Activity-based protein profiling of diacylglycerol lipases

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CHAPTER 2

2-Arachidonoylglycerol: a Signaling Lipid

1. Introduction

2-Arachidonoylglycerol (2-AG) is one of the most extensively studied monoacylglycerols. It is an important signaling lipid as well as an intermediate in lipid metabolism.[1,2] It was isolated from canine gut and identified as an endogenous ligand for the cannabinoid receptors in 1995.[3,4] 2-AG behaves as a full agonist for the cannabinoid CB_1 and CB_2 receptors,[5-8] which are the main receptors through which 2-AG exerts its physiological effects. 2-AG and the cannabinoid receptors are part of an endogenous signaling system termed "the endocannabinoid system", which is also modulated by Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive constituent of the plant *cannabis sativa*.[9] Both CB receptors are associated with the regulation of many physiological processes, including inflammation,[10] food intake,[11-13] locomotor activity,[14,15] pain sensation,[16] mood,[17,18] addiction and reward.[19] The cannabinoid receptors share ~44% sequence homology, and show different expression profiles.[20] The CB_1 receptor is expressed throughout the body, but it is most extensively studied in the central nervous system where it is one of the highest expressed GPCRs in the brain.[21,22] While there is a strong debate about CB_2 receptor expression in the brain, its predominant localization on immune cells is well established, which is in line with its role in modulating immune responses.[23-25] While several other endogenous biomolecules interact with the CB_1 and CB_2 receptors, including *N*-arachidonoylethanolamine (anandamide; AEA)[26], virodhamine, *N*-arachidonoyldopamine (NADA) and noladin ether (see Table 1 for a complete list, and Pertwee *et al.*[27] for their in vitro binding affinities), 2-AG, together with AEA, is considered to be one of the most important endocannabinoids. Of note, brain 2-AG levels are ~170 times higher than those of AEA.[28] 2-AG is involved in neurogenesis, synapse formation and synaptic transmission, thereby mediating various forms of long- and short-term plasticity.[29] 2-AG is synthesized

"on demand" and acts as a retrograde messenger that inhibits neurotransmitter release at both inhibitory and excitatory synapses.[30-32]

Several reports indicate that 2-AG produces physiological effects independent of the CB_1 and CB_2 receptors. 2-AG has been shown to interact with a number of other receptors, including $GABA_A$,[33] TRPV1,[34] Adenosine A_3,[35] GPR55,[36] and PPARγ.[37]Interactions with these proteins are important to consider when studying the physiological role of 2-AG. The levels of 2-AG, and thereby the interaction with its target receptors, are tightly regulated by its biosynthetic and catabolic enzymes (Figure 1). Multiple enzymes in different pathways contribute to the biosynthesis and catabolism of 2-AG. Notably, multiple precursors and metabolites of 2-AG have signaling functions as well. This chapter describes the physiological role of 2-AG in the brain, and how disrupting 2-AG biosynthesis and catabolism facilitates insight in these physiological functions.

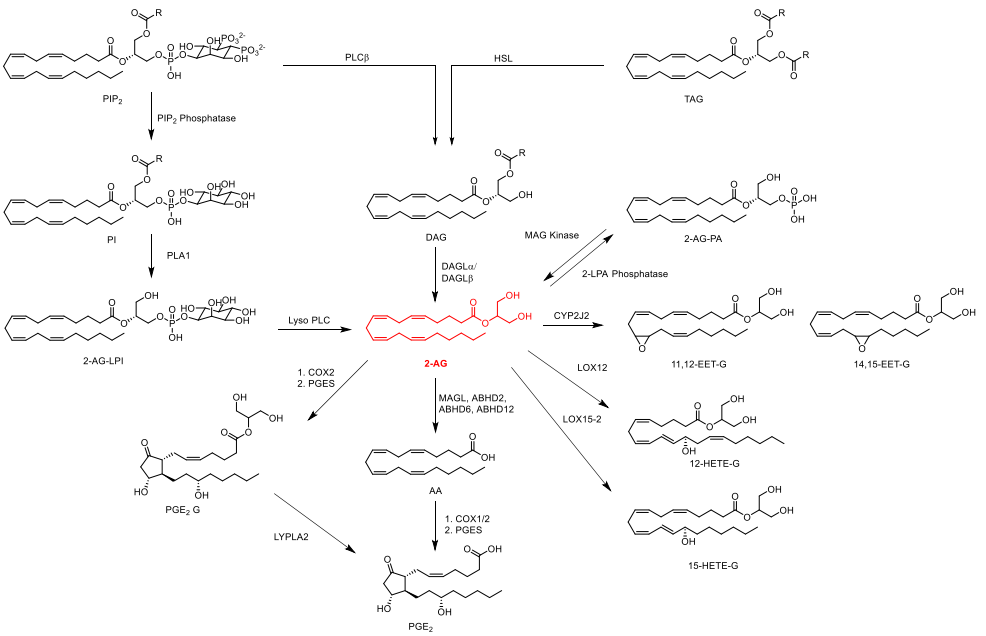

Figure 1. Overview of the major biosynthetic and metabolic pathways for 2-AG. PLCβ: phospholipase Cβ, HSL: hormone sensitive lipase, PIP_2: phosphatidylinositol-4,5-bisphosphate, PI: phosphatidylinositol, TAG: triacylglycerol, DAG: diacylglycerol, PLA1: phospholipase A1, 2-AG-LPI: 2-arachidonoyl lyso-phosphatidylinositol, Lyso-PLC: lyso-phospholipase C, MAG kinase: monoacylglycerol kinase, 2-LPA phosphatase: 2-lysophosphatidic acid phosphatase, 2-AG: 2-arachidonoylglycerol, 2-AG-PA: 2-arachidonoylglycerol phosphatidic acid, CYP2J2: cytochrome P2J2, LOX-12: lipoxygenase 12, LOX-15-2: lipoxygenase 15-2, MAGL: monoacylglycerol lipase, ABHD2, 6, 12: alpha/beta hydrolase domain containing 2, 6, 12, COX1/2: cyclooxygenase 1/2, LYPLA2: lysophospholipase 2, 11, 12-EET-G: 11, 12-epoxyeicosatrienoic acid glycerol ester, 14, 15-EET-G: 14, 15-epoxyeicosatrienoic acid glycerol ester, PGE_2-G: prostaglandin E_2 glycerol ester, PGE_2: prostaglandin E_2, 12-HETE-G: 12-hydroxyeicosatetraenoic glyceryl ester, 15-HETE-G: 15-hydroxyeicosatetraenoic glyceryl ester.

2. 2-AG biosynthesis

The biosynthesis of 2-AG can be divided in two main pathways with different functionalities (Figure 1). A signaling pathway, starting from phosphatidylinositol-4,5-bisphosphate (PIP_2), and a metabolic pathway using *sn2*-arachidonate containing triglycerides. PIP_2 is converted into two important second messengers by phospholipase Cβ (PLCβ): diacylglycerol (DAG) and inositol-1,4,5-triphosphate.[28,38] PLCβ uses Ca^{2+} as a cofactor and acts as a coincidence detector by integrating the signals coming from G_q-coupled receptor activation and the influx of extracellular Ca^{2+} via ionotropic receptors and voltage gated calcium channels.[28,39-42] The metabolic pathway involves the hydrolysis of triglycerides by hormone-sensitive lipase, carboxyl esterase and other lipases to *sn-2*-arachidonoylglycerol containing diglycerides.[28] At this point the two pathways converge and the diglycerides are further processed by two isoforms of a *sn-1*-specific diacylglycerol lipase, diacylglycerol lipase-α and -β (DAGL-α and -β).[43] The DAG lipases hydrolyze diacylglycerols at the *sn-1* position, generating 2-AG and a fatty acid. DDHD domain-containing protein 2 (DDHD2) has recently been reported to possess also the ability to hydrolyze DAG.[44] Partially purified DDHD2 from rat brain, and rat DDHD2 expressed in Chinese hamster ovary cells (CHO cells) exhibited diacylglycerol lipase activity in vitro. However, Inloes *et al.* generated a DDHD2 KO mouse, which revealed that DDHD2 was a principal triglyceride lipase *in vivo*.[45] Thus, further research is required to establish the physiological relevance of this pathway with regard to 2-AG biosynthesis in the brain.

2-AG can also be generated from PIP_2 by PIP_2 phosphatase towards phosphathidyl inositol (PI), followed by hydrolysis of the *sn1*-ester by phospholipase A1 to generate 2-arachidonoyl-LPI. In a final step the phosphate is removed by lysophospholipase C (LysoPLC) to form 2-AG.[46] In 2002, lysophosphatidic acid was found in rat brain. This lipid could be quickly converted to 2-AG by 2-LPA phosphatase.[47] Although these alternative pathways may provide 2-AG, diacylglycerol lipases are considered to be the most important enzymes. These proteins are the main contributors to 2-AG biosynthesis as identified by selective DAGL inhibitor studies, as well as by DAGL-α and DAGL-β KO studies.[48-50]

2.1 Diacylglycerol lipase alpha and beta

The enzymatic activity that hydrolyses the *sn*-1-acyl chain from 1,2-diglycerides yielding 2-AG in human platelets was first detected by Prescott and Majerus in 1983.[51] Substrates containing *sn-1*-palmitate and stearate showed the same rate of hydrolysis. After the identification of this transformation, considerable efforts were undertaken to characterize and isolate the enzyme from bovine brain.[52,53] It was not until 2003 that two enzymes were identified and cloned, and designated as diacylglycerol lipase-α and -β.[43] The human genes show a high homology compared with those of mouse, 97% for DAGL-α and 79% for

DAGL-β. DAGL-α and DAGL-β structurally differ by the absence of a large C-terminal tail in DAGL-β compared to DAGL-α. Transfection of the DAGL-α and DAGL-β genes in COS cells resulted in enzymes with a molecular weight of ~120 kD for DAGL-α and ~70 kD for DAGL-β. Molecular characterization of these two enzymes revealed that they are plasma membrane bound serine hydrolases that show specificity for hydrolysis at the *sn-1*-position of diacylglycerols.[43]

The C-terminal tail of DAGL-α plays an important regulatory function. The activity of DAGL-α is modulated by calcium/calmodulin dependent protein kinase II (CaMKII) which phosphorylates Ser^{782} and Ser^{808} on the *C*-terminal tail of DAGL-α.[54]. DAGL-α phosphorylation significantly reduced its activity. The C-terminal tail of DAGL-α contains also a consensus motif (PPxxF) for binding with the coiled coil domain of homer proteins. Homers are adaptor proteins that interact with many different proteins, including the metabotropic glutamate receptor. An interaction between homer-1b and homer-2 with DAGL-α was shown to be important for DAGL-α localization at the plasma membrane, but is not required for its catalytic activity.[55] Moreover, the surface expression of DAGL-α is dynamic, undergoing endocytosis and recycling back to the postsynaptic membrane.[56] DAGL-α cycling between the cell surface and intracellular endosomal compartments is regulated by protein kinase C (PKC). Rapid degradation and replenishment of DAGL-α upon inhibition with covalent inhibitor DH376 indicates a short protein half-life.[57] These observations indicate that the activity, expression and localization of DAGL-α have a tight spatiotemporal control on 2-AG synthesis and release.

Two landmark studies have shown the importance of the contribution of DAGL-α and DAGL-β to the *in vivo* biosynthesis of 2-AG. Congenital deletion of DAGL-α or -β reduced 2-AG levels in the brain with 80% and 50%, respectively.[48,49] These studies also revealed a tissue specific contribution of the DAG-lipases to the biosynthesis of 2-AG. In the liver, the roles were reversed with a 50% reduction of 2-AG in DAGL-α KO mice compared to a 90% reduction in DAGL-β KO mice. DAGL-α is highly expressed in neurons and is found at (peri)post-synaptic sites. 2-AG acts as a retrograde neurotransmitter, suppressing synaptic transmission at central synapses. Stimulation of 2-AG release by depolarization of postsynaptic neurons, activation of $G_{q/11}$-coupled receptors and a combination of $G_{q/11}$-coupled receptor activation and Ca^{2+} elevation resulted in suppression of synaptic transmission at both excitatory and inhibitory synapses (DSE and DSI respectively). This suppression was absent in DAGL-α KO brain slices, but DAGL-β knockout brain slices maintained retrograde suppression of synaptic transmission.[58,59] This is in line with the higher activity of DAGL-α in the brain and the cellular distribution of both isoforms. DAGL-α displays higher activity in neurons, while DAGL-β is relatively more active in microglia.[60]

Over the years multiple pharmacological tools have been developed to modulate DAGL activity, see Kohnz *et al.*[61] and Janssen *et al.*[62] for recent reviews on DAGL inhibitors. Subtype selective inhibitors for the diacylglycerol lipases have not been identified to date.

Dual DAGL-α and DAGL-β inhibitors in combination with DAGL-α and DAGL-β knockout mice have greatly facilitated the study of the physiological role 2-AG in health and disease models. Recently, Ogasawara *et al.* reported the first CNS active dual DAGL-α/β inhibitors.[57] Lipid analysis in the brain revealed an extensive rewiring of lipid metabolic pathways in the brain upon inactivation of DAGL-α and DAGL-β. Not only levels of *sn-1*-stearyl-2-arachidonoyl-glycerol, but also levels of *sn1*-palmitoyl-2-arachidonoyl-glycerol, *sn-1*-palmitoyl-2-stearyl-glycerol, *sn-1*-oleyl-2-stearyl-glycerol were elevated in DAGL-α KO, and DAGL inhibitor treated mouse brains. Downstream, not only levels of 2-AG were reduced, but also levels of palmitoyl-glycerol, oleoyl glycerol and docosahexaenoyl-glycerol. These lipids are important signaling lipids in their own right, which could complicate interpretation of physiological effects observed upon DAGL blockade to 2-AG.

3. 2-AG Catabolism

2-AG is catabolized by multiple different pathways and enzymes (Figure 1). All of these lead to a decrease in the 2-AG levels and signaling. The predominant pathway for 2-AG catabolism is the hydrolysis of the ester bond into arachidonic acid (AA) and glycerol. Blankman *et al.* showed that MAGL was responsible for ~85%, ABHD12 (~9%) and ABHD6 (~4%) of 2-AG hydrolysis in mouse brain.[63] Together they are responsible for more than 98% of the 2-AG hydrolysis in the brain. Cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450s (CytP450) have been reported use 2-AG also as a substrate. Recently ABHD2 has been reported to hydrolyze 2-AG in spermatozoa. This enzyme has a relatively high expression in the brain compared to other tissues. Therefore this enzyme could be an important player to consider in this context. In the next section the enzymes responsible for the metabolism of 2-AG will be discussed in more detail.

3.1 Monoacylglycerol Lipase (MAGL)

In the early sixties, it was demonstrated that two proteins distinct from lipoprotein lipase, were involved in the hydrolysis of triacylglycerol towards fatty acids in adipose tissue of both rats and rabbits. Hormone sensitive lipase (HSL) was believed to hydrolyze triacylglycerol to monoacylglycerols, and a dedicated lipase subsequently hydrolysed monoacylglycerols towards free fatty acids and glycerol.[64] Partial purification of the monacylglycerol lipase activity convincingly showed its preference towards monoacylglycerols over diacylglycerols and triacylglycerols.[65] It took until 1997 before a specific monoacylglycerol lipase (MAGL) was cloned for the first time.[66] The enzyme consists of 302 amino acids and has a molecular weight of 33 kD.[66] It is an ubiquitously expressed serine hydrolase containing the typical GXSXG motif with Ser-122, Asp-239 and His-269 forming the catalytic triad. [61,64] The active site of the enzyme resides in the cytosol, but the enzyme also associates with membranes. MAGL exists in several splice forms in the

brain and testes.[67-69] MAGL has a broad substrate specificity and hydrolyses multiple monoacylglycerols with different chain lengths and degrees of unsaturation, including 2-palmitoylglycerol, 2-stearoylglycerol and 2-oleoylglycerol.[70-72]

The interest in MAGL boosted when, next to its original role in mobilizing free fatty acids in adipose tissue, the enzyme was linked to the endocannabinoid system.[27] This was demonstrated by overexpression of the enzyme in HeLa cells, which significantly increased 2-AG hydrolysis. In line with this observation, RNA interference in the same cell line elevated 2-AG levels.[27] Immunodepletion of MAGL reduced 2-AG hydrolyzing activity in rat brain with ~50%.[27,33]

The development of mouse models with congenital deletion of MAGL has significantly advanced our understanding of the role of MAGL *in vivo*.[73-76] Chronic disruption of MAGL activity severely decreased the mouse brain ability to hydrolyse 2-AG. This leads to a >10 fold increase of 2-AG in the brain and less pronounced but significant reduction of 2-AG in the thymus, spleen and liver.[76] Noteworthy, levels of other monoacylglycerols were also increased *in vivo*. Tonic elevation of 2-AG levels by MAGL knockout in the brain causes CB_1 receptor adaptations. MAGL knockout mice exhibited impaired endocannabinoid-dependent synaptic plasticity, physical dependence and desensitization of brain CB_1 receptors.[74] Opposite to the increase in 2-AG levels, AA abundance in the brain dropped significantly in the absence of MAGL.[74,76] This indicates that a large extent of the metabolic route towards AA travels through MAGL. Therefore, the activity of MAGL affected levels of pro-inflammatory oxidative metabolites of AA, and designated MAGL as a key enzyme in regulating neuro-inflammatory responses.[77]

Several selective and *in vivo* active MAGL inhibitors have been developed to study the role of MAGL in 2-AG metabolism and signaling.[69,78-81] MAGL is relatively abundant in neurons compared to astrocytes and microglia. In contrast to the major 2-AG biosynthetic enzyme DAGL-α, MAGL is positioned presynaptically along with the cannabinoid CB_1 receptor. Evidence for the role of MAGL in retrograde 2-AG signaling was shown in electrophysiology experiments in cultured neurons and brain slices, where both MAGL inhibitors and genetic deletion of MAGL enhanced DSI/DSE.[82-88] Hydrogen peroxide mediated MAGL sulfenylation of C-201 and C-208 reduces MAGL activity and thereby increases endocannabinoid signaling in neurons.[89] However, the effect of this posttranslational modification on DSI and DSE remains to be investigated.

The tissue specific role of MAGL was further investigated using the MAGL inhibitor JZL184. The levels of 2-AG were 8-fold increased in the brain, whereas in peripheral tissues, such as liver, kidney, spleen, BAT and heart, the change was less pronounced. No significant changes were observed in the testis, lung and white adipose tissue.[69] Interestingly, C16:0 and 18:1 MAGs were more increased in peripheral tissues compared to the brain. This suggests that MAGL has a more general metabolic function in peripheral tissues.[69,76]

Repeated administration of the selective MAGL inhibitor JZL184 mirrored the observations in the knockout mice and caused CB_1 receptor desensitization, tolerance to

CB_1 receptor agonists and downregulation of CB_1 receptors.[74] This points towards a major role for MAGL in regulating bulk 2-AG levels and raises the question whether a therapeutic window to elevate 2-AG levels can be achieved without causing desensitization of CB_1 signaling. Several studies have addressed this question and investigated the effect of partial blockade of MAGL using low doses of JZL184.[90-93] Chronic JZL184 dosing for ~7 days up to 8 mg/kg per day did not lead to CB_1 receptor desensitization or behavioral tolerance, while chronically treated animals with 16 mg/kg JZL184 per day did show tolerance.[92,93] Thus, it appears to be possible to elevate 2-AG levels without desensitization of the CB_1 receptor.

3.2 Alpha/beta hydrolase domain containing 6 (ABHD6)

The α,β-hydrolase domain-containing protein 6 (ABHD6) is part of a superfamily of proteins having an α,β-hydrolase fold.[94] It is a membrane bound protein consisting of 337 amino acids and has a mass of 38 kD.[63] It is a serine hydrolase that contains a catalytic triad with the typical GXSXG motif surrounding the catalytic Ser-148; the other two members of the catalytic triad are Asp-278 and His-306.[95] mRNA expression analysis in mice showed that ABHD6 is ubiquitously expressed throughout the body with relatively high expression in brown adipose tissue and the brain.[96] In the human cortex mRNA expression of ABHD6 steadily increased from neonatal age until adulthood.[97] It is postulated that ABHD6 is membrane bound, with its active site facing the cytosol. ABHD6 is responsible for ~4% of the total 2-AG hydrolysis in mouse brain homogenate.[63] Several selective inhibitors have been developed to study ABHD6 function.[98-101] In microglial (BV-2) cells ~50% of 2-AG degradation is mediated by ABHD6. In Neuro2A cells, 2-AG levels are increased after inhibition with the selective ABHD6 inhibitor KT195.[50] Both Neuro2A and BV-2 cells lack MAGL activity, therefore ABHD6 can be an important player in 2-AG hydrolysis in specific cell types. Many studies have focused on the role of ABHD6 in 2-AG metabolism and endocannabinoid signaling in the brain. Electron microscopy and immunofluorescence experiments revealed localization of ABHD6 in postsynaptic dendrites in adult mouse brain, whereas MAGL is predominantly expressed pre-synaptically.[102] Positioning ABHD6 at the site of 2-AG production. Inhibition of ABHD6 in prefrontal cortical brain slices by the selective inhibitor WWL70 could reduce CB_1 receptor mediated long term depression (LTD).[102] Depolarization induced suppression of inhibition (DSI) or excitation (DSE) in autaptic hippocampal neurons were not reduced by inhibition of ABHD6.[87,103,104] ABHD6 is considered an interesting drug target for moderate elevation of 2-AG levels. Inhibition of ABHD6 had anti-inflammatory and neuroprotective effects in a mouse model of traumatic brain injury, exerted antiepileptic activity and reduced neuro-inflammation and neurodegeneration in a mouse model of multiple sclerosis.[105-107] Interestingly, ABHD6 has recently been reported to negatively regulate surface delivery and synaptic function of the

AMPA receptors, ionotropic glutamate receptors that mediate synaptic transmission. ABHD6 overexpression in neurons could drastically reduce excitatory neurotransmission mediated by the AMPA receptor independent of its hydrolytic activity.[108]

Several studies have assessed the role of ABHD6 in peripheral tissues. ABHD6 inhibition raises 2-AG levels in macrophages and is subsequently converted by COX2 to PGD_2-G, which demonstrated an anti-inflammatory effect.[109] Targeted antisense oligonucleotides (ASOs) to knockdown ABHD6 *in vivo* in peripheral tissues (liver, white adipose tissue and kidney) did not affect monoacylglycerol levels, however, lysophospholipid levels were strongly elevated. Knocking down ABHD6 in the liver exerted a protective effect against high-fat diet induced obesity in mice.[47] Studies with ABHD6 knockout and inhibitor treatment showed a role of ABHD6 in insulin secretion by pancreatic β-cells and identified Munc13-1 as the intracellular receptor for MAG-induced exocytosis.[110,111] Recently, it was demonstrated that genetic deletion of ABHD6 elevates 1-MAG concentrations and plays a role in fuel homeostasis, BAT-function and WAT browning via PPARα/γ activation.[112] Furthermore, ABHD6 has been shown to degrade bis(monoacylglycero)phosphate (BMP).[113] ABHD6 is responsible for 90% and 50% of the BMP hydrolysis in the liver and brain, respectively. ABHD6 co-localizes with the late endosomes/lysosomes. It would be interesting to see whether changes in lipid sorting or cargo recycling by inhibition of ABHD6 can affect cannabinoid signalling or steatosis. The role of 2-AG in these metabolic effects might be small, since ABHD6 has been shown to have a broad substrate specificity.[95,112]

3.3 Alpha/beta hydrolase domain containing 12 (ABHD12)

The α,β-hydrolase domain-containing protein 12 (ABHD12) belongs to the same α, β-hydrolase fold containing superfamily as ABHD6.[94] ABHD12 is a membrane bound protein with an extracellular orientation of its active site.[63,114] It is a 398 amino acid containing enzyme with a molecular weight of 45 kD. It is a serine hydrolase having a lipase motif (GTSMG) and the catalytic triad is formed by Ser-246, Asp-333 and His-372.[95] ABHD12 contributes approximately ~9% of the total 2-AG metabolism in mouse brain homogenates.[63] Null mutations in ABHD12 cause the human neurodegenerative disorder PHARC (polyneuropathy, hearing loss, ataxia, retinosis pigmentosa, and cataract).[115] ABHD12 transcripts are highly expressed in the brain, with a significant enrichment in microglia.[116] No selective inhibitors of ABHD12 are available, but the generation and analysis of the metabolic and behavioral phenotype of a mouse model with congenital deletion of ABHD12 has greatly facilitated the understanding of its biological role.[117] ABHD12 knockout mice showed PHARC like symptoms such as hearing disruptions, muscle weakness and ataxia. Importantly, these mice showed high elevations in lysophosphatidylserines (LPS), whereas the 2-AG hydrolyzing capacity of ABHD12 knockout brain homogenate did not significantly differ compared to wild type brain

homogenate. Interestingly, after incubation with a MAGL inhibitor, a significant reduction in 2-AG hydrolyzing capacity was observed compared to wild type brain homogenate. A possible explanation for this observation could be that ABHD12 plays a more specialized role and is recruited when there is insufficient 2-AG hydrolyzing capacity. This is in line with the observation that ABHD12 overexpression did not affect DSE.[87] No data supporting endogenous ABHD12 hydrolysis of 2-AG in *in situ* or *in vivo* have been generated so far. Identification of selective inhibitors that allow spatiotemporal control over ABHD12 activity will be a crucial tool to elucidate the role of ABHD12 in 2-AG metabolism.

3.4 ABHD2

The α,β-hydrolase domain-containing protein 2 (ABHD2) is another member of α,β-hydrolase fold containing superfamily. The enzyme is a serine hydrolase with a classical Ser-207, His-376, Asp-345 catalytic triad. ABHD2 is predicted to be a single pass type-II membrane protein. The expression of the enzyme is highest in the lung, brain and adrenal glands.[96] Knockdown of ABHD2 using antisense oligonucleotides and gene trapping techniques demonstrated an essential role of 2-AG in Hepatitis B virus propagation.[118] ABHD2 appears to play an important role in chronic diseases involved in monocyte/macrophage recruitment, such as atherosclerosis and emphysema.[119,120] It was not until recently that ABHD2 was linked to 2-AG metabolism.[121] The enzyme is highly expressed in spermatozoa and acts as a progresterone-dependent hydrolase. Activation of ABHD2 reduces inhibition of sperm calcium channel CatSper by 2-AG, which leads to calcium influx and stimulates sperm activation. No inhibitors for ABHD2 have been reported to date, and the contribution of ABHD2 in 2-AG metabolism in the brain requires further investigation.

3.4 Cyclooxygenase-2 (COX-2)

Cyclooxygenases exist in two isoforms cyclooxygenase-1 and -2 (COX-1 and COX-2). Both enzymes play an important role in inflammation. The expression of COX-2 is increased in inflammatory processes and is essential for the oxidation of AA as part of its transformation into prostaglandins.[109] In contrast to the constitutively expressed COX-1, which has a strong preference for substrates with a free carboxylate group, COX-2 metabolizes both AA and 2-AG with similar K_{cat} and K_m.[122,123] Several papers suggest that COX-2 plays a role in terminating 2-AG signaling in the CNS. Kim and Alger showed that inhibition of COX-2 prolonged DSI in hippocampal slice preparation.[124] A later study in cultured hippocampal neurons showed that the duration of DSI in a subpopulation of interneurons was influenced by both MAGL and COX-2.[103] Substrate selective inhibitors for COX-2 have been developed, which selectively block the oxidation of 2-AG but not

AA.[125,126] The substrate selective inhibitor LM-4131 could increase 2-AG levels in RAW264.7 cells and intraperitoneal (IP) injection of this inhibitor led to an *in vivo* increase of 2-AG levels in the brain.[126]

COX-2 mediated oxidation of 2-AG produces the prostaglandin glyceryl ester PGE_2-G (Figure 2). Most of the effects of substrate selective blockade of COX-2 appear to be caused by changes in PGE_2-G concentrations rather than minor alterations in 2-AG levels.[126] PGE_2-G is a multifunctional signaling molecule that plays a role in pain, immunomodulation, and synaptic plasticity.[127-129] The receptor for PGE_2-G has not been identified, but the actions of PGE_2-G are mediated through ERK, p38 mitogen-activated protein kinase (MAPK), IP(3), and NF-κβ.[129] In contrast to the anti-nociceptive and anti-inflammatory role of 2-AG, the COX-2 metabolite PGE_2-G produces hyperalgesia and enhances inflammation induced neurodegeneration.[127,129,130] Important to note is that 2-AG suppresses elevation of COX-2 expression via a CB_1 receptor mediated mechanism involving PPARγ and the MAPK/NFκ-B signaling pathway.[131,132] PGE_2-G can be further processed by lysophospholipase A2 (LYPLA2) towards PGE_2.[133]

3.5 Lipoxygenase

Lipoxygenases are a family of non-heme iron containing dioxygenases that catalyze the oxidation of polyunsaturated fatty acids that contain one or more (1*Z*,4*Z*)-pentadiene moieties.[134] This oxidation yields the corresponding (1*S*,2*E*,4*Z*)-hydroperoxides. Moody *et al.* showed that lipoxygenase-12 (LOX-12) oxygenates glycerol esters towards 12-hydroxyeicosatetraenoic (12-HETE) glyceryl ester.[135] Kozak *et al.* screened a panel of 6 lipoxygenases for their 2-AG oxygenating properties. They found that LOX-15-1 and LOX-15-2 also catalyse the oxygenation of 2-AG towards 15-HETE-glyceryl ester.[136] Moreover, among a series of structural related arachidonoyl esters, 2-AG was the most optimal substrate for LOX-15 oxygenation. 2-AG treatment of COS-7 cell transfected with 15-LOX led to the biosynthesis and excretion of 15-HETE-G. Van der Stelt *et al.* investigated the affinity of LOX metabolites of 2-AG for the cannabinoid receptors. It was found that 15-HETE-1-AG showed moderate affinity for the CB_2 receptor, but not for the CB_1 receptor.[137] No data on *in vivo* conversion of 2-AG by LOX-15 or LOX-12 has been reported. Therefore, the physiological role of this pathway has yet to be established.

3.6 Cytochrome P450

Cytochrome P450 (CYP450) are heme-containing oxidative enzymes involved in drug metabolism, but they can also use endogenous lipids, such as 2-AG, as substrates.[138,139] Two epoxide regioisomers (2-11, 12- and 2-14, 15-epoxyeicosatrienoic acid glycerol ester (EET-EG)) of CYP metabolites of 2-AG have been isolated from rat kidney and spleen,

whereas 2-14, 15-EET-EG was also detected in the brain.[140] These metabolites had a higher binding affinity for the CB_1 and CB_2 receptors compared to 2-AG, whereas the corresponding oxidized metabolites from AA (11,12-EET and 14,15-EET) did not display any CB receptor affinity. Incubation of 2-AG with the predominant CYP450 in the heart, CYP2J2 epoxygenase produces 2-11, 12- and 2-14, 15-EET-EG in vitro.[141] In addition, heart microsomes derived from bovine and porcine tissues also produced 2-11, 12- and 2-14, 15-EET-EG. The physiological role of these CYP450 metabolites remains to be elucidated.

4. 2-AG storage and transport

2-AG is a retrograde messenger. It is produced in the postsynaptic cell, and travels across the synapse to the presynaptic nerve terminal where it inhibits neurotransmitter release by activation of the CB receptors. There has been an outstanding question whether 2-AG is synthesized "on demand" or stored and released from preformed pools. DSI was robustly absent in DAGL-α, but not DAGL-β knockout models.[48,58] However, pharmacological inhibition gave inconsistent results.[142] Several studies found a reduction in DSI,[84,143-146] where others did not.[147,148] Recently new chemotypes were identified as DAGL inhibitiors that could reduce DSI,[57,149] supporting the hypothesis of on demand synthesis. A possible explanation for the discrepancies between the earlier studies is poor penetration of lipophilic inhibitors in brain slices. The localization, expression and activity of DAGL-α is highly regulated, and the site of 2-AG synthesis in combination with transport might be a pivotal determinant for its fate. Therefore, understanding whether and how 2-AG is stored and transported is crucial for understanding the physiological role of 2-AG.

In contrast to polar neurotransmitters that are stored in vesicles, AEA and 2-AG are neutral lipids that have the intrinsic tendency to associate with membranes. It is poorly understood how 2-AG and AEA move across the synapse, traverse within the cellular interior and how cellular uptake of 2-AG and AEA is regulated. Possible routes for 2-AG and AEA transmembrane transport are passive diffusion, endocytosis, transporter proteins, or a combination of these mechanisms. The transport of AEA has been subject of intensive research, but the mechanisms of AEA uptake and transport are still under debate (for reviews see Niculussi & Gertsch[150], and Fowler[151,152]). Fewer studies have addressed the transport process of 2-AG, because its metabolism complicates the analysis of the transport processes.

Ben Shabat *et al.* found the first indication of cellular 2-AG uptake.[153] Co-incubation of 2-AG with 2-lineoyl-glycerol reduced 2-AG depletion from media, while 2-palmitoyl-glycerol had no effect.[153] 2-AG accumulation in rat basophilic RBL-2H3 and mouse neuroblastoma N18TG2 cells was also observed by Di Marzo *et al.*[154] Saturable 2-AG uptake by human astrocytoma cells with a Michaelis-Menten constant (K_M) of 0.7 ± 0.1

μM and a V_{max} of 28 ± 6 pmol/min/mg, indicated the existence of a specific transporter protein.[155] In addition, 2-AG uptake could be inhibited by AEA and the putative AEA transport inhibitor AM404.[156,157] Recently, it was found that UCM707 and OMDM-2 could also inhibit 2-AG and AEA uptake with equal potency. A common carrier mechanism was proposed for both AEA and 2-AG.[158] Importantly, not only the uptake could be blocked, but also 2-AG release. Suggesting that the putative transporter is responsible for bidirectional trafficking of 2-AG. Identification and functional characterization of the putative transporter protein(s) will be highly important for a better understanding of 2-AG storage, release and transport.

In addition to transport across the membrane, it is also unclear how intracellular transport of 2-AG is regulated. Recently, the first evidence for intracellular 2-AG transport was demonstrated. [159] A fluorescence polarization assay with a 12-N-methyl-(7-nitrobenz-2-oxa-1,3-diazo) aminostearic acid (NBD-stearate) probe showed that both 2-AG and AEA bind to cytosolic carrier protein FABP5. Further evidence for this binding was provided in a co-crystalization study of FAB5 and 2-AG.[160] It would be interesting to see to what degree FABP5 regulates the physiological role of 2-AG.

5. Non CB_1/CB_2 receptors targeted by 2-AG

The CB_1 and CB_2 receptors are not the only receptors that have been shown to interact with 2-AG. Interactions with non-classical cannabinoid receptors may be responsible for some of the physiological functions of 2-AG. 2-AG potentiates $GABA_A$ receptor function at low GABA concentrations.[33] The $GABA_A$ receptor is a ligand-gated ion channel that is activated by GABA, the most widely distributed inhibitory neurotransmitter in the CNS. Activation of the $GABA_A$ receptor allows conductance of Cl^- ions, causing an inhibitory effect on neurotransmission. The $GABA_A$ receptor is involved in many physiological functions, including amnesia, motility, sedation, anxiety and insomnia.[161] Potentiation of the $GABA_A$ receptor by 2-AG inhibits motility. It was shown that 2-AG binds at the M4 transmembrane region of the β2 subunit, and the observed effect was still present in CB_1/CB_2 double knockout mice.[33] The interaction of 2-AG can have important implications on studies involving 2-AG on sedation and locomotion.[33] In addition, an interaction between 2-AG and the peroxisome proliferator-activated receptor-γ (PPAR-γ) has been

Table 1. 2-AG interacting proteins.

	Receptor	Ligand	K_i, IC_{50} or EC_{50}	Additional ligands	physiological effect	Reference[n]
Receptor interaction	CB1	2-AG	K_i = 34.6 -472 nM[a]	AEA, Virodhamine, Noladin ether[170], NADA[171], oleamide[172], DTEA[173], DLEA[173], EPEA[174], DHEA[174], Hemopressin[175]	Anxiety, pain, metabolism, addiction, inflammation	176, 153
	CB2	2-AG	K_i =1400 nM[a]	AEA, Noladin ether[170], NADA[171], DLEA[173], EPEA[174], DHEA[174]	Inflammation	176
	TRPV1	2-AG	EC_{50} = 2.5 μM[b]	AEA, NADA[177]	Pain	34
	$GABA_A$	2-AG	EC_{50} = 2.1 μM[c]	GABA		33
	A_3	2-AG	IC_{50} = 12.6 μM[d]	Adenosine		35
	GPR55	2-AG	EC_{50} = 3 nM[e]	Lyso-PI, AEA, Noladin ether, virodhamine		36
	PPARγ	2-AG	EC_{50} = 10 μM[f]	Prostaglandins	Inflammation	37
	Enzyme	substrate	K_m	Additional substrates	physiological effect	Reference
Biosynthesis	DAGL-α	DG (18:0/20:4)	155 μM[g]	DG(18:1/18:1), DG(18:1/18:0), DG (16:0/18:0), DG(16:0/20:4), DG(18:1/20:4).	metabolism, anxiety, inflammation	178
	DAGL-β	DG (18:0/20:4)	74 μM[g]	DG(18:1/18:1), DG(18:1/18:0)	Inflammation	178
	DDHD2	DG (18:0/20:4)	248 μM[h]	TAG species with combinations of C16:0, C18:0, C18:1, C20:4, C22:5, C22:6 fatty acyl chains.[45] (DG 18:0/18:2), DG (18/22:6).	-	44,179
	2-LPA Phosphatase	2-AG-LPA	-	Oleoyl-LPA	-	180
	Lyso PI-PLC	2-AG-LPI	-	LysoPC, LysoPS[181]	-	4,46
	Enzyme	Metabolite	K_m	Additional substrates	physiological effect	Reference
Metabolism	MAGL	AA	110 μM[i]	MG (18:1), MG(18:2)[76] MAG (16:0), MAG (18:1), MG (18:2), MG (22:6)[74]	Anxiety, pain, addiction, metabolism	95
	ABHD6	AA	159 μM[i]	MG(14:0), MG(16:0) , MG(18:0) ,MG(18:1)[110] in vivo in BAT ABHD6 KO.	Inflammation, metabolism	95
	ABHD12	AA	117 μM[i]	LPS(16:0), (18:0), (20:4), (22:4), (22:1), (22:0), (18:0), (18:0) and PS(16:0/18:1), (18:1/18:1), (16:1/20:4), (18:1/20:4), (18:0/20:4), (20:1/20:4), (20:0/20:4), (22:1/20:4), (22:0/20:4) , (24:1/20:4)[117]	Inflammation	95
	COX2	PGE_2-G	4.4 ±μM[j]	AA, AEA[126]	Inflammation	122
	LOX12	12-HpETE-G	6 μM[k]	AA, AEA, linoleic acid, docohexanoic acid	Inflammation	135
	LOX15-2	15-HpETE-G	9 μM[l]	AA, AEA	Inflammation	136
	CYP2J2	11,12-EET-G	13.6 μM[m]	AA, AEA	Inflammation	138,141
		14,15-EET-G	32.6 μM[m]			

[a]Range of K_i's measured by ligand displacement assay with [^{3}H]HU-243 in membrane fractions of COS cells transfected with the corresponding receptor.[153,176] [b]Effect on intracellular calcium concentration in TRPV1 expressing HK293 cells, maximum responses were normalized to the 10 μM CAP induced response. [c]Dose dependent potentiation of currents elicited by 1 μM GABA. [d]Ligand displacement assay with [^{125}I] AB MECA in CHO membranes expressing the hA_3 receptor. [e]GTPγS binding assay. [f]IC50 competition for fluorescent ligand

occupancy the purified PPARγ ligand binding domain. [g]Membrane of COS cells overexpressing DAGL-α or DAGL-β respectively. [h]Recombinant rDDHD2. [i]Fluorescent glycerol assay for 2-AG hydrolase activity in HEK293 cells transiently transfected with the corresponding hydrolase. [j]Oxygenation by purified human COX2.[122] [k]UV assay for the conjugated diene (leukocyte LOX12).[135] [l]Hexahistidine-tagged human 15 LOX-2 expressed in Sf-9 cells and purified on Ni-NTA agarose. [m]Formation kinetics for CYP2J2 nanodisc incubations with 2-arachidonoyl glycerol. [n]Reference regarding the K_i, K_m, IC_{50} or EC_{50}.

reported. However it is not completely clear if this interaction is mediated by 2-AG itself, via the CB_1/CB_2 receptors or through COX-2 metabolites of 2-AG.[162]

Adenosine is an orthosteric endogenous ligand for the adenosine A_3 receptor. This GPCR plays an important role in modulation of inflammation.[163] 2-AG acts as a negative allosteric modulator of human adenosine A_3 receptor ligand binding. Therefore, the adenosine A_3 receptor can be a potential alternative pathway for 2-AG to mediate inflammatory responses.[164] In addition, 2-AG has also been reported to act as a ligand for the TRPV1 receptor, which is an outwardly rectifying Ca^{2+} permeable nonselective cation channel. The receptor is activated by stimuli such as heat ($T > 42\ ^oC$) and low extracellular pH, and also by endogenous ligands including lysophosphatidic acid and AEA. TRPV1 plays an important role in the regulation of core body temperature and inflammatory pain.[165] Therefore, TRPV1 is an important target to consider when studying the relation between 2-AG and pain sensation.[34,166-168] GPR55 has been called the type 3 cannabinoid receptor, because of its affinity for multiple cannabinoids. Rydberg *et al.* showed that 2-AG has a ~150 fold higher affinity for the GPR55 receptor compared to the CB_1 and CB_2 receptor.[36] In addition to 2-AG, also other endogenous endocannabinoids showed affinity for GPR55, including virodhamine, AEA, oleamide and noladin ether. GPR55 has been linked to energy balance and is a potential drug target for various cancer types.[169] Together, these studies indicate that 2-AG has a wide variety of targets distinct from the classical cannabinoid receptors. To a large extent, the physiological relevance of these non-CB_1/CB_2 receptors has yet to be established *in vivo*. Further research will reveal the extent of physiological effects governed by interactions between 2-AG and the other proteins.

6. *In vivo* physiological effects of 2-AG

This section aims to map the physiological role of 2-AG, and whether an effect can be directly or indirectly ascribed to 2-AG. To gain insight in the role of 2-AG, multiple tools and techniques have been developed and applied during the past decades. These include pharmacological blockade and genetic disruption of its target receptor(s), catabolic and metabolic enzymes, modulation of 2-AG localization, and administration of exogenous 2-AG. A single approach is often not sufficient to gain information on the role of 2-AG. A combination of these approaches is often required because:

1) 2-AG biosynthetic and metabolic enzymes have multiple substrates and roles. Thus, disruption of these enzymes results in decreased 2-AG levels, but levels of other substrates or metabolites of targeted enzymes will also be altered.

2) 2-AG precursors and metabolites have important signaling functions and are intermediates for multiple lipid metabolic pathways.

3) Receptors targeted by 2-AG recognize multiple ligands. Additionally, 2-AG is a promiscuous ligand that interacts with multiple receptors, resulting in a considerable increase of possible interactions through which 2-AG can mediate its physiological effects.

Intensive research has provided insight in the physiological role of 2-AG, which will be discussed below.

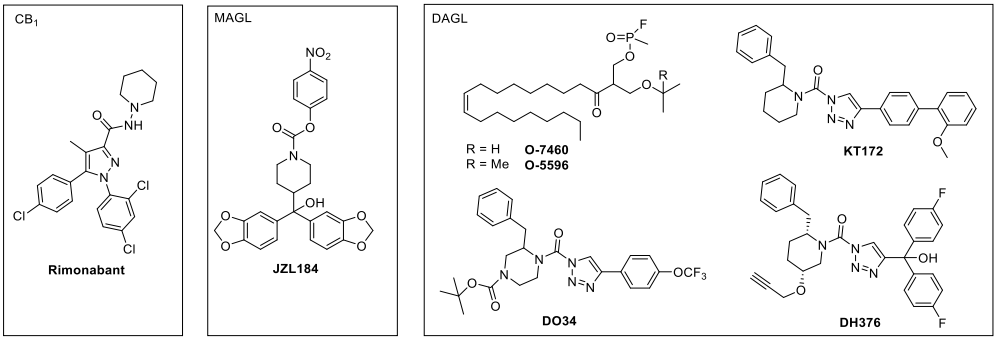

Figure 2. Tool compounds used to study the physiological role of 2-AG.

6.1 Food intake and metabolism

The intimate link between the endocannabinoid system and energy balance is well established.[182] CB_1 knockout mice display a lean phenotype, reduced adiposity and feeding efficiency. When fed a high fat diet, they display decreased triglyceride, plasma insulin, cholesterol, and leptin levels, while having increased adiponectin levels compared to their wild type littermates.[183,184] This phenotype can be mirrored by treatment with the CB_1 inverse agonist rimonabant.[185] Importantly, rimonabant was approved on the European market in 2006 as a drug for the treatment of obesity. However, rimonabant was withdrawn from the European market in 2008 due to serious psychiatric side effects, in particular depression.

Several lines of evidence indicate that 2-AG is, to a large extent, responsible for CB_1 mediated regulation of food intake and energy metabolism. Fasting increases levels of 2-AG in the limbic forebrain and the hypothalamus. Additionally, bilateral injection of 2-AG into the nucleus acumbens stimulated increased feeding, and pretreatment with rimonabant could reduce the effect elicited by 2-AG infusion.[186] DAGL-α knockout mice showed a similar phenotype with decreased body weight, low triglyceride, cholesterol, and insulin levels as observed in the CB_1 knockout mice.[187] This phenotype was absent in

DAGL-β knockout mice, indicating that 2-AG biosynthesized by DAGL-α is responsible for the observed effects. Acute disruption of DAGL activity by (i.p.) administration of O-7460, a fluorophosphonate-based DAGL inhibitor, dose dependently inhibited intake of high fat diet in mice.[188] This is in line with a previous report where the DAGL inhibitor O-5596 reduced the intake of palatable food in mice.[189]

Jung *et al.* provided further evidence for the involvement of 2-AG in metabolism.[190] Upregulation of 2-AG hydrolysis by overexpression of MAGL in the mouse forebrain led to a 50% decrease of 2-AG levels in this brain region, but did not affect AEA nor AA levels. These mice were lean, hyperphagic, resistant to diet induced obesity, hyperthermic and hypersensitive to β_3-adrengenic-stimulated thermogenesis. Inhibition with the MAGL inhibitor JZL184 showed that recovering 2-AG signaling at the CB_1 receptors normalized β_3-adrengenic-dependent thermogenesis. This indicated that 2-AG mediated CB_1 signaling helps to conserve body energy by moderating heat production.

6.2 Neuro-inflammation

2-AG plays a central role in multiple neuro-inflammatory processes. In 2001, Panikashvili *et al.* reported a neuroprotective role of 2-AG in a traumatic brain injury model.[191] After infliction of closed head injury, a strong elevation, reaching a ten-fold increase after 4h, of mouse brain 2-AG levels was observed. Injection of exogenous 2-AG after closed head injury elicited a neuroprotective effect. This effect was reduced in CB_1 knockout models and after pretreatment with rimonabant. Neuro-inflammation is one of the early neurochemical responses after closed head injury.[192] Elevations of pro-inflammatory mediators, such as TNF-α and IL-lβ, at early stages after injury are observed. 2-AG inhibited NF-κB transactivation and reduced acute expression of the pro-inflammatory cytokines TNF-α, IL1β and IL-6 via a CB_1 dependent mechanism.[193]

Of note, metabolites of 2-AG are also involved in the modulation of neuro-inflammatory processes. Previously, phospholipase A_2 (PLA_2) was considered to be the primary source of AA for COX-mediated biosynthesis of prostaglandins, but this model has significantly changed in recent years. Nomura *et al.* showed that MAGL hydrolysis of 2-AG provides the major pool of AA for the generation of neuro-inflammatory eicosanoids in specific tissues, such as the brain, liver and lungs.[77] Chemical inhibition or genetic disruption of MAGL activity lowered lipopolysaccharide (LPS)-induced pro-inflammatory eicosanoid production in the brain, such as prostaglandin E_2 (PGE_2), PGD_2, PGF_2, and thromboxane B2 (TXB2), via a CB_1 and CB_2 receptor independent mechanism. In addition, abolishment of MAGL activity did not affect basal cytokine levels, but almost completely blocked LPS-induced elevation of interleukin-1α (IL-1α), IL-1β, IL-6 and TNF-α. Specific knockdown of MAGL in astrocytes moderately elevated 2-AG levels and did not cause CB_1 receptor desensitization nor any behavioral effect, but decreased cytokine and prostaglandin levels via a CB_1/CB_2 independent mechanism.[194] This is in line with the anti-inflammatory

effects observed with blocking 2-AG biosynthesis. DAGL-α/β inhibitors DO34 and DH376 reduced brain levels of 2-AG, AA and AEA.[57] The pro-inflammatory prostaglandins PGE_2 and PGD_2 and cytokine IL-1β were also reduced in LPS-treated animals. In line, Viader *et al.* showed that DAGL-β is important for regulation of 2-AG levels in microglia without change in global 2-AG and prostaglandin levels in the brain.[60] Finally, LPS-induced anapyrexia, i.e. lowering of core body temperature, was substantially reduced in DAGL-α and DAGL-β KO and inhibitor-treated mice,[60] which is consistent with increased anapyrexia observed in MAGL inhibitor treated mice.[195] This implies an important role for 2-AG in the regulation of body temperature.

Multiple neurological disorders, including multiple sclerosis (MS), Parkinson's and Alzheimer's disease, have a neuro-inflammatory component. Consequently, modulation of MAGL activity has been tested in various animal models of neurodegenerative diseases. Nomura *et al.* used a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model for Parkinson's disease.[77] JZL184 provided neuroprotective effects, which were not reversed by the CB receptor antagonists, but were recapitulated by COX inhibition. This indicated that the neuroprotective effects were not mediated via the CB receptors, but due to reductions in AA and pro-inflammatory prostaglandins. Mounsey *et al.* confirmed these findings.[196]

Perturbation of MAGL activity has been investigated in animal models of Alzheimer's disease, such as 5XFAD and $PS1/APP^+$ models. Genetic and pharmacological inactivation of MAGL suppresses proinflammatory responses and amyloidosis in a $PS1/APP^+$ mouse model.[197] In the 5XFAD mouse model, inhibition of MAGL reduces production and accumulation of β-amyloid (Aβ) and improves cognitive function.[198] In mice suffering from experimental autoimmune encephalitis (EAE), a mouse model for MS, inhibition of MAGL activity reduced the severity of the clinical symptoms.[199,200] Inhibition of DAGL with the fluorophosphonate-based inhibitor O-3841 was neuroprotective in a malonate model of Huntington's disease in rat brain.[201] In contrast, inhibition of MAGL activity and administration of PGE_2-G worsened malonate-induced injury. Therefore oxidative metabolites of 2-AG have been suggested to play a role in the toxic effects.

6.3 Anxiety

Endocannabinoid signaling via the CB_1 receptor has a primary role in modulating anxiety and depressive behaviors.[202,203] The effects of CB_1 receptor agonists on anxiety are complex and show biphasic effects. Low agonist doses lead to anxiolytic effects,[204,205] while high doses lead to anxiogenic effects.[206] Pharmacological blockade and genetic deletion of the CB_1 receptor has been shown to increase anxiety.[204,207-210]

2-AG plays an important role in stress and anxiety. Studies have generally shown a bidirectional effect of stress on endocannabinoid levels, AEA levels decrease, while 2-AG

levels increase.[211,212] Shonesy *et al.* investigated the role of DAGL-α in anxiety.[213] An increase in anxiety and depressive behavior in DAGL-α KO mice was observed. This effect was reversed by normalization of deficient 2-AG by pharmacological inhibition of MAGL. This normalization experiment provides extra confidence that the observed effects are 2-AG mediated and not caused by reduction downstream metabolites. Jenniches *et al.* observed a similar anxiogenic phenotype in DAGL-α knockout mice.[214] Currently, it is unknown whether acute blockade of DAGL-α also results in anxiogenic behavior. In line with anxiogenic effects when 2-AG levels are lowered by disruption of 2-AG biosynthesis, anxiolytic effects are observed when 2-AG levels are increased.

Elevation of 2-AG levels by inhibition of MAGL with JZL184 at relatively low doses did not affect motility, induced anxiolytic like effects in in a marble burying test,[78] and in elevated plus mazes.[90,215-217] Sumislawski *et al.* showed that chronic administration of JZL184 prevented chronic stress induced anxiety like behavior, but that acute MAGL inhibition has little effect on anxiety like behaviors. This could indicate that long term synaptic adaptations play an import role. Additionally, it was shown that elevated 2-AG tone prevents behavioral and synaptic adaptations to chronic stress.[92] The anxiolytic effect in most studies could be blocked by rimonabant, indicating it is likely CB_1 mediated.

6.4 Addiction

The endocannabinoid system has been implicated in multiple aspects of addiction.[218-220] CB_1 receptor agonists have been shown to alleviate withdrawal symptoms,[221,222] while the inverse agonist and antagonists reduce nicotine self-administration.[220] Several lines of evidence indicate an important role of 2-AG in addictive behavior and withdrawal symptoms. Injection of exogenous 2-AG was moderately effective in reducing the intensity of precipitated withdrawal signs in morphine-dependent mice.[222] Ramesh *et al.* investigated the effect of MAGL inhibition by JZL184 on naloxone-precipitated morphine withdrawal symptoms.[223] Selective elevation of 2-AG levels over AEA levels completely blocks behavioral effects of spontaneous and precipitated withdrawal, including paw flutters, diarrhea, jumps, and weight loss. Reduction of precipitated withdrawal effects by 2-AG elevation is blunted by rimonabant, suggesting a CB_1 receptor mediated effect. The role of 2-AG in relation to nicotine addiction was also studied.[224] Somatic and aversive withdrawal signs in nicotine-dependent mice were reduced by both genetic and pharmacological inactivation of MAGL via a CB_1 receptor mediated mechanism.[224]

In agreement with experiments with CB_1 receptor antagonists,[225] reduction of 2-AG levels by inhibition of its biosynthetic enzymes DAGL-α and DAGL-β reduced nicotine self-administration of mice. Release of the inhibitory neurotransmitter GABA is diminished upon chronic nicotine exposure.[220,226] To gain insight in the underlying mechanism, the authors studied the effect of DAGL inhibition on GABA signaling. Inhibition of DAGL by the 1,2,3-triazole urea KT172 rescues GABAergic signaling at

dopaminergic neurons in the ventral tegmental area (VTA).[226] Conversely, increasing 2-AG signaling by blocking the 2-AG metabolizing enzyme MAGL recapitulates the loss of nicotine-induced GABA signaling following chronic nicotine exposure.

6.5 Pain

Cannabinoids have been used for their ability to reduce pain for many centuries.[227] In addition, various studies have demonstrated the analgesic efficacy of local administration of 2-AG.[228,229] Elevation of 2-AG has also been observed in preclinical models of inflammatory pain, indicating a role of endogenous 2-AG in pain perception.[230,231] Anti-nociceptive effects by elevation of 2-AG via MAGL inhibition has been well established. Robust analgesic effects by MAGL inactivation are demonstrated in neuropathic pain,[232] peripheral inflammatory pain,[93] gastrointestinal pain[233] and chemotherapy-induced neuropathy.[234,235] For a comprehensive review on the endocannabinoid system in pain see S. G. Woodhams *et al.*[236] Anti-nociceptive effects in thermal, noxious chemical, and neuropathic pain sensation by MAGL inhibition were blocked by the CB_1 receptor inverse agonist rimonabant.[78,234] Intracerebroventricular injection of JZL184 produced TRPV1-dependent anti-nociception in the mouse formalin test, which indicates that anti-nociceptive effects are not only mediated by CB receptors, but that TRPV1 can also contribute to anti-nociceptive effects.[168] Recently, inhibition of DAGL was reported to reduce nociception in preclinical models of inflammatory and neuropathic pain.[237] A likely explanation for this effect is a decrease in AA levels. Lower AA levels result in decreased levels of prostaglandins and subsequent modulation of inflammation and pain.

Taken together, these data demonstrate an important role for 2-AG in pain sensation via multiple mechanisms, including TRPV1, CB_1, and CB_2 signaling and as a precursor for prostaglandins that modulate inflammation and pain.

7. Conclusions

A major mechanism through which 2-AG exerts its physiological effects in the brain are the CB_1 receptors. Activation of the CB_1 receptor by 2-AG regulates multiple physiological processes. This chapter described important roles of 2-AG signaling in food intake, neuroprotection, neuro-inflammation, addiction, anxiety and pain. In addition to classical CB receptors, 2-AG has shown remarkable affinity for multiple other receptors, including $GABA_A$, adenosine A_3, TRPV1, PPARγ, and GPR55 receptor. To date it is, however, unknown whether these proteins are involved in physiological effects of 2-AG *in vivo*. Next to its function as a signaling lipid, 2-AG is also a key intermediate many lipid metabolic pathways. A number of 2-AG metabolites have important signaling functions in their own right. 2-AG is a key precursor for the biosynthesis of eicosanoids in the brain, which in

turn, act on several G-protein coupled receptors to propagate inflammation.[123] Its involvement in multiple important (patho)physiological processes makes modulation of 2-AG levels an interesting strategy from a therapeutic perspective.

Modulation of 2-AG levels *in vivo*, in particular by blocking MAGL or DAGL activity, triggers multidirectional effects in various (patho)physiological processes (Figure 3). Reduction of 2-AG levels by blocking its major, DAGL-α/β biosynthetic pathway can be beneficial for treatment of the metabolic syndrome, addiction, and neuro-inflammatory disorders, while elevation of 2-AG levels by blocking MAGL, the major hydrolytic pathway towards AA, reduces neuro-inflammation, anxiety related behavior, withdrawal symptoms and nociception. However, its prime role in many important physiological pathways makes modulation of 2-AG levels sensitive to untoward effects (Figure 3). The essential role of cannabinoid signaling in the brain was emphasized by the retraction of the CB_1 inverse agonist and anti-obesity drug rimonabant from the European market because of severe neuropsychiatric side effects.

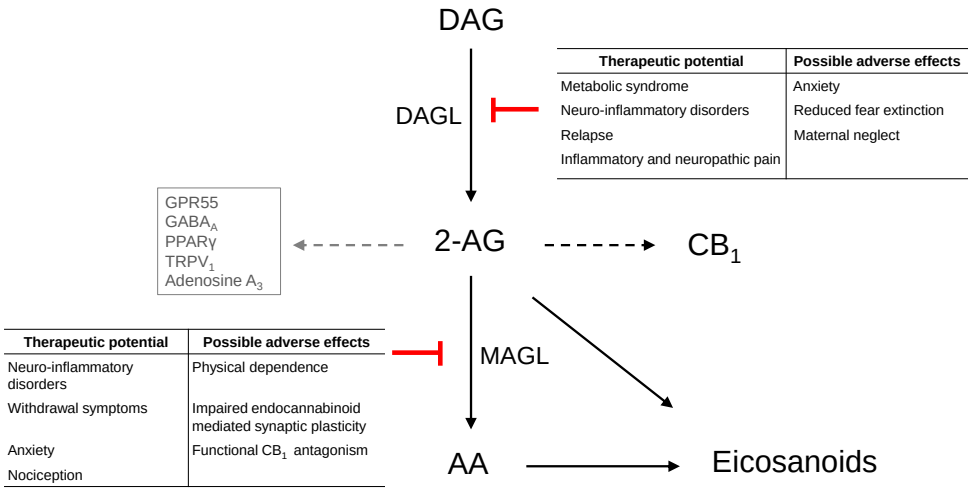

Figure 3. 2-AG plays a central role in multiple pathophysiological processes. Both elevation and reduction of 2-AG has therapeutic potential. However, untoward neurological effects could pose a serious threat for the use 2-AG modulation for the treatment of human disorders.

It is important to note that not all behavioral phenotypes of CB_1 KO mice were mirrored in DAGL-α KO mice, for example, they showed less anxiogenic behavior compared to CB_1 KO mice in open field test and platform tests.[187] These differences can partly be explained by considering some CB_1 signaling pathways (e.g. AEA) remain intact when modulating 2-AG levels. Therefore, selective modulation of 2-AG metabolism can help to dissect the signaling roles of 2-AG and AEA, and potentially circumvent adverse effects observed by direct blockade of the CB_1 receptor. In addition, elevation of 2-AG

levels by blockade of MAGL did not cause the full spectrum of cannabinoid-behavioral effects such as hypothermia and catalepsy as observed with direct cannabinoid agonists.[69,238]

Although modulation 2-AG levels does not reproduce all untoward effects observed with direct CB_1 interference, several adverse effects have been observed upon pharmacological or genetic disruption of the major biosynthetic and metabolic pathways of 2-AG. Chronic blockade of MAGL causes desensitization and down regulation of the CB_1 receptor, while disruption of DAGL increases anxiety, maternal neglect and reduced fear extinction. Therefore fine-tuning rather than complete disruption of 2-AG metabolism and biosynthesis could be a promising approach to establish a therapeutic window. This therapeutic window can be achieved via several strategies: *1)* Partial blockade of major biosynthetic (DAGL) or metabolic (MAGL) pathways. *2)* Targeting 2-AG metabolizing enzymes that have a smaller contribution to bulk 2-AG regulation compared to DAGL and MAGL.

Lowering of 2-AG levels by partial blockade of the DAGL pathway can be achieved by subtype selective DAGL inhibitors. DAGL-β has been shown to reduce inflammatory responses *in vivo* without affecting synaptic transmission.[60] In addition, incomplete inhibition of the DAGL enzymes can be used to tweak endocannabinoid tone. In opposite direction, moderate increase of 2-AG could be achieved by partial inhibition of MAGL. For partial inhibition of both MAGL and DAGL reversible inhibitors would be highly valuable tools. The *in vivo* role with respect to 2-AG of enzymes with a less defined contribution to 2-AG metabolism (ABHD2, 6 and 12, COX2, LOX and CytP450), transport (FABP5) and signaling ($GABA_A$, adenosine A_3, TRPV1, PPARγ, and GPR55) requires further investigation. Future research on these enzymes might uncover new physiological roles of 2-AG and novel approaches to modulate 2-AG in health and disease.

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