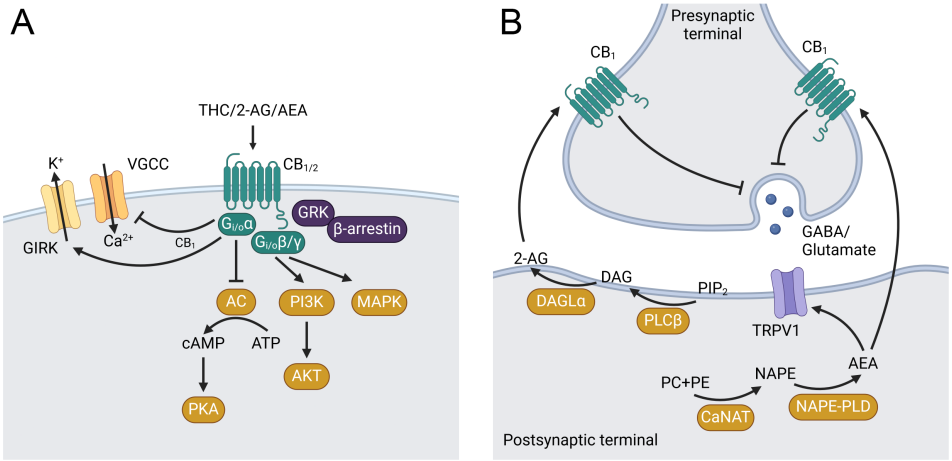

Figure 1.1 CB receptor signaling and synaptic modulation by endocannabinoids. (A) Activation of cannabinoid (CB) receptors by (endo)cannabinoids modulates ion channel activities and intracellular signaling cascades through $G_{i/o}$ proteins. CB receptors also couple to β-arrestins. **(B)** Sequential action of CaNAT and NAPE-PLD generates AEA. Both enzymes are found on intracellular membranes, with NAPE-PLD locating to presynaptic neurons. PLCβ and DAGLα associate with the plasma membrane and DAGLα is found on postsynaptic neurons. Anandamide and 2-AG activate CB_1 receptors on the presynaptic plasma membrane. Anandamide also activates TRPV1. AC: Adenylyl cyclase, 2-AG: 2-arachidonoyl glycerol, AEA: Anandamide, CaNAT: Calcium-dependent *N*-acyltransferase, DAG: Diacylglycerol, DAGLα: Diacylglycerol lipase α, GIRK: G protein-coupled inwardly rectifying potassium channel, GRK: G protein-coupled receptor kinase, MAPK: Mitogen-activated protein kinase, NAPE: *N*-acylphosphatidylethanolamine, NAPE-PLD: *N*-acylphosphatidylethanolamine phospholipase D, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PIP_2: Phosphatidylinositol-4,5-bisphosphate, PKA: Protein kinase A, PLCβ: Phospholipase Cβ, TRPV1: Transient receptor potential cation channel subfamily V member 1, VGCC: Voltage-gated calcium channel. Created with biorender.com.

anandamide[17–19]. Inactivation of the ligand through hydrolysis into arachidonic acid and ethanolamine is mainly catalyzed by fatty acid amide hydrolase (FAAH)[20] but can also be catalyzed by *N*-acylethanolamine hydrolyzing acid amidase (NAAA), a lysosomal hydrolase[21].

The main precursors for 2-AG formation are diacylglycerols (DAGs), which are formed by hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by phospholipase Cβ (PLCβ) and cleaved at the *sn*-1 position by DAG lipase α or β (DAGLα or β) to release 2-AG (**Figure 1.2**)[22,23]. Alternative pathways involve the formation of lysophosphatidylinositol, which can be hydrolyzed to 2-AG[24], and the sequential hydrolysis of triacylglycerols to diacylglycerols and 2-AG by hormone sensitive lipase as part of metabolic lipid mobilization[25]. The majority of 2-AG is inactivated by monoacylglycerol lipase (MAGL) through hydrolysis into arachidonic acid and glycerol[26,27]. α,β-hydrolase domain–containing proteins 6 and 12 (ABHD6 and ABHD12) are also able to hydrolyze 2-AG[28].

Synaptic modulation by endocannabinoids

2-AG and anandamide modulate various physiological processes by activating the cannabinoid receptors and triggering downstream signaling events. The effect of receptor activation largely depends on where the activated cannabinoid receptor is located. In the central nervous system, CB_1 receptors are found on presynaptic axon terminals in multiple brain regions such as hippocampus, cerebellum, amygdala, hypothalamus and others[8].

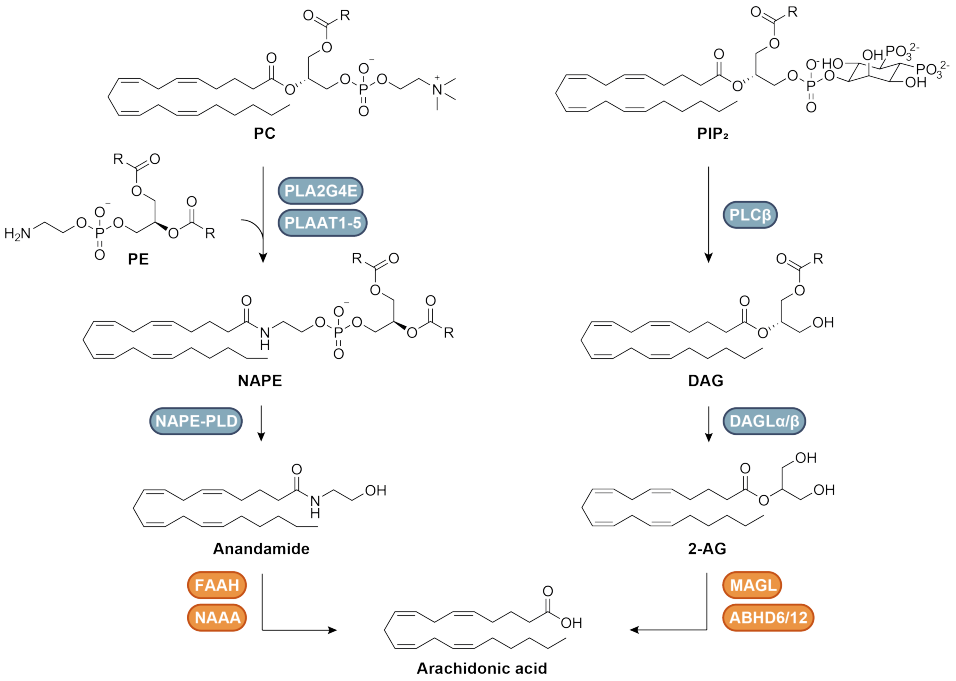

Figure 1.2 Major biosynthetic and degradation pathways of anandamide and 2-AG. Enzymes involved in endocannabinoid synthesis are depicted in purple. Enzymes degrading 2-AG and anandamide are shown in orange. 2-AG: 2-arachidonoyl glycerol, ABHD6/12: α/β-hydrolase domain–containing protein 6/12, DAG: Diacylglycerol, DAGLα/β: Diacylglycerol lipase α/β, FAAH: Fatty acid amide hydrolase, MAGL: Monoacylglycerol lipase, NAAA: *N*-acylethanolamine acid amide hydrolase, NAPE: *N*-acylphosphatidylethanolamine, NAPE-PLD: *N*-acylphosphatidylethanolamine phospholipase D, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PIP_2: Phosphatidylinositol-4,5-bisphosphate, PLCβ: Phospholipase Cβ.

Both excitatory and inhibitory neurons express CB_1 receptors, but expression is especially high in specific cholecystokinin-positive GABAergic interneurons[29]. CB_1 receptor activation modulates several forms of synaptic transmission, induced by the retrograde action of endocannabinoids. Binding to presynaptic CB_1 receptors leads to a reduction (depression) of neurotransmitter release. This is achieved through inactivation of presynaptic calcium channels, preventing the influx of calcium, which is necessary for synaptic vesicle exocytosis and neurotransmitter release[7]. If the reduction in neurotransmitter release is short (seconds to minutes), this is called short-term depression.

The most commonly found forms of endocannabinoid-mediated short-term plasticity are depolarization-induced suppression of inhibition (DSI) and excitation (DSE)[30]. In DSI and DSE, depolarization of the post-synapse leads to the release of endocannabinoids, more specifically 2-AG, which then activates CB_1 receptors of presynaptic terminals to reduce the release of γ-amino butyrate (GABA) or glutamate, respectively. Endocannabinoid-mediated short-term plasticity was first observed in the hippocampus[31,32] and cerebellum[33] but is found in many brain regions at synapses that express CB_1 receptors[34]. This demonstrates that endocannabinoids are important for the regulation of synaptic transmission throughout the brain[35].

In addition to short-term plasticity, endocannabinoids also mediate long-term plasticity, which lasts minutes to several hours. Long-term plasticity by endocannabinoids is induced post-synaptically but requires additional integration of presynaptic signals[36]. Anandamide acts as both an autocrine and retrograde messenger. In addition to its activity at CB receptors, anandamide induces long-term depression via autocrine signaling at TRPV1[37–39].

On-demand hypothesis

Due to their lipophilic nature, the endocannabinoids differ from regular neurotransmitters in that they cannot be stored in synaptic vesicles and freely diffuse across the extracellular space once released. Together with the observation that endocannabinoid levels increase with stimulation, this led to the formulation of the 'on-demand' hypothesis. This model postulates that endocannabinoids are only produced and released if a stimulus, such as depolarization or stimulation of G_q/11-coupled metabotropic receptors, dictates the need for endocannabinoids[30,40,41]. Within this framework, the amount of endocannabinoid that is produced determines the amount that is released and activates the CB_1 receptor. In turn, endocannabinoid inactivation through enzymatic hydrolysis terminates signaling. Support for the 'on-demand' model came from the observation that DSI, which is mediated by 2-AG, is abolished in DAGL KO systems or following DAGL inhibition[42,43]. However, this model does not provide a complete molecular understanding of endocannabinoid signaling. While basal levels of 2-AG in the brain are high, widespread CB_1 receptor activation is not observed. Additionally, 2-AG is a monoacylglycerol and therefore there is constant turnover of 2-AG as part of metabolic lipid and fatty acid recycling[44]. Moreover, MAGL inhibition leads to an increase in tissue, but not extracellular, 2-AG levels, unless there is depolarization[45]. Together, this suggests that 2-AG is produced in cells without being immediately released to activate CB_1 receptor signaling, which does not fit with the 'on-demand' dogma.

Regulation of DAGLα

Central to the on-demand model and the synaptic functions of 2-AG is its main biosynthetic enzyme DAGL. Two isoforms of DAGL are known, DAGLα and DAGLβ. In DAGLα, but not DAGLβ, knockout mice DSI is absent from hippocampal neurons[43,46]. This indicates that DAGLα is the isoform responsible for the synaptic functions of 2-AG[40]. The main difference between the two isoforms is the presence of a long unstructured C-terminal tail in DAGLα[47]. This tail contains a proline rich recognition sequence for Homer scaffolding proteins, which anchor DAGLα close to the postsynaptic density and in proximity to metabotropic glutamate mGluR1/5 receptors. It also contains several phosphorylation sites that influence the enzymes activity and interaction with other proteins. Phosphorylation by CamKII at Ser782 and Ser808 was shown to reduce enzyme activity and influence 2-AG levels and endocannabinoid signaling in the striatum[48]. Striatal 2-AG signaling was also modulated by PKA, which phosphorylates DAGLα at multiple sites

to increase enzymatic activity[49]. Additionally, phosphorylation of Ser738 regulates the interaction with the scaffolding protein ankyrin-G and influences dendritic spine morphology[50]. DAGLα is further regulated by PKC, which was shown to control cycling of DAGLα between the plasma membrane and endosomal compartments[51]. Together, this demonstrates that regulation of enzymatic activity and cellular location of DAGLα is important for 2-AG function. Therefore, an increased understanding of how DAGLα is regulated could help uncover the molecular mechanisms governing 2-AG signaling.

Tools to study endocannabinoid signaling and transport

A significant reason for the poor understanding of endocannabinoid signaling regulation at the molecular level is the lack of knowledge about the mechanisms behind the release and intercellular transport of 2-AG and anandamide[52]. Proposed mechanisms include passive diffusion, facilitated diffusion, protein carriers and extracellular vesicles (EVs)[53]. Studying the cellular and intercellular movement of endocannabinoids and lipids more broadly has proven to be challenging. The inherent chemical diversity of lipids leads to a vast number of lipid species contained within each cell. Since lipids do not amend themselves to genetic modification, studying a specific lipid species requires different approaches.

Recent technological advances for the study of lipid signaling and transport include their chemical modification to equip them with bioorthogonal handles and photoactivatable groups. Niphakis *et al*. developed bifunctional anandamide analogues to study anandamide-interacting partners which revealed NUCB1 as a protein involved in endocannabinoid metabolism[54]. Photocaged anandamide was used to investigate the dynamics of endocannabinoid signaling in hippocampal slices[55] and a photocaged 2-AG enabled the study of its effect on β-cell signaling. A similar strategy was used to study transport of DAGs across the lipid bilayer, including 1-stearoyl-2-arachidonoylglycerol (SAG), the substrate for 2-AG biosynthesis[56]. These approaches, however, either report indirectly on lipid function or require fixation before analysis. Visualization of lipids by live-cell imaging was achieved for the naturally occurring lipid sterculic acid in combination with live-cell compatible click chemistry[57], but application of this approach to CB receptor ligands is currently limited by the challenging chemical synthesis of these lipid derivatives.

Another technique for the study of endocannabinoid signaling was introduced with the development of GPCR activation-based (GRAB) sensors for a number of bioactive molecules, including endocannabinoids[67]. $GRAB_{eCB2.0}$ is based on the CB_1 receptor and contains a circular permutated enhanced green fluorescent protein (cpeGFP) in the intracellular loop 3 of CB_1. Binding of its ligands leads to a fluorescent response[67]. This is proposed to be induced by conformational changes upon ligand binding. Therefore, the sensor can report on changes in endocannabinoid levels in a spatiotemporal manner. It has enabled the study of endocannabinoid dynamics in *in vitro* models such as organotypical slices and primary cultures[58], as well as *in vivo* in behaving animals[59].

Aim and outline of this thesis

The aim of the research described in this thesis is to study endocannabinoid transport. To this end, novel assays were developed and used to investigate the mechanisms regulating endocannabinoid release.

Chapter 2 describes the development of an endocannabinoid transport assay based on the endocannabinoid sensor $GRAB_{eCB2.0}$. This assay enabled the study of stimulus-dependent intercellular transport of 2-AG by both live-cell confocal microscopy and in a plate-reader format. In **Chapter 3** a focused pharmacological screen is performed to discover novel regulators of 2-AG transport. This revealed that endocannabinoid transport inhibitors interfere with 2-AG release and suggested the involvement of Arf6-regulated microvesicles in 2-AG transport. Additionally, kinases PKC and ROCK were found to be regulators of 2-AG mobilization. In **Chapter 4**, the isolation and characterization of Neuro2A extracellular vesicles (EVs) is described. ATP stimulation was found to increase EV production and proteomic profiling showed that the isolated EVs contained both EV marker proteins as well as proteins related to endocannabinoid production and release. Furthermore, lipidomic analysis of EVs revealed that 2-AG is specifically released in EVs in a process regulated by DAGL and Arf6. In **Chapter 5**, the endocannabinoid transport assay is used to study release of 2-AG from primary hippocampal neurons. This showed that activation of metabotropic glutamate receptors mobilizes 2-AG via DAGL- and Arf6-dependent activities. **Chapter 6** summarizes the research presented in this thesis and provides future prospects.

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