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DINA NAVIA-PALDANIUS

PHARMACOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF ABHD6, ABHD11 AND ABHD12

Pharmacological and biochemical characterization of ABHD6, ABHD11 and ABHD12

DINA NAVIA-PALDANIUS

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ABSTRACT

A retrograde lipid signaling system, the endocannabinoid system (ECS), influences various functions in the CNS (central nervous system) and periphery. The ECS consists of two G-protein coupled cannabinoid receptors (CB1 and CB2), their endogenous ligands called endocannabinoids, as well as three hydrolases MAGL (monoacylglycerol lipase), ABHD6 and ABHD12 (α/β-hydrolase domain containing 6 and 12) which regulate the lifetime of the endocannabinoids. These hydrolases belong to the serine hydrolase family which is the largest group of enzymes having an important role in many biological processes. Interestingly, although about half of these enzymes have been well characterized and their importance recognized, much less is known about the others.

The ABHD proteins belong to the serine hydrolase family of enzymes; they are potential regulators of lipid metabolism and signal transduction. Dysregulation of this system may be involved in many chronic diseases such as diabetes, cardiovascular disease and cancer. Their predicted role in lipid metabolism has made ABHD hydrolases interesting drug targets. Despite this potential, the uncertainties surrounding their physiological function as well as the paucity of endogenous substrates and even the lack of a reliable way to assay enzyme activity have impaired the development of novel ABHD inhibitors.

This study had three aims: (I) to explore regional CB1 receptor-mediated signaling in (MAGL)-KO mouse brain, (II) to develop sensitive cell-based activity assays that could be used for characterizing ABHD6, ABHD11, ABHD12 in comparison to the well-characterized MAGL, (III) to identify inhibitors and substrates for these hydrolases.

The results emerging from these studies indicated that MAGL-KO mouse brain undergoes a regional desensitization of the CB1 receptor in response to overload with the MAGL substrate, 2-AG. We set up and optimized a sensitive glycerol-based hydrolase activity assay that made it possible to profile the substrate preferences for two novel endocannabinoid hydrolases, ABHD6 and ABHD12, in comparison to MAGL, and were able to define ABHD6 and MAGL as genuine MAG hydrolases. With respect to ABHD11, we managed to establish an activity assay using a chromogenic substrate, para-nitrophenyl butyrate, and in conjunction with activity-based protein profiling (ABPP), we discovered several potent inhibitors that could be used in the further characterization of this hydrolase.

National Library of Medicine Classification: QU 136, QU 143, QU 375, QV 126
Medical Subject Headings: Hydrolases; Monoacylglycerol Lipases; Enzyme Inhibitors; Endocannabinoids; Receptor, Cannabinoid, CB1; Signal Transduction; Brain; Mice, Knockout

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TIIVISTELMÄ

Endokannabinoidijärjestelmä on yksi elimistön lipidisignalointijärjestelmistä, joka vaikuttaa sekä keskushermostossa että perifeerisissä kudoksissa. Järjestelmään luetaan nykyisin kaksi G-proteiinikytkentäistä reseptoria (CB1 ja CB2), niitä aktivoivat ligandit (endokannabinoidit), sekä endokannabinoidien elinikää säätelevät entsyymit MAGL (monoasyyliglyseroli lipaasi), ABHD6 ja ABHD12 (α/β-hydrolaasi domain 6 ja 12). Nämä entsyymit kuuluvat suurimpaan entsyymiperheeseen, seriinihydrolaaseihin, jotka osallistuvat moniin eri biologisiin toimintoihin. Tästä huolimatta noin puolet tämän perheen entsyymeistä on huonosti tunnettuja.

ABHD proteiiniperhe kuuluu myös seriinihydrolaaseihin. Niiden on esitetty säätelevän lipidimetaboliaa ja solujen signalointia. Ongelmat näissä säätelyjärjestelmissä saattaa johtaa moniin kroonisiin sairauksiin kuten diabetekseen, sydän- ja verisuonitauteihin sekä syöpään. ABHD entsyymien oletettu rooli lipidimetaboliassa on tehnyt niistä kiinnostavia lääkekehityskohteita. Tästä huolimatta näiden entsyymien fysiologisia rooleja eikä luonnollisia substraatteja tunnetta. Sen lisäksi näille entsyymeille ei ole spesifisiä inhibiittoreita, eikä sopivia entsyymiaktiivisuusmittausmenetelmiä, mitkä hidastavat näiden entsyymien tutkimista.

Tällä tutkimuksella oli kolme tavoitetta: (I) tutkia CB1-kannabinoidireseptorin signalointia MAGL poistogeenisten hiirien aivoleikkeissä, (II) kehittää herkkä solupohjainen menetelmä entsyymiaktiivisuusmäärityksiin, jolla karakterisoida huonosti tunnettuja ABHD6, ABHD11, ABHD12 entsyymejä sekä hyvin tunnettua MAGL ja lisäksi (III) löytää inhibiittoreita ja substraatteja näille entsyymeille.

Tutkimuksessa havaittiin, että MAGL-poistogeenisten hiirten aivot sopeutuvat tilanteeseen vähentämällä eri aivoalueilla CB1-reseptorien kykyä signaloida. Kahdessa muussa osatyössä käytettiin useita eri menetelmiä huonosti tunnettujen ABHD-perheen entsyymien karakterisoimiseen. Työssä kehitettiin herkkä entsyymiaktiivisuuteen perustuva menetelmä, jolla määritettiin substraattimieltymykset ABHD6- ja ABHD12-entsyymeille rinnan hyvin tunnetun MAGL-entsyymin. Työssä osoitettiin, että ABHD6 ja MAGL ovat varsin samankaltaisia monoglyseridipohjaisten substraattimieltymysten suhteen. Näitä tuloksia ja kehitettyä menetelmää käytettiin myöhemmin spesifisten inhibiittorien kehittämiseen näille entsyymeille. Lisäksi karakterisoimme huonosti tunnettua ABHD11-entsyymiä useilla eri menetelmillä. Vaikka proteiinin fysiologinen rooli jäi selvittämättä, löysimme sille lipaasien yleisesti käyttämän keinotekoisen substraatin. Tämä voisi viitata ABHD11 mahdollisesti toimivan lipaasina. Käyttämällä kemoproteomista entsyymiaktiivisuusmääritysmenetelmää löysimme ABHD11:lle kolme potenttia inhibiittoria, joita voidaan käyttää jatkotutkimuksissa tämän entsyymin kohdalla.

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Kuopio, September 2018

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II Navia-Paldanius D, Savinainen JR and Laitinen JT. Biochemical and pharmacological characterization of human α/β-hydrolase domain containing 6 (ABHD6) and 12 (ABHD12). *J Lipid Res. 53:2413-2124, 2012*.

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Abbreviations

2-AG	2-arachidonoylglycerol
AA	Arachidonic acid
ABHD2	α/β hydrolase domain 2
ABHD3	α/β hydrolase domain 3
ABHD5	α/β hydrolase domain 5
ABHD6	α/β hydrolase domain 6
ABHD11	α/β hydrolase domain 11
ABHD12	α/β hydrolase domain 12
ABHD16A	α/β hydrolase domain 16A
AFMID	Arylformamidase
AtABHD11	Arabidopsis homolog of a human ABHD11 gene
ATGL	Adipose triacylglycerol lipase
AEA	Ananamide (*N*-arachidonoyl-ethanolamine)
ABPP	Activity-based protein profiling
AM251	CB1 receptor antagonist
BAT5	Known also as ABHD16A
BSA	Bovine serum albumin
BMP	bis(monoacylglycerol) phosphate
Ca^{2+}	Calcium ion
CGI-58	Comparative gene identification-58
CNS	Central nervous system
CP55,940	CB1/CB2 receptor agonist
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DDHD2	DDHD Domain Containing 2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DOG	1,2-Dioleoyl-rac-glycerol
DTNB	5,5′-dithiobis-2-nitrobenzoic acid
Δ^9-THC	Δ^9-tetrahydrocannabinol
FA	Fatty acid
FAAH	Fatty acid amide hydrolase
FBS	Fetal bovine serum
G-1-P	Glycerol-1-phosphate
GK	Glycerol kinase
$G_{i/o}$	Inhibitory class of heterotrimeric G proteins
GPO	Glycerol phosphate oxidase
GPR55	G-protein coupled receptor 55
GSH	Glutathione
GTPγS	Guanosine-5′-O-(3-thio)-triphosphate
[^{35}S]GTPγS	Guanosine-5′-O-(3-[^{35}S]thio)-triphosphate
H_2O_2	Hydrogen peroxide
HDSF	Hexadecylsulfonyl fluoride
HEK293	Human embryonic kidney293 cells
HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidase
HSL	Hormone sensitive lipase
IDFP	Isopropyldodecyl-fluorophosphonate
JJKK-048	MAGL inhibitor
JW651	MAGL inhibitor
JZL184	MAGL inhibitor
KO	Knockout
LC/MS/MS	Liquid chromatography/ tandem mass spectrometry
LNCaP	Androgen-sensitive human prostate adenocarcinoma cell line
LPA	Lysophosphatidic acid
LPI	Lysophosphatidyl inositol
LPS	Lysophosphatidyl serine
LYPLA2	Lysophospholipase II
MAFP	Methylarachidonoylfluoro-phosphonate

MAG	Monoacylglycerol
MAGL	Monoacylglycerol lipase
ML211	LYPLA1/2 and ABHD11 inhibitor
NAPE	N-acyl phosphatidyl ethanol amine
NLSDI	Neutral lipid storage disease
NMBA	2-nitro-5-mercaptobenzoic acid
PBS	Phosphate-buffered saline
PC3	Human prostate cancer cell line
PHARC	Polyneuropathy, hearing loss, ataxia, retinis pigmentosa and cararact
PLA	Phospholipase A
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
pNP	para-nitro phenol
RHC80267	DAGL inhibitor
RT	Room temperature
TAG	Triacylglycerol
TAMRA-FP	Active-site-directed serine hydrolase probe
THL	Tetrahydrolipstatin (orlistat), lipase inhibitor
URB597	FAAH inhibitor
VCaP	Human prostate cancer cell line
WAT	White adipose tissue
WT	Wild-type
WWL70	ABHD6 inhibitor

1 Introduction

Proteins are responsible for cellular functions. Some proteins act as either receptors or enzymes. They are inert until they are activated by the presence of a ligand or substrate stimulating a specific cellular response. Most receptors are membrane-bound proteins that participate in signaling transduction. Thus, receptors are the triggering parts of cell signaling pathways; in contrast, enzymes are proteins that catalyze chemical reactions and participate in many biological processes. The actual chemical reaction where the substrate is converted to one or more end products occurs in the active site of the enzyme. Due to their crucial physiological roles, enzymes are important pharmaceutical targets, there are many approved drugs which act as enzyme inhibitors (Overington et al., 2006).

The human genome sequence project was one of the greatest achievements of the biological sciences (International Human Genome Sequencing Consortium 2004). The full sequence of the human genome revealed not only a huge number of unknown proteins, but also revealed how far we are from understanding the metabolic networks in humans (Medina-Cleghorn and Nomura 2014). The human metabolic network consists of enzymes converting substrates to products. Enzymes can function together can also produce metabolic pathways such as glycolysis, where multiple enzymes are involved in catalyzing the conversion of a metabolite, in this case glucose, into the end-product, i.e. pyruvate (Medina-Cleghorn and Nomura 2014). One metabolic pathway can interact with other biochemical pathways producing numerous metabolites that the cell can use as building blocks, regulatory metabolites and signaling molecules.

Serine hydrolases are one of the largest and the most diverse enzyme families. This enzyme family has two subclasses, proteases and metabolic serine hydrolases, and at present is thought to consist of about 200 enzymes (Long and Cravatt 2011; Simon and Cravatt 2010). Serine hydrolases are therapeutic targets for several diseases. For example, the dementia associated with Alzheimer's Disease is treated by inhibiting the enzyme which breaks down acetylcholine, obesity can be alleviated by inhibiting several gastric enzymes such as hormone-sensitive lipase (HSL), and type II diabetes can be treated by inhibiting dipeptidyl peptidase (Bachovchin and Cravatt 2012). They also modulate lipid signaling systems like the endocannabinoid system that is involved in a wide range of physiological processes in mammals, such as in the responses to pain and inflammation, feeding and energy regulation, learning, memory, and emotions (Fowler 2006; Pacher et al. 2006). While the importance of serine hydrolases is recognized, it is appreciated that many, perhaps half, of these enzymes are still poorly characterized with respect to their physiological functions, endogenous substrates and end-products. Most of them also lack specific inhibitors, which are crucial in the characterization of the physiological function of these unknown enzymes.

If one wishes to understand the processes coordinating lipid metabolism, we need to better understand the molecular mechanisms behind the cellular effects and the consequences when these become disrupted, leading to human diseases. It is widely accepted that most of the serine hydrolases are lipid-metabolizing enzymes, playing important role(s) in (patho)physiology (Thomas et al. 2014) which means that serine hydrolases are attractive drug targets. Nonetheless, most of these proteins need to be better characterized in order to provide a foundation for understanding their physiological functions.

The complete human genome sequence and the large number of the still uncharacterized genes that it identified provided us with a workframe. At the same time, it has posed a major challenge. In order to understand human physiology and to learn more about the

development of diseases (pathophysiology), it is desirable to find a function and a physiological relevance for these unknown proteins and the first step is to identify physiologically important substrates and end-products. This can only be achieved when more efficient assay methods are devised. Furthermore, we need to develop specific inhibitors to uncover the specific role of the enzymes and their possible role in pathophysiology. In addition, these inhibitors can often be used as lead structures in drug design programs. One of the aims of this study was to characterize both biochemically and pharmaceutically several of the relatively recently found endocannabioid hydrolyzing metabolic serine hydrolases and to discover inhibitors for the mitochondrial metabolic serine hydrolases at present lacking a physiological function.

2 Review of the literature

2.1 INTRODUCTION TO PROTEIN CHARACTERIZATION

We have multiple methods which can be exploited in the characterization of a protein. Proteins can be characterized by isolating them from biological materials or the protein of interest can be cloned when one knows the sequence of its coding gene. The used methods depend on the strategy. One of these protein characterization strategy is described in Figure 1.

In the pre-genomic era, assays were developed for *in vitro* characterization. In the ideal case, these assays led to the recognition of the protein which, after cloning, was sequenced to determine the gene behind the observation. Now, the knowledge of the complete human genome sequence has reversed the traditional strategy. The availability of the sequences and genome maps of each gene has switched the focus so that instead of *in vitro* observations, we are now able to begin from the protein coding gene and clone the protein. In the ideal case, we can develop an assay and inhibitor(s) that can be used in further experiments for characterizing the protein.

2.1.1 Isolation and separations of protein of interest

Whether we are interested in protein from some biological material or a cloned protein, the first step in the protein's characterization is to isolate and separate the protein of interest. For example, proteins can be separated by size using centrifugation, chromatography or gel electrophoresis. The drawback of using these methods is that they cannot separate different proteins with the same size. This is a problem especially when isolating proteins from biological materials. One way to overcome this problem is to use 2D-PAGE (2-dimension polyacrylamide gel electrophoresis) which distinguishes between different proteins based on their isoelectric point and size.

The greatest advantage of 2D-PAGE is its capability of separating mixtures of proteins. The first dimension is called isoelectric focusing where the proteins are separated in an immobilized pH gradient. Proteins migrate to their isoelectric point where their net charge, based on their amino acid composition, is zero. SDS (sodium dodecyl sulfate) PAGE further separates the proteins based on their size. This separation performed with SDS-PAGE is called the second dimension. 2D-PAGE enables the separation of thousands of proteins simultaneously. However, the method is unreliable for protein identification. Additionally,

it is not useful for proteins with low abundances and furthermore the quantification of the protein is difficult.

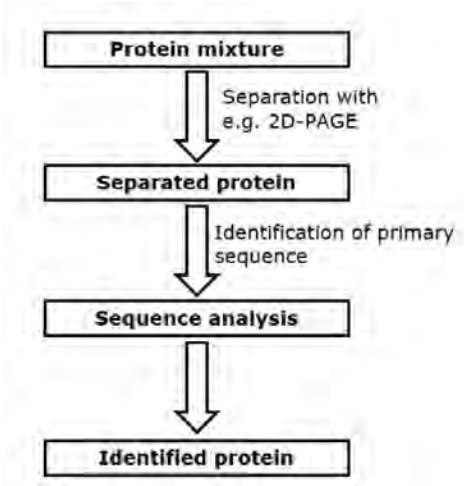


Figure 1. Schematic representation of how a protein is identified from a biological material.

2.1.2 Identifying protein of interest

If the separated protein is unknown, the next step is to identify the protein by determining its primary sequence. The primary sequence can be determined using either chromatography or mass spectrometry (Lodish et al. 2000; Mallick and Kuster 2010). One option is to use *Edman degradation* where the N-terminal is labeled and then the protein is degraded in a stepwise manner and subsequently analyzed with liquid chromatography. Although the method has its advantages, mass spectrometry-based methods are more commonly used. In mass spectrometry, the peptides are ionized after they have been separated, with the identification being based on their mass-to-charge (m/z) ratio (Mallick and Kuster 2010). The results are analyzed by comparison with a sequence database. The advantage of the mass spectrometry is its sensitivity. Additionally, it achieves a quantitative determination and qualitative structural information of a large range of samples. Even though mass spectrometry is by far the most useful method, its relatively expensive cost obstructs its widespread use.

2.1.3 Defining protein structure

The three-dimensional 3D structure needs to be defined if one wishes to understand how the protein functions at the molecular level. When investigating a protein's structure, it is necessary to produce a sufficient amount of the full length and active form of the protein of interest. Proteins vary with respect their structure and stability, hence there is no universal production method or protein characterization scheme that works for every protein. One option to produce a sufficient amount of protein is to clone the gene, for example in *E. coli*, after which the protein needs to be purified.

The 3D structure can be defined from a high quality purified protein. The methods most commonly used to define the 3D structure are NMR spectroscopy, X-ray crystallography and cryoelectron microscopy (Lodish et al. 2000; Zheng et al. 2015). NMR can be used to study small proteins (~20 kDa) (Marion 2013). In NMR, the proteins are in solution whereas in X-ray crystallography, the protein needs to be crystallized (Zheng et al. 2015). Crystallized proteins allow the determination of 3D structure from more complex and

larger proteins than can be achieved with NMR. For proteins that are particularly large or hard to crystallize, cryoelectron microscopy can be applied. In this method, the protein is rapidly frozen in liquid ethane (Carroni and Saibil 2016). The resulting low resolution views are analyzed by computer programs to reconstruct the 3D protein structure. It is also possible to combine X-ray crystallography and cryoelectron microscopy. This combination helps to determine the protein's location as well as protein–protein interfaces especially in complex models (Wang and Wang 2017).

Defining 3D structure can be challenging and especially crystallization is demanding, since the protein may be sensitive to temperature, pH and ionic strength. Moreover, these experimental methods are time consuming and laborious and this probably explains why 3D structures are lacking for so many proteins. Methods capable of elucidating protein structures have proceeded relatively slowly, therefore computational approaches for predicting the 3D structures of proteins have been often considered as being more efficient than their experimental alternatives (Venkatesan et al. 2013).

2.1.4 Homology modeling in functionalizing unknown enzymes

Even with the known sequence, it is hard to predict the function of the protein since even proteins with closely related amino acid sequences may have different functions. On the other hand, proteins with very low amino acid sequence similarities may perform similar functions. This can be explained by structural similarities between folded proteins such that related functions can be almost identically coded by two distinct sequences with limited or no homology (Kaczanowski and Zielenkiewicz 2010). Structural biology is an approach which has been used to explore the physiological function of uncharacterized proteins by predicting their 3D shapes.

One option is to use molecular modeling, which is a useful methodology for analyzing the 3D structures of macromolecules. The aim of molecular modeling is to provide an explanation for the biological activity of a structure (Forster 2002). Molecular modeling can be used to hypothesize the ligand binding site, the substrate specificity and an annotation of function (Vyas et al. 2012). It has been shown that protein structures are more conserved than the amino acid sequences of which they are made (Kaczanowski and Zielenkiewicz 2010). One approach used in homology modeling is to predict the 3D structure from sequence, using information gained from a homologous protein with a known structure. This can provide valuable clues about the 3D structure of the protein, but it becomes even more valuable when it can be combined with experimental data. The quality of the model depends on the sequence identity. If the similarity between the target sequence and the template is over 25 %, the similarity is typically thought to be useful (Vyas et al. 2012).

2.1.5 Genetic alteration for recognizing the endogenous substrate

One major challenge encountered in investigating poorly known enzymes is designing enzyme activity assays. Most methods used to explore poorly characterized proteins concentrate on simply the amount of the protein instead of its function or activity. Enzyme assays are used to study enzyme kinetics and enzyme inhibition. One common feature of all enzyme assays is that they measure either the consumption of substrate or the production of an end-product with respect to time. In other words, all enzyme activity assays need to have a known substrate in order to function. There are several approaches to develop assays for orphan enzymes such as mass-based method which monitors the binding of ligands (Yuan et al. 2009); this approach can be used for screening of biological activity (Guengerich et al. 2010). However, the field of protein characterization would benefit from new enzyme activity assays.

Knock-out models

By far the most powerful method to study a protein's function is genetic alteration, where the gene of interest has been inactivated, so-called knocked-out. Inactivating a gene produces a knock-out (KO) model if the gene is not vital. With a vital gene, KO-models cannot be produced. If the gene is not vital, the KO model can reveal the cellular pathways in which the gene product, often a protein, operates. There are several ways of achieving a KO model such as mutagenesis, targeted mutations, CRISPR/Cas9, silencing RNA or antibody binding. A KO model can be created in cell culture *in vitro* as well as *in vivo* in different organisms. The principal concept is simple; after disabling the gene of interest, changes can be observed in either behavior and/or biochemical function. In KO models, the inhibition of enzyme activity should lead to an increased amount of a physiologically relevant substrate. The substrate may be identified using lipidomics. This approach has been used to reveal the endogenous substrates for ABHD6 (Thomas et al. 2014), ABHD12 (Blankman et al. 2013) and ABHD3 (Long et al. 2011).

The first step taken with a KO model is to analyze the phenotype of the animals, as this could give a clue of the function of the protein (Thomas et al. 2014). The KO model can make it possible to analyze tissues where the enzyme is normally widely expressed using untargeted LC/MS (liquid chromatography-mass spectrometry). This method can identify a wide range of metabolites without the need for internal standards. The assumption is that eliminating enzymatic activity will increase the levels of the endogenous substrate in the KO animal. These levels can be compared to the corresponding compound in the WT animals. For example, after some potential lipid substrates have been discovered, it may be possible to determine the catalytic activity towards the discovered substrates with purified/over-expressed enzyme, and to develop a suitable enzyme activity assay. When one possesses an enzyme activity assay, then it is possible to screen a small molecule library to identify active and selective inhibitors for the enzyme of interest. Selective and potent inhibitors can be further evaluated in preclinical studies. This approach is powerful but rather laborious and screening all enzymes would take a relatively long time.

It is important to take into consideration a few aspects which may have an impact on the interpretation of results. First, even though genes are orthologous between human and other mammals, there is no guarantee that the same gene functions similarly in humans as in other species (Guengerich et al. 2010). Furthermore, interfering with one gene may cause alterations in other genes. It is also possible that organisms have compensatory systems which can overcome the functional loss of the gene of interest. It may also be difficult to decide which tissue should be analyzed. Nonetheless, without doubt, the KO model has proved to be an unsurpassed way of identifying the function of unknown proteins. However, because of both economical and time-related reasons, other methodologies are often used.

2.1.6 Activity-based protein profiling (ABPP)

There are suitable methods for quantifying proteins, such as SDS-PAGE or LC/MS. However, many proteins including the serine hydrolases are often regulated by post-translational mechanisms which means that the expression levels measured by conventional proteomic or genomic methods are only estimations and do not always correlate with a change in enzymatic activities (Kilpinen et al. 2008; Wiedl et al. 2011). The chemoproteomic approach called activity-based protein profiling (ABPP) is a method capable of determining the active enzyme. The method has proved useful with a large range of enzyme classes. In ABPP, a specific small-molecule probe is designed to target a certain enzyme class that shares a conserved mechanism and this procedure can be conducted from practically any biological material (Cravatt et al. 2008). The probe has a reactive group that binds covalently to the active site of the enzyme (Fig 2.) (Niphakis and

Cravatt 2014). The probe has also a reporter tag such as biotin/fluorophore which enables the detection, enrichment, and identification of the labelled enzyme (Fig. 2). For the family of serine hydrolases, fluorophosphonate (FP) has been shown to potently and irreversibly label > 80 % of the members of the serine hydrolase family (Bachovchin et al. 2010). The commercially available probe TAMRA (tetramethyl-6-carboxyrhodamine)-FP attacks the active site of the serine hydrolase and the reporter tag fluorophore TAMRA allows visualization of the labelled protein.

The power of ABPP lies in its ability to label active serine hydrolases in native biological samples (Cravatt et al. 2008). Unlike methods determining changes in gene or protein levels, ABPP assesses changes in enzyme activity. Even in highly complex samples, it can detect changes in the activities of very low-abundance enzymes. ABPP can also be used in a competitive mode where proteomes are first treated with inhibitors and then labeled with the activity-based probe that reacts with most enzymes in the class under investigation (Fig. 2). Using this methodology, researchers have been able to screen for enzyme inhibitors or to identify the target(s) of each inhibitor (W. Li et al. 2007). Competitive ABPP can be used for screening inhibitor selectivity and it can also be exploited for uncharacterized enzymes.

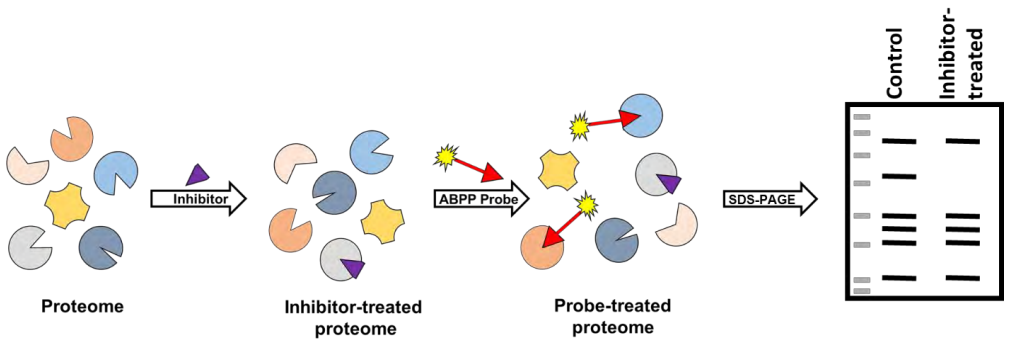


Figure 2. Principle of competitive ABPP. Cell lysates/ tissue preparations are incubated with an inhibitor and then with a fluorescent probe, TAMRA-FP, whose binding to serine hydrolases is visualized by in-gel scanning after a SDS-PAGE separation of proteins.

Although ABPP is very useful, it does not provide information about the substrate and, as such, it is not suitable for incorporation into a high throughput screen (HTS) format. There are enzyme activity methods available for those enzymes with known substrates, such as the endocannabinoid hydrolases. The substrate hydrolysis of endocannabinoid hydrolases has been commonly studied using radiolabeled monoacyl glycerol (MAG) substrates (Dinh et al. 2002; Goparaju et al. 1999; Karlsson et al. 1997) or MAG substrates combined with MS-based (Blankman et al. 2007) and HPLC-based (Saario et al. 2004) detection. However, when characterizing unknown serine hydrolases, these methodologies have limitations such as being laborious and requiring the use of radioactive material. Additionally, they are not suitable for an HTS format, which explains why more useful HTS enzyme activity methods are urgently needed.

2.2. INTRODUCTION TO THE SERINE HYDROLASES

Serine hydrolases comprise one of the largest and most diverse groups of enzymes. They regulate a wide range of biological processes. Nearly half of the serine hydrolases are relatively well known but the other 50 % of human serine hydrolases are lacking any known physiological function or endogenous substrates, and the majority of these enzymes lack selective inhibitors that could be exploited in their further characterization.

2.2.1. The family of serine hydrolases

The family of serine hydrolases consists of about 200 enzymes (Long and Cravatt 2011; Simon and Cravatt 2010). This enzyme family uses nucleophilic serine in the catalytical core to achieve the hydrolysis of the substrate. Many well known proteins belong to this family, for example proteases, lipases, phospholipases, cholinesterases, thionesterases and amidases. Due to their role in many important physiological processes such as neurotransmission and digestion (Bachovchin and Cravatt 2012), the activity of these proteins is tightly regulated. For example, serine hydrolases regulate the life cycle of signaling lipids under normal physiological circumstances (Long and Cravatt 2011). However, it has been postulated that imbalances and dysfunction of these processes can contribute to many human diseases (Wymann and Schneiter 2008). This is not surprising keeping in mind that lipid signaling is a delicate system involving networks with multiple pathways.

Serine hydrolases are divided into two subclasses, proteases and metabolic serine hydrolases. Serine proteases are found in most genomes (Di Cera 2009) and catalyze the hydrolysis of the polypeptide chain of a protein; metabolic serine hydrolases hydrolyze small molecules such as lipids (Bachovchin and Cravatt 2012). The α/β-hydrolase domain (ABHD) fold is often thought to characterize the metabolic serine hydrolases (Bachovchin and Cravatt 2012) and proteins with an ABHD-fold have been detected in every reported genome (Lord et al. 2013). The metabolic serine hydrolases typically use a Ser-His-Asp (SHD) triad in the catalytic reaction (Dodson and Wlodawer 1998). The main role of the metabolic serine hydrolases is thought to be to hydrolyze small molecules such as fatty acyl esters, phospholipids, lipid amines and acylthioesters, e.g. acyl CoA (Long and Cravatt 2011).

2.2.2. Lipid degrading serine hydrolases

Lipid metabolism is a biochemical process which is controlled by hormones (e.g insulin and growth hormone). In the process, lipids are synthesized, broken down by lipases, prior to being incorporated and used by several cells. Most lipases belong to the serine hydrolases (Long and Cravatt 2011) highlighting the critical contribution of the serine hydrolases in lipid metabolism. For example, serine hydrolases can act as extra- and intra-cellular lipases that are responsible for absorbing dietary lipids, mostly triacylglycerols (TAGs), into cells as well as producing energy for the cell (Long and Cravatt 2011). Changes in the nutritional and physiological conditions alter the levels of hormones regulating TAG hydrolysis (Zechner et al. 2012). When the need for energy is increased, this initiates the hydrolysis of TAGs. The process where TAGs are hydrolyzed by various lipases to fatty acids (FAs) and MAG is known as lipolysis. As shown in Fig. 3, lipolysis in adipose tissue is initiated by a serine hydrolase called ATGL (adipose triacylglycerol lipase) (Zimmermann et al. 2004). ATGL hydrolyzes TAG to DAG (diacylglycerol) and subsequently, a well characterized set of enzymes convert DAG into glycerol and FAs.

Hydrolases participating in lipolysis serve as ideal targets for the treatment of some metabolic diseases. Therefore, these hydrolases have been under intense investigation and their properties are rather well understood.

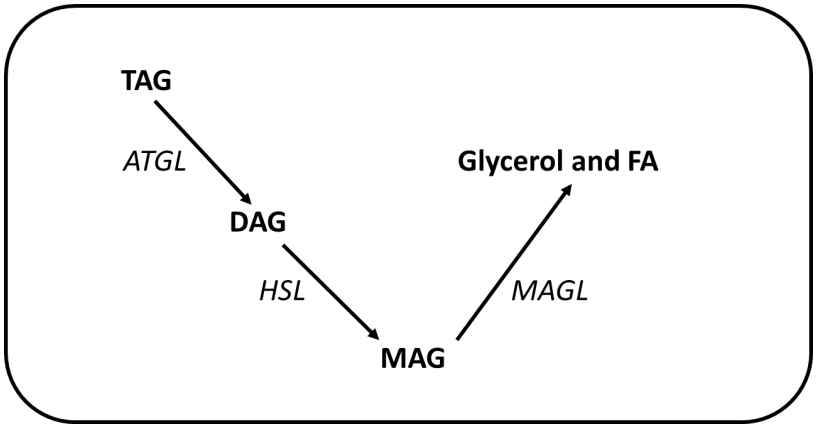


Figure 3. Schematic of lipolysis in adipose tissue. DAG is produced by ATGL hydrolysis of TAG. DAG is further hydrolyzed to MAG and finally to glycerol and FA by HSL and MAGL, respectively.

Hormone-sensitive lipase (HSL) and ATGL

The highest expression of HSL is present in the adipose tissue, with lower expression in the cardiac and skeletal muscles (Holm et al. 1988). As the name hormone-sensitive lipase indicates, certain hormones regulate HSL. In adipose tissue, HSL activity is stimulated by catecholamines and suppressed by insulin (Egan et al. 1992; Kraemer and Shen 2002) while in the skeletal muscle, HSL is controlled by adrenaline (Yeaman 2004).

The generation of HSL-KO mice highlighted the central role of HSL as the main DAG lipase. The HSL-KO mice displayed elevated DAG levels, their adipocytes were increased in size and there were alterations in the distribution of their adipose tissue (Yeaman 2004; Zimmermann et al. 2009). Moreover, the generation of HSL-KO mice established that the lack of HSL had no effect on the hydrolysis of TAGs (Osuga et al. 2000). The discovery that HSL hydrolyzed DAG instead of TAG (Haemmerle et al. 2002) lead to the discovery of ATGL (Zimmermann et al. 2004).

The current view is that activation of ATGL is the initial step in lipolysis. The availability of ATGL-KO mice has clarified the physiological role of ATGL in hydrolyzing TAG (Haemmerle et al. 2006). ATGL-KO mice display a clear decrease in TAG hydrolytic activity in white adipose tissue (WAT), skeletal muscle and liver. Their total cholesterol, TAG and FA levels were decreased as compared to WT mice (Haemmerle et al. 2011) and these KO mice exhibited mild obesity and as expected, an increased fat mass. Most likely, because of their inability to release FAs for heat and energy production, ATGL-KO mice could not tolerate cold exposure or fasting and died prematurely from cardiac dysfunction caused by the accumulation of TAG in their hearts (Haemmerle et al. 2011).

Monoacyl glycerol lipase (MAGL)

MAGL is the main enzyme involved in the degradation of MAGs in most tissues (Long, Nomura et al. 2009). Membrane-associated MAGL is responsible for the last step of lipolysis where the MAG is degraded into glycerol and fatty acid (Fig. 3 and 5). MAGL is widely expressed in the body with the highest expression levels in adipose tissue. MAGL is also recognized as the main hydrolase degrading the major endocannabinoid, 2-arachidonoyl glycerol (2-AG), which is responsible for endocannabinoid signaling (Howlett 2002). The generation of MAGL-KO mice established the major *in vivo* role of MAGL as a MAG lipase in various tissues (Chanda et al. 2010; Taschler et al. 2011). These studies indicated that MAGL-KO mice had reduced 2-AG (2-arachidonoylglycerol) hydrolase activity, which increased 2-AG levels in the brain, white adipose tissue (WAT) and liver (Taschler et al. 2011). Furthermore, the pharmacological blockade of MAGL caused an

accumulation of 2-AG in many tissues, especially in the brain. This excess amount of 2-AG reduced the number and functionality of CB1 receptors in cell membranes, which led to desensitization (Chanda et al. 2010; Zhong et al. 2011), as well as to functional and behavioral tolerance (Chanda et al. 2010; Schlosburg et al. 2010). Interestingly mice lacking MAGL showed signs of enhanced memory and learning (Pan et al. 2011). Furthermore, a lack of MAGL increased MAG levels and decreased plasma glycerol levels; however, MAGL deficiency had no effect on food intake or fat mass (Taschler et al. 2011). Based on these studies, MAGL seems to affect energy metabolism by hydrolyzing MAG and has a major impact on endocannabinoid signaling, which also affects energy metabolism.

2.2.3. ABHD proteins

The ABHD (α/β-hydrolase domain) protein family is a subclass of the serine hydrolases and it has been detected in all reported genomes (Hotelier et al. 2004; Nardini and Dijkstra 1999). This family contains at least 22 members, including proteases, lipases, esterases, dehalogenases, peroxidases and epoxide hydrolases, making it one of the most diverse protein families (Nardini and Dijkstra 1999). The physiological function and endogenous substrates of the ABHD proteins are mostly unknown (Bachovchin and Cravatt 2012; Lord et al. 2013). Intensive research has provided potential functions for some ABHD family members, such as ABHD3, ABHD6, ABHD12 and ABHD16A (also known as a BAT5) whose endogenous substrates have also been identified (Blankman et al. 2007; Blankman et al. 2013; Kamat et al. 2015; Long et al. 2011; Marrs et al. 2010; Simon and Cravatt 2006).

The known substrates for ABHD hydrolases are listed in Table 1. It is evident that most of the ABHD proteins lack defined endogenous substrates and specific inhibitors. Interestingly, those ABHD hydrolases that have a known endogenous substrate (Table 1.) seem to share a common ability to regulate glycerophospholipid metabolism. This could point to a common role for the ABHD family in lipid and energy metabolism (Lord et al. 2013). This has been established by mutations and gene knockdowns of ABHD hydrolases which have strengthened the hypothesis that the ABHD family shares a common role in lipid metabolism and certain diseases (Lord et al. 2013; Thomas et al. 2014) (Table 1.). There are some medical conditions in which there is altered lipid metabolism associated with the ABHD hydrolases such as PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa and cataract) (Chen et al. 2013; Fiskerstrand et al. 2010), Chanarin-Dorfman syndrome and neutral lipid storage disease (NLSDI) (Lefevre et al. 2001) (Table 2.). This is further support for the hypothesis that ABHD hydrolases are involved in the control of lipid synthesis and degradation (Blankman et al. 2007; Long and Cravatt 2011; Simon and Cravatt 2006). Moreover, ABHD6 has been linked to a metabolic disorder (Thomas et al. 2013) and it was shown that inhibition of ABHD6 could provide benefits in diet-induced obesity and type 2 diabetes (Taschler et al. 2011; Thomas et al. 2013; Zhao et al. 2014) however, the detailed mechanism responsible for these beneficial effects needs to be explored. Additionally, several publications have reported altered expression of ABHD proteins in different cancer cells (F. Li et al. 2009; Max et al. 2009; Yoshida et al. 2010). Most of the ABHD enzymes linked to cancer have been shown to have increased activity or expression (Lord et al. 2013). This could be compatible with the suggested role of ABHD enzymes as mediators of glycerolipid metabolism. It is possible that ABHD enzymes could be recruited to provide small molecules to be used in pre-tumorgenic signaling or as an energy source as has been shown for MAGL (Nomura et al. 2010). However, the exact role of ABHD proteins in cancers is still unknown.

Table 1. ABHD hydrolases

Enzyme	Highest expression	Potential functions	Known endogenous substrates	Selective inhibitors	References
ABHD1	Heart and small intestine Lung, liver	Has a protective role during oxidative stress.	-	-	(Edgar and Polak 2002; Lord et al. 2013; Stoelting et al. 2009; van Roon-Mom et al. 2008)
ABHD2	Ubiquitously expressed in peripheral tissues	TAG lipase and ester hydrolase. Acts in stress response. Controls sperm activation.	TAG	-	(Lord et al. 2013; Miller et al. 2016; Miyata et al. 2008; Naresh Kumar et al. 2016)
ABHD3	Brain and small intestine	Phospholipase. Hydrolyzes phosphatidylcholines in conditions of oxidative stress which is important in disease progression e.g. atherosclerosis	Lysophoshatidyl-choline	-	(Long et al. 2011; Lord et al. 2013)
ABHD4	Brain, small intestine, kidney and testis	Responsible for N-acylphospholipid metabolism in CNS. Inhibits cell proliferation and cell cycle.	NAPE[1] Lyso-NAPE N-acyl phospholipids	-	(Brady et al. 2011; Katz-Jaffe et al. 2009; Lee et al. 2015; Liu et al. 2008; Simon and Cravatt 2006)
ABHD5	Testis and adipose tissue	Coactivator of adipose tissue TAG lipase. Ligand-regulated lipase activator involved in glycerol-phospholipid metabolism. Generates lipid mediators that regulate TAG metabolism and insulin sensitivity. Coenzyme A-dependent lysophosphatic acid acyltransferase.	Long-chain acyl-CoA	SR-4995	(Lass et al. 2006; Lord et al. 2013; Montero-Moran et al. 2010; Sanders et al. 2015)
ABHD6	Ubiquitously expressed in CNS and peripheral tissue	Role in neurotransmission by regulating 2-AG signaling. Acts as a MAG and lysophospholipase. Controls the levels of anti-inflammatory bioactive lipid PGD2GE. Regulates fuel homeostasis. Regulates *de novo* FA synthesis.	2-AG, MAG, BMP	WWL70 JZP-169 JZP-430 KT182 KT203	(Alhouayek et al. 2013; Blankman et al. 2007; Hsu et al. 2013; W. Li et al. 2007; Long and Cravatt 2011; Lord et al. 2013; Marrs et al. 2010; J. Z. Patel et al. 2015; J. Z. Patel, van Bruchem et al. 2015; Pribasnig et al. 2015; Thomas et al. 2014; Zhao et al. 2014; Zhao et al. 2015; Zhao et al. 2016)
ABHD7	Brain	Reduces the bioactivity of epoxide-containing lipids.	-	-	(Lord et al. 2013; Morisseau 2013)
ABHD8	Testis and brain	Unknown	-	-	(Lord et al. 2013)
ABHD9	Skeletal muscle, white adipose tissue and heart	Acts as an epoxide hydrolase. May negatively regulate angiogenesis.	FA epoxides, Epoxy-eicosatrienoic acids, leukotoxin	-	(Lord et al. 2013)

[1]N-acyl phosphatidylethanolamine

Table 1. Continued

Enzyme	Highest expression	Proposed functions	Known endogenous substrates	Selective inhibitors	References
ABHD10	Testis and brown adipose tissue	Esterase responsible for deglucuronidation of acyl-glucuronides	probenecid acyl glucuronide	7j ABL303	(Adachi et al. 2015; Lajkiewicz et al. 2014; Lord et al. 2013)
ABHD11	Ubiquitously expressed	Mitochondrial enzyme	-	ML226 WWL222 AA44-2	(Adibekian, Hsu et al. 2010; Adibekian et al. 2011; Bachovchin et al. 2010; Long and Cravatt 2011; Lord et al. 2013)
ABHD12A	Brain and ubiquitously expressed	Hydrolyzes 2-AG. Participates in LPS homeostasis	2-AG, LPS, BMP	Triterpenoids Maslinic acid	(Blankman et al. 2007; Blankman et al. 2013; Kamat et al. 2015; Long and Cravatt 2011; Lord et al. 2013; Parkkari et al. 2014)
ABHD12B	Not characterized	Unknown	-	-	(Lord et al. 2013)
ABHD13	Testis, small intestine and ubiquitously expressed	Unknown	-	-	(Lord et al. 2013)
ABHD14A	Testis and ubiquitously expressed in adult mice	Possible role in granule neuron development	-	-	(Hoshino et al. 2003; Lord et al. 2013)
ABHD14B	Ubiquitously expressed in peripheral tissues	Unknown	-	-	(Lord et al. 2013)
ABHD15	Adipose tissue, liver and skeletal muscle	Required for adipogenesis. Regulates the role and size of adipocytes. Possible role in apoptosis.	-	-	(Lord et al. 2013; Walenta et al. 2013)
ABHD16A	Skeletal muscle, brain and ubiquitously expressed	Phosphatidylserine hydrolase. Participates in LPS homeostasis.	Medium and long-chain unsaturated MAG	-	(Blankman et al. 2013; Kamat et al. 2015; Lord et al. 2013; Savinainen et al. 2014)
ABHD16B	Testis, skeletal muscle and brown adipose tissue	Unknown	-	-	(Lord et al. 2013; Savinainen et al. 2014)
ABHD17A/B/C	Brain	Regulates PSD-95 palmitoylation cycles in neurons	Palmitate	-	(Lin and Conibear 2015)

2.2 ENDOCANNABINOID SYSTEM

The endocannabinoid system (ECS) is a retrograde lipid signaling system influencing a variety of functions in the CNS and the periphery. The ECS consists of two G-protein coupled cannabinoid receptors (CB1 and CB2), their endogenous activating ligands, endocannabinoids, and hydrolases regulating the life-time and signaling functions of the endocannabinoids. Compared to the other neurotransmitters systems, the ECS displays a few unique properties. For example, instead of storing endocannabinoids in synaptic vesicles, they are produced on demand by activity-driven synthesis (Fig. 4). In endocannabinoid signaling, the endocannabinoids are released from post-synaptic cells into the synaptic cleft, from where they retrogradely act on the pre-synaptic cells to cause activation of their cannabinoid receptors, eventually causing a suppression of transmitter release.

2.2.1 Basic features of the ECS

Cannabinoid receptors and their endogenous ligands
The receptors activated by cannabinoid drugs are called cannabinoid (CB) receptors. So far, two CB receptors have been identified. Devane and coworkers (Devane et al. 1988) reported the first evidence of the CB receptors. A few years later both cannabinoid receptors had been identified and cloned, CB1 in 1990 (Matsuda et al. 1990) and CB2 in 1993 (Munro et al. 1993). Later, a novel non-cannabinoid receptor was suggested to participate in endocannabinoid signaling. This suggestion was based on the fact that cannabimimetic effects remained after either pharmacological or genetic blockade of CB1 and CB2 receptors (Begg et al. 2005; Mackie and Stella 2006). GPR55 (G protein-coupled receptor 55) was identified in *in silico* screening to have a similar amino acid sequence in its binding region (Baker et al. 2006) making it a strong candidate for a receptor capable of being activated by cannabinoids. The hypothesis was established after several cannabinoid ligands were shown to activate GPR55 (Ryberg et al. 2007). Similar to the CB receptors, GPR55 is a G protein-coupled receptor (GPCR) but with 15 % sequence similarity with the cannabinoid receptors (Henstridge et al. 2011). The GPR55 receptor was thus seen as a highly promising drug target since it was thought to lack the psychotropic effects associated with the CB receptors and thus the research around GPR55 has been intense, but provided a confusing pharmacological profile mainly with ligands being reported as agonists or antagonists (Oka et al. 2007; Ryberg et al. 2007). Although GPR55 can be activated by several cannabinoids, the current opinion is that LPI (lysophosphatidyl inositol) is the endogenous ligand for GPR55 (Henstridge et al. 2011; Makide et al. 2014; Oka et al. 2007). Most known GPR55 ligands are cannabinoid related and thus there is a need for more potent and especially more selective ligands. This need was satisfied in a recent publication introducing new ligands with nanomolar potencies (Yrjola et al. 2016). Nonetheless, many open questions need to be answered before we have a thorough understanding of the physiology of GPR55.

CB1 and CB2 receptors are seven transmembrane receptors belonging to the rhodopsin-class of GPCRs (Howlett et al. 2002). Both CB receptors can signal through $G_{i/o}$ proteins. The activation of CB receptors by endocannabinoids inhibits the adenylyl cyclases (Howlett et al. 2002) and CB1 receptors have been shown also to control the release of GABA by inhibiting N-type calcium channels (Szabo et al. 2014). Activation of CB1 or CB2 receptors has diverse consequences on cellular physiology, including alterations in synaptic function, gene transcription, and cell motility. CB1 and CB2 receptors have different tissue distributions. The CB1 receptor is widely expressed in brain; these receptors are located in

axons and in presynaptic sites. The location of the CB1 receptor together with its inhibitory effect on voltage-dependent calcium channels and adenylyl cyclase indicated that the main function of CB1 receptors was to inhibit neurotransmitter release (Mackie 2006).

CB1 receptors are more abundantly expressed in CNS while CB2 receptors are expressed in the periphery. However, there is evidence that CB2 receptors are upregulated in microglia under certain pathological conditions such as after a nerve injury, or in the presence of inflammation and neurogenerative diseases (Fernandez-Ruiz et al. 2008; Sagredo et al. 2009). These studies have indicated that CB2 receptors that are sparingly expressed in healthy brain are upregulated in pathological conditions (Fernandez-Ruiz et al. 2008; Fernandez-Ruiz et al. 2015; Ramirez et al. 2005). CB2 receptors have been shown to be present in tissues and cells of the immune system such as thymus, tonsils, B lymphocytes, NK cells and granulocytes (Howlett et al. 2002).

The endogenous ligands of CB receptors are called endocannabinoids. So far, at least 5 endocannabinoids have been identified. The most widely investigated endocannabinoids are 2-AG (Mechoulam et al. 1995; Sugiura et al. 1995) and anandamide (AEA) (Devane et al. 1992). Endocannabinoids activate CB1 and CB2 receptors with cell specific biochemical responses (Silvestri and Di Marzo 2013). In the CNS, endocannabinoid activation of CB receptors leads to the inhibition of neurotransmitter release (Fig. 4). The life cycle of endocannabinoids is tightly regulated by their synthesizing and hydrolyzing enzymes (Fig. 5).

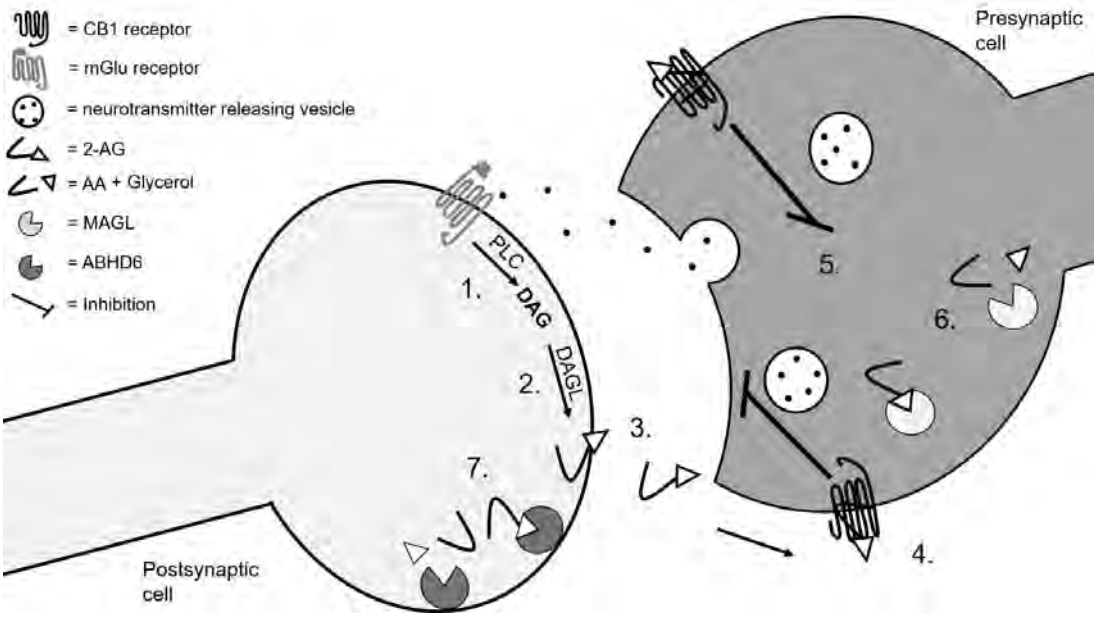


Figure 4. Lifecycle of 2-AG. Released neurotransmitters activate receptors in post-synaptic cells which leads to an increase in the Ca2+ concentration activating PLC. 1) PLC generates DAG from phospholipid precursors such that the postsynaptically located DAGLα 2) is converted into 2-AG. 3) The liberated 2-AG travels to the presynaptic cell membrane and 4) activates CB1 receptors leading to 5) inhibition of neurotransmitter release. After activating the CB1 receptor, 2-AG is 6) degraded by MAGL in the presynapse and 7) ABHD6 is suggested to control the 2-AG levels in postsynaptic cells.

2.2.2 Life cycle of the endocannabinoids

Biosynthesis of endocannabinoids
The endocannabinoid 2-AG is a lipid mediator widely present throughout the CNS. For example, the activation of glutamate receptors increases the post-cellular Ca^{2+} concentration which activates PLC (phospholipase C) that cleaves DAG from membrane phospholipids and then diacylglycerol lipase (DAGL) α or β further catalyzes 2-AG production FA release (Bisogno et al. 2003) (Fig. 4 and 5). Interestingly, both DAGLs are expressed in the same brain regions as CB1 receptors, making them ideal for generating 2-AG for retrograde signaling. The KO models of DAGLs have indicated that with respect to retrograde signaling, DAGLα seems to be the major enzyme responsible for 2-AG synthesis (Tanimura et al. 2010; Yoshino et al. 2011). In contrast to the synthesis of 2-AG, which is relatively straightforward, several pathways have been suggested to be capable of synthesizing the second endocannabinoid, AEA (Fig. 5) (Di Marzo 2011). It has been shown that mice lacking NAPE-PLD (N-acylphosphatidylethanolamine-specific phospholipase D) expressed normal AEA levels, suggesting that different pathways could substitute for the loss of one enzyme pathway (Di Marzo 2011; Leung et al. 2006).

Degradation of endocannabinoids
After the endocannabinoids have activated the CB receptors in the presynaptic cells, they are quickly degraded. Several potential serine hydrolases are postulated to be responsible of their degradation (Alger 2002; Freund et al. 2003). The degradation of AEA has been shown to be accomplished through FAAH activity where AEA is hydrolyzed to ethanolamide and AA (Fig. 5). However, several enzymes are known to participate in the degradation of 2-AG to glycerol and AA (Fig. 5). MAGL is the main 2-AG hydrolase. Additionally, two recently identified hydrolases, ABHD6 and ABHD12, also have been found to hydrolyze 2-AG (Blankman et al. 2007; Blankman et al. 2013). It is not known why several enzymes should be involved in the hydrolysis of 2-AG. There is evidence that ABHD6 and MAGL have distinct distributions (Marrs et al. 2010) and it has been speculated that especially ABHD6 could be responsible for 2-AG hydrolysis in tissues and brain areas lacking MAGL (Alhouayek et al. 2014; Marrs et al. 2010; Zhao et al. 2014).

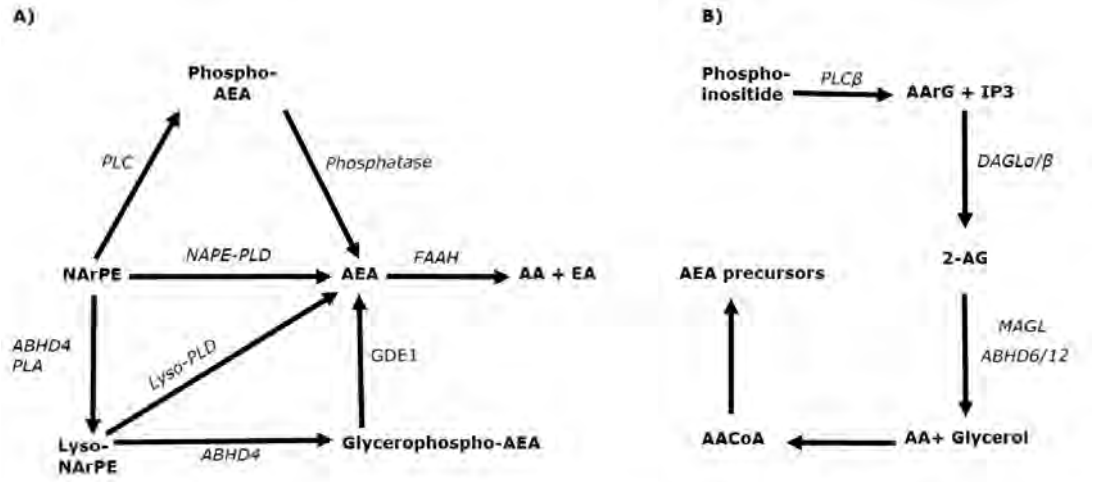


Figure 5. A simplified schematic of anandamide (A) and 2-arachidonoylglycerol (2-AG) biosynthesis (B) according to Di Marzo et al. (2011) and Fezza et al. (2014). It is notable that AA resulting from the hydrolysis of 2-AG can be converted into phospholipid precursors for anandamide biosynthesis indicating that there is an interconnection between these two biosynthetic pathways (Di Marzo 2011). Abbreviations: NArPE, *N*-arachidonoyl

phosphatidylethanolamine; NAPE-PLD, *N*-acylphosphatidylethanolamine-specific phospholipase D; LysoPLD, lysophospholipase D; Lyso-NAPE lysoarachidonoyl phosphatidylethanolamine; GDE1, glycerophosphodiesterase 1; AArG, sn-1-acyl-2arachidonoyl-glycerol; AACoA, arachidonoyl-coenzyme A

2.4 SERINE HYDROLASES AND ENDOCANNABINOID SYSTEM AS DRUG TARGETS

Today there are more proteins than functions that can be ascribed to them. There are over 20000 genes from which it has been estimated that the human genome contains about 2000-6000 drugged or druggable targets (Finan et al. 2017; Hopkins and Groom 2002; Landry and Gies 2008). This leads to the assumption that there are thousands of druggable targets waiting to be exploited for pharmaceutical use and it is likely that the majority of the serine hydrolases belong to this group. Their evident roles in many chronic diseases (Lord et al. 2012; Nomura and Casida 2016; Zechner et al. 2012) have made the serine hydrolases promising targets for therapeutic intervention.

2.4.1. Serine hydrolases as a drug target

The significant role of serine hydrolases in lipid metabolism has made them attractive drug targets for metabolic disorders and obesity. In 2013, obesity was categorized as a disease by the American Medical Association and it is well known that obesity increases the risks of many other metabolic diseases (Pollack, 2013). Obesity is a global public health problem contributing to morbidity and mortality, as well as causing deteriorations in the quality of life. The research emphasis has been in obesity and obesity-induced metabolic diseases such as cardiovascular disease, cancer and diabetes. Much effort and financial resources have been spent in searching for a drug that would cause a reduction in body weight when combined with improved eating habits and physical activity (Kim et al. 2014). Many drugs for obesity have been successfully designed, however severe side-effects have prevented their use. At present, there are only three anti-obesity drugs (Orlistat, Naltrexone/bupropion and Liraglutide) in clinical use in Europe and two more (Phentermine/topiramate extended-release and Lorcaserin) in the USA (D. K. Patel and Stanford 2018); one of them, Orlistat (trade names Alli and Xenical), acts through serine hydrolases and other lipases (Kim et al. 2014). The biologically active compound of this drug is also known as a broadly acting inhibitor of lipases, THL (Tetrahydrolipstatin). The non-specific inhibitor Orlistat targets secreted TAG lipases (pancreatic, carboxylesterase and gastric lipases) in the intestine (Nomura and Casida 2016); it inhibits TAG hydrolysis, which prevents the absorption of dietary fats leading to reduced energy intakes and eventually to weight loss.

Lipolysis offers many possibilities for drug discovery since it should be possible to target lipases acting in different phases of lipid metabolism. In fact, these possibilities have been intensively investigated and as a result, several inhibitors have been developed. One example is the ATGL inhibitor, Atgl statin, that has been shown to selectively inhibit TAG hydrolase activity in WAT resulting in a reduction of FA release both *in vitro* and *in vivo* (Mayer et al. 2013). This beneficial effect revealed that ATGL was a suitable target for drug intervention of obesity and that this could be achieved with small molecules. Unfortunately, the lack of ATGL in ATGL-KO mice leads to cardiomyopathy (Haemmerle et al. 2006; Hirano 2009), and this reduced the potential interest towards ATGL as a clinical target. Since the small molecule enzyme inhibitors are neither tissue- nor function-selective, such challenges are unfortunately common with multifunctioning proteins acting in multiple tissues.

All of the serine hydrolases have not been studied in detail although they have a definite importance in the (patho)physiology of lipid metabolism and cell signaling. For example, if we consider the putative regulatory role of ABHD proteins in glycerophospholipid homeostasis, and the fact that the ultimate cause of several diseases is the inflammation caused by disrupted glycerophospholipid homeostasis, then the therapeutical potential of these enzymes is undeniable.

The potential physiological functions for ABHD hydrolases are described in Table 1. Table 2. lists the ABHD hydrolases associated with diseases. It is interesting that although about half of ABHD enzymes are linked to diseases (Table 2.), their physiological function still needs to be confirmed.

Table 2. The ABHD enzymes associated with pathophysiology

Enzyme	Pathogenical role/Disease	References
ABHD2	Promotes prostate cell proliferation in mice. It is over-expressed in prostate cancer tissue and has a role in macrophage infiltration to atherosclerotic lesions Progesterone-activated endocannabinoid hydrolase stimulating sperm motility	(Miller et al. 2016; Miyata et al. 2008; Obinata et al. 2016)
ABHD3	Up- or downregulated in several cancer models	(Thomas et al. 2014)
ABHD5	Loss of ABHD5 causes Chanarin Dorfman Syndrome also known as Neutral lipid storage disease (NLSD) as well as promoting colorectal tumor development and progression by reducing fatty acid oxidation and enhancing the production of growth-promoting metabolites from glycolysis.	(Igal et al. 1997; Lass et al. 2006; Lefevre et al. 2001; Ou et al. 2014)
ABHD6	Endocannabinoid hydrolase Associated with obesity, metabolic syndrome, and diabetes. Participates in whole-body energy homeostasis.	(Blankman et al. 2007; Thomas et al. 2013; Zhao et al. 2014; Zhao et al. 2015; Zhao et al. 2016)
ABHD9	Hypermethylation of its promoter region has been observed in gastric cancer, prostate cancer and melanoma. Mutation of ABHD9 is predicted to lead to human autosomal recessive nonlamellar, non-erythrodermic congenital ichthyosis. Activity toward leukotoxin produces metabolites that mediate the acute respiratory distress syndrome (ARDS).	(Ala et al. 2008; Cottrell et al. 2007; Decker et al. 2012; Furuta et al. 2006; Lord et al. 2013; Yamashita et al. 2006)
ABHD11	One of the deleted genes in Williams-Beuren syndrome	(Merla et al. 2002)
ABHD12	Endocannabinoid hydrolase in vitro, LPS hydrolase *in vivo* Loss of ABHD12 cause PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract) syndrome.	(Blankman et al. 2007; Blankman et al. 2013; Fiskerstrand et al. 2010)
ABHD14A	One of the deleted genes in Williams-Beuren syndrome and a gene associated with a genetically complex autism spectrum disorder	(Casey et al. 2012; Henrichsen et al. 2011; Lord et al. 2013)

If one wishes to develop successful treatments for obesity and other chronic metabolic diseases, such as diabetes and cardiovascular disease (Lord et al. 2013), it will be crucial to understand the metabolic roles of enzymes controlling the lipid metabolic network. Additionally, it will be necessary to define the physiological roles and endogenous

substrates of these poorly characterized proteins as well as pinpointing their locations in the metabolic network.

2.4.2. Inhibitors of serine hydrolases

The emerging role in lipid metabolism has expanded the interest in the ABHD family of hydrolases as drug targets (Bachovchin and Cravatt 2012). Even although some of these enzymes are targets of approved drugs, there are major reasons why it has been difficult to achieve a physiological characterization of these hydrolases i.e. the lack of a suitable enzyme activity assay and the fact that there are no selective inhibitors. From the drug discovery point of view, the stumbling block has been the poor understanding of the mechanisms underpinning lipid metabolism. To overcome this obstacle, it is essential to develop selective inhibitors which could be used to investigate these hydrolases. It is apparent that potent and selective inhibitors would represent much needed tools to increase our understanding of the basic biology of these uncharacterized enzymes and eventually to offer new targets for disease treatment. Although there has been a clear progress in developing inhibitors for serine hydrolases, often these inhibitors have either lacked selectivity or activity *in vivo*.

Four different strategies have been proved successful in the development of novel inhibitors, (Banchovchin & Cravatt 2012). 1) Inhibitors have been found through screening natural products, such as peptides/proteins, polysaccharides and small molecules; 2) optimizing lead scaffolds obtained from screening compound libraries; 3) converting endogenous substrates into inhibitors and 4) tailoring compounds which would react covalently with the serine in the active site of the enzyme.

The main problem in developing inhibitors for serine hydrolases is their similarity. Furthermore, it has proved challenging to design a drug capable of selectively inhibiting one single enzyme. Most likely, there will always be off-targets causing side-effects. Interestingly, it has been proposed that unknown enzymes may control alternative pathways regulating certain distinct conditions, which might allow interventions with fewer side-effects. For example, ABHD6 may control the hydrolysis of MAG in tissues lacking MAGL (Alhouayek et al. 2014; Marrs et al. 2010) e.g. in pancreas (Zhao et al. 2014). Therefore, inhibition of ABHD6 could be beneficial since it acts more locally as compared to the broadly-acting MAGL whose inhibition might cause more undesired effects in the whole body.

MAGL inhibitors have been proposed to be used to enhance tissue levels of endocannabinoids, lower those of eicosanoids, or even achieve both properties which could be beneficial in the treatment of several pathophysiological conditions such as pain, inflammation or mental disorders such as anxiety and depression (Cao et al. 2013). Several potent inhibitors targeting MAGL have been developed in recent years. JJKK-048 (Aaltonen et al. 2013; Aaltonen et al. 2016), JZL184 (Long, Li et al. 2009), KML29 (Chang et al. 2012) and JW651 (Chang et al. 2013) have been tested also *in vivo* in rodents and have been shown to evoke tissue specific differences in MAG metabolism. Inhibition of MAGL increased the endocannabinoid levels and displayed analgesic properties in mouse models. Additionally, the novel inhibitor, JZL184, was shown to prevent restraint stress-induced anxiety in mice (Bedse et al. 2018) emphasizing the potential of MAGL as a clinical treatment.

2.4.3. Endocannabinoid system as a drug target

Marijuana has been used for millennia and the plant *Cannabis sativa* has a long history of self-medication, especially for pain relief, although it may be better known for its hedonistic properties. The *Cannabis sativa* plant contains around 80 phytocannabinoids (Ahmed et al. 2008; Elsohly and Slade 2005); Δ^9-tetrahydrocannabinol (THC) is its major psychoactive compound. THC is mostly responsible for the psychoactive effects mediated by CB1 receptors. The ECS is an attractive drug target because of its role in emotional functioning, cognition (e.g. learning and thinking) (Zanettini et al. 2011), as well as in the regulation of food intake and pain sensations (Huang et al. 2016). In the peripheral nervous system, it regulates the action of cardiovascular, immune, metabolic or reproductive function (Mouslech and Valla 2009).

The ECS undoubtedly participates in energy homeostasis and there seem to be promising possibilities of finding compounds acting through this lipid signaling system. One of the initial discoveries was that after pharmacological blockade of CB1 receptors, the food intake of mice was reduced (Cota et al. 2003), which led to the hypothesis that inhibition of the CB1 receptor would be a suitable treatment for obesity and subsequently several antagonists were developed. One CB1-antagonist, called rimonabant, was found to inhibit CB1 receptor activity in animal models and, after encouraging *in vivo* results, also in obese patients. Patients treated with rimonabant indeed showed a decrease in food intake and body weight (Cota 2007; Despres et al. 2005; Pi-Sunyer et al. 2006). The drug also improved lipid and glucose metabolism in obese patients (Cota 2007; Pi-Sunyer et al. 2006) and had a positive impact on cardiovascular risk factors (Pacher and Kunos 2013; Pi-Sunyer et al. 2006). These positive impacts were considered sufficiently useful that rimonabant was released as an anti-obesity drug into the European market in 2006. Despite the benefits, rimonabant was withdrawn from the European market in 2009 because of its psychiatric side-effects. Clinical trials targeting ECS for diabetes and pain were performed as well. However, they all failed due to the cardiovascular and metabolic side-effects (Pacher and Kunos 2013).

The greatest challenge in developing drugs modulating CB1 receptors is finding ways to avoid their psychotropic effects. Several strategies have been proposed to overcome this challenge e.g. by focusing on selective either activation or inactivation of CB2 receptors (Pacher et al. 2006) or CB1 receptors in the periphery instead of CNS. The psychotropic side-effects have also driven the research focus away from modulating receptors towards the enzymes controlling and regulating endocannabinoid signaling since this approach is expected to be associated with minimal side-effects. The blockade of 2-AG hydrolysis has provided beneficial effects *in vivo* without severe side-effects (Blankman and Cravatt 2013; Lambert and Fowler 2005; Saario and Laitinen 2007).

Several pharmaceutical companies have conducted clinical trials targeting the enzymes regulating endocannabinoid signaling and several FAAH inhibitors have undergone clinical trials. Excluding the BIA 10-2474 trial that led to death of one volunteer and affected five participants (MacDonald 2016), the conclusions emerging from three clinical trials is that the FAAH inhibitors are well tolerated and have not been associated with the adverse effects commonly encountered with exogenous cannabis (Mallet et al. 2016). However, these trials have been halted because of the lack of useful clinical effects as well as precautions after the disaster of the BIA 10-2474 trial. Abide Therapeutics has initiated new clinical trials with the MAGL inhibitor ABX-1431 for the treatment of pain and neuroinflammatory diseases.

3 *Aims of the study*

Their predicted role in lipid metabolism has made the ABHD hydrolases interesting drug targets. Despite their potential, the uncertainties surrounding their physiological function as well as the lack of endogenous substrates and suitable enzyme activity assays have slowed down the development of novel inhibitors of these enzymes (Bachovchin and Cravatt 2012; Lord et al. 2013). The aims of this thesis were to

I. Explore regional CB1 receptor-mediated signaling in MAGL-KO mice brain.

II. Better characterize ABHD6 and ABHD12, two proteins with postulated roles as endocannabinoid hydrolases, by determining their substrate and inhibitor preferences in comparison to MAGL, a well-characterized endocannabinoid hydrolase.

III. Investigate ABHD11 and to undertake activity-based approaches to discover inhibitors for this poorly-characterized enzyme. To achieve aims 2 and 3, it was necessary to develop a sensitive cell-based enzyme activity assay and to discover inhibitors and substrates for these hydrolases.

4 *Experimental procedures*

4.1 MATERIALS

The materials utilized in this study are summarized in Tables 3-10. More information can be found in the original publications (I-III). All chemicals were of the highest purity available.

Table 3. Commercial hydrolase inhibitors and receptor agonists/antagonists

Name	Description	Source	Original publications
MAFP	Broadly acting serine hydrolase inhibitor Methyl arachidonoylfluorophosphonate	Cayman Chemicals	I, II
IDFP	Isopropyl dodecylfluorophosphonate	Cayman Chemicals	II
HDSF	Hexadecane-1-sulfonylfluoride	Calbiochem	II
WWL70	ABHD6 inhibitor [4-(4-carbamoylphenyl)phenyl] *N*-methyl-*N*-[(3-pyridin-4-ylphenyl) methyl] carbamate	Cayman Chemicals	II
Palmostatin B	Non-specific LYPLA1 inhibitor	Calbiochem	III
URB597	FAAH inhibitor cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester	Cayman Chemicals	III
ML211	ABHD11, LYPLA1 and LYPLA2 inhibitor	Cayman Chemicals	III
RHC 80267	DAG lipase inhibitor	Biomol	II
AM251	CB1 antagonist *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide	Tocris Cookson Ltd.	I
CP55,940	CB1/CB2 agonist (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol	Tocris Cookson Ltd. Bristol,UK	I

Table 4. Chemicals

Name	Description	Source	Original publications
TAMRA-FP	ActivX Fluorophosphonate Probes	Thermo Fisher Scientific	II, III
DTT	Dithiothreitol	Sigma	I
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine	Sigma	I
GDP	Guanosine diphosphate	Sigma	I
[^{35}S]GTPγS	[^{35}S]GTPγS initial specific activity 1250 (Ci/mmol)	NEN Life Science Products Inc.	I
AEA-d8	Arachidonoylethanolamide-d8	Cayman Chemicals	I
2-AG-d8	2-Arachidonoyl glycerol-d8	Cayman Chemicals	I
X-tremeGENE Hp	DNA Transfection reagent	Roche	II, III

Table 5. Commercial substrates

Name	Description	Source	Original publications
2-AG	2-Arachidonoylglycerol [C20:4]	Cayman Chemical	II, III
1-OG	1-Oleoylglycerol [C18:0]	Sigma	II, III
2-OG	2-Oleoylglycerol [C18:0]	Sigma	II, III
1-LG	1-Lauroyl-*rac*-glycerol [C12:0]	Sigma	II, III
2-LG	2-Lauroyl-*rac*-glycerol [C12:0]	Cayman Chemical	II, III
1-CG	1-Capryloyl-*rac*-glycerol [C8:0]	Sigma	II, III
1-DG	1-Decanoyl-*rac*-glycerol [C10:0]	Sigma	II
1-LaurG	1-Lauroyl-rac-glyserol [12:0]	Sigma	II, III
1-MG	1-Myristoyl-*rac*-glycerol [C14:0]	Sigma	II
2-PG	2-Palmitoyl-*rac*-glycerol [C16:0]	Tocris	II
1-SG	1-Stearoyl-*rac*-glycerol [C18:0]	Sigma	II, III
15-deoxy-PGJ2-2GE	15-deoxy-Δ12,14-Prostaglandin J2-2-glycerol ester	Cayman Chemical	II
PGE2-1GE	Prostaglandin E2 -1-glyceryl ester	Cayman Chemicals	II
PGD2-1GE	Prostaglandin D2 -1-glyceryl ester	Cayman Chemicals	II
PGF2α-1GE	Prostaglandin F2 -1-glyceryl ester	Cayman Chemicals	II
LPA	1-Oleoyl(C18:1)-*sn*-glycero-3-phosphate	Sigma	II
DOG	1,2-Dioleoyl-*rac*-glycerol (DAG)	Cayman Chemicals	II, III
TOG	Glyceryl trioleate	Sigma	II, III
POLG	1-Palmitoyl-2-oleoyl-3-linoleoyl-*rac*-glycerol	Cayman Chemicals	II, III
-	Acetyl Coenzyme A	Sigma	III
-	Arachidonoyl Coenzyme A	Avanti Polar Lipids	III

4.2 METHODS

4.2.1 Animals and tissue preparations

Animals (I & III)

The tissues were dissected from the animals described in Table 6; these were supplied by the National Laboratory Animal Centre (University of Eastern Finland, Kuopio, Finland). Approval for harvesting of the animal tissues was obtained from the animal welfare officer of the University of Eastern Finland. No *in vivo* treatments were conducted in these studies.

Table 6. Rodents

Animal	Source	Original publication
4-week-old male Wistar rats	National Laboratory Animal Centre (University of Eastern Finland, Kuopio, Finland)	II
4-month-old mice	National Laboratory Animal Centre (University of Eastern Finland, Kuopio, Finland)	III
14-week-old MAGL-KO mice	Taschler et al. 2011) (University of Graz, Graz, Austria)	I

Harvesting tissues (I & III)

Animals were decapitated and whole brains were dissected within 5 minutes, dipped in isopentane, chilled on dry ice and stored at – 80 °C. Tissues were harvested immediately after brain dissection. After a brief rinse in 0.9 % NaCl, the dissected tissues were frozen on dry ice and stored at – 80 °C until use.

The generation of the MAGL-KO mice has been described by Taschler et al. (2011). Breeding of MAGL-KO animals was approved by the Ethics committee of the University of Graz, the Austrian Federal Ministry of Science and Research, and is in accordance with the council of European Convention (ETS 123). Fourteen-week-old animals were anaesthetized with IsoFlo/Isoflurane and euthanized by cervical dislocation and whole brains were dissected as described above.

Preparation of mouse/rat whole brain membranes (I & III)
A glass Teflon homogenizer was used to mechanically homogenize frozen brains in Tris-buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl) after which the homogenized brains were centrifuged at low speed (1000 x g, 10 min, +4 °C) and then the supernatant was centrifuged at high speed (145000 × g, 45 min +4 °C). The pellet was resuspended in Tris buffer followed by sonication and centrifuged at high speed (145000 × g, 45 min, +4 °C) with the washing step being repeated. The final pellet (membrane proteome) was resuspended in Tris buffer and aliquoted for storage at –80 °C. Pierce BCA Protein Assay Kit was used to determine the protein concentration with bovine serum albumin (BSA) as a standard.

Preparation of mouse brain cryosections (I)
A Leica cryostat was used to cut horizontal brain sections (20 μm) at -20 °C and then the sections were thaw-mounted onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany). Brain sections were started to be collected at the interaural level of 6.90 mm of the brain rat atlas of Paxinos and Watson, 1998). Cutting was ended at the interaural level of 6.14 mm or interaural 4.18 mm (Paxinos and Watson, 1998). Ten slides, each having three consecutive brain sections were collected. Twenty-five slides, each having five brain sections, were collected for the endocannabinoid analysis. Brain sections were dried at room temperature (RT) for 1–2 h under a constant stream of air and stored at -80 °C until use.

Isolating mitochondria from mice tissues (III)
Mitochondria were purified as previously described (Reyes et al. 2005). Briefly, fresh mouse tissues were homogenized in Tris-buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl) and centrifuged (1000 × g, 10 min, +4 °C). The mitochondrial pellets were re-suspended in ice-cold PBS and briefly sonicated, followed by centrifugation at 100,000 × g, 45 min, +4 °C. The pellet was resuspended and centrifugation was repeated. The resulting pellets were resuspended in PBS and aliquoted for storage at –80 °C. Protein concentrations were determined with the Pierce BCA Protein Assay Kit (Pierce, Rockford, IL) using BSA as a standard. Enrichment of mitochondrial outer membrane transporter (TOMM20) in the pellets was verified using Western Blot.

4.2.2 Functional [^{35}S]GTPγS autoradiography experiments (I)

Functional [^{35}S]GTPγS autoradiography was used to explore brain regional CB1 receptor-dependent $G_{i/o}$ protein activity as previously described (Aaltonen et al. 2014; Palomaki and Laitinen 2006; Palomaki et al. 2007). Shortly, this three-step assay protocol was performed at RT in a Tris-based assay buffer (50mM Tris–HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, and 5 mM $MgCl_2$). The protocol consisted of a pre-incubation for 20 min (step 1), GDP-loading for 60 min (step 2) with 10 μM methylarachidonylfluorophosphonate (MAFP) in DMSO to prevent MAGL-dependent hydrolysis of 2-AG (Saario et al. 2004; Savinainen et al. 2003) or with vehicle (DMSO), and [^{35}S]GTPγS labeling for 90 min using ~50 pM of [^{35}S]GTPγS (step 3). In step 3, the brain sections were incubated with the cannabinoid agonist CP55,940 and the antagonist/inverse agonist AM251 at the indicated concentrations or with vehicle. In each experiment, 10 μM GTPγS was used to determine non-specific binding.

4.2.3 LC/MS/MS analysis of endocannabinoid levels (I)

To obtain comparable results with functional [^{35}S]GTPγS autoradiography experiments, triplicate slides each with five horizontal brain sections underwent a three-step autoradiography assessment mimicking the protocol described by Aaltonen et al. (2014). Additionally, endocannabinoid levels were determined from unprocessed slides, which did not go through the autoradiography mimicking protocol (three slides/genotype).

Brain sections were treated with 10 μM of the broad-spectrum serine hydrolase inhibitor MAFP (methyl arachidonoylfluorophosphonate) (Fig. 8) or vehicle (DMSO) for 60 min (step 2) and thereafter for 90 min (step 3) in the assay buffer. Endocannabinoid extraction from tissue and assay buffer was performed as previously described (Aaltonen et al. 2014; Lehtonen et al. 2011).

4.2.4 Generation of recombinant protein lysates and membranes (II & III)

Cell culturing of HEK293, LNCaP, VCaP and PC3 (III)

All cell lines were grown in a humified atmosphere of 5 % CO_2/ 95 % air at + 37 °C with the medium described in Table 7; supplemented with 10 % of FBS, 25 U/ml penicillin and 25 μg/ml streptomycin.

Table 7. Cell lines and cell culturing media used

Cell line	Cell culturing media	Source	Original publication
HEK293	DMEM	Euroclone	II, III
LNCaP	RPMI1640	Gibco	III
VCaP	DMEM	Euroclone	III
PC3	F-12 Nut Mix medium	Gibco	III

Cells over-expressing the gene of interest

HEK293 cells were transiently transfected with plasmids containing either WT cDNA (ABHD6, ABHD11, ABHD12, MAGL, LYPLA and AFMID), mutant cDNA (ABHD6 mutants S148A; D278A/E/N; H306A/S/Y; and ABHD12 mutants S246A; D333N; H372A) or empty control vector (Mock) (Table 8.) according to the manufacturer's instructions using the X-tremeGENE Hp DNA Transfection reagent. For transfection, HEK293 cells were cultured in DMEM and 10 % FBS without antibiotics. Untransfected HEK293 and/or mock-transfected cells (transfected with empty vector) were cultured in parallel for controlling the expression and activity in later experiments.

Table 8. cDNAs and primers used for mutating the catalytical triad

Name	Description	Source	Original publication
pCMV6-AC-hABHD6	Expression vector for ABHD6	Origene Technologies	II
pCMV6-XL4-hABHD12	Expression vector for ABHD12	Origene Technologies	II
pCMV6-XL-hABHD11	Expression vector for ABHD11	Origene Technologies	III
pCMV6-XL5-hMAGL	Expression vector for MAGL	Origene Technologies	II
pCMV6-XL5-hLYPLA2	Expression vector for LYPLA	Origene Technologies	III
pMCV-Entry-hAFMID	Expression vector for AFMID	Origene Technologies	III

Harvesting cells (II & III)
Cell were harvested by washing the cells twice with ice-cold PBS after which the cells were scraped and pelleted at 250 × g, 10 min, + 4°C.

Preparation of cell lysates and membranes (II & III)
After harvesting of the cells, either cell lysates or cell membranes were prepared. To generate cell lysates, cell pellets were three times freeze-thawed, resuspended in ice-cold PBS and briefly sonicated before storage at –80 °C.

Cell membranes were prepared by resuspending the cell pellet in ice-cold PBS, followed by a brief sonication and centrifugation at 100,000 × g, 45 min, +4 °C. The pellet was resuspended in ice-cold PBS by a brief sonication and aliquoted for storage at –80 °C. Protein concentrations were measured with the Pierce BCA Protein Assay Kit (Pierce, Rockford, IL) using BSA as a standard.

Generation of SH-triplet (III)
To facilitate inhibitor screening, a serine hydrolase (SH)-triplet preparation was generated by pooling cell lysates of AFMID, ABHD11, LYPLA2. The SH-triplet contained 1 mg/ml of each HEK293 cell lysate overexpressing the SH of interest.

4.2.5 Site-directed mutagenesis of the postulated catalytic triads (II)

In site-directed mutagenesis, we introduced mutations in plasmids pCMV6-AC-hABHD6, pCMV6-XL4-hABHD12, and pCMV6-XL5-hMAGL transcript variant 1 [313 amino acid residues] to generate ABHD6 mutants S148A, D278A/E/N, and H306A/S/Y, and ABHD12 mutants S246A, D333N, and H372A. Mutagenesis was performed as described before (Laitinen et al. 2014) with the QuikChange® Site-Directed Mutagenesis Kit following the manufacturer's instructions using the primers defined in Table 9. Mutated DNA was transformed into competent bacterial *E. coli* cells. Plasmid DNA was isolated and purified with the Qiagen plasmid purifying kit. The sequences of the constructs were verified by fully sequencing the purified plasmids. The plasmids were transiently transfected into HEK293 cells as detailed in section 4.2.1.

Table 9. Primers used for mutating the catalytical triad of ABHD6 and ABHD12

Name	Description	Source	Original publication
Fwd: S246A primer for ABHD12	5'- CATCTGGGGCCACGCTCTGGGCACTGGCG-3'	Oligomer	II
Rev: S246A primer for ABHD12	5'- CGCCAGTGCCCAGAGCGTGGCCCCAGATG-3'	Oligomer	II
Fwd: D333N primer for ABHD12	5'-GCACGCTGAGGACAACCCGGTGGTGCCC-3'	Oligomer	II
Rev: D333N primer for ABHD12	5'-GGGCACCACCGGGTTGTCCTCAGCGTGC-3'	Oligomer	II
Fwd: H372A primer for ABHD12	5'-CCTTGGCTACAGGGCCAAATACATTTACAAGAGCCC-3'	Oligomer	II
Rev: H372A primer for ABHD12	5'-GGGCTCTTGTAAATGTATTTGGCCCTGTAGCCAAGG-3'	Oligomer	II

Fwd: S148A primer for ABHD6	5′-CCTGGTAGGCACCGCCATGGGTGGCCAGG-3′	Oligomer	II
Rev: S148A primer for ABHD6	5′-CCTGGCCACCCATGGCGGTGCCTACCAGG-3′	Oligomer	II
Fwd: D278N primer for ABHD6	5′-GGGGGAAACAAAACCAGGTGCTGGATGTG-3′	Oligomer	II
Rev: D278N primer for ABHD6	5′-CACATCCAGCACCTGGTTTTGTTTCCCCC-3′	Oligomer	II
Fwd: H306A primer for ABHD6	5′-CTGGAAAACTGTGGGGCCTCAGTAGTGATGGAAAG-3′	Oligomer	II
Rev: H306A primer for ABHD6	5′-CTTTCCATCACTACTGAGGCCCCACAGTTTTCCAG-3′	Oligomer	II

4.2.6 Western Blotting (II & III)

Western blotting was used to verify the expression level of WT and mutant hABHD6/12 in cell lysates, as well as to confirm the enrichment of mitochondria. Cell lysates or cell membranes (20-50 μg of total protein), with molecular weight markers, were separated by SDS-PAGE (10% gels) and transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubating the nitrocellulose membranes with Tris-buffered saline containing 0.1% Tween (TBS-T) and 0.5% (w/v) BSA for 1 h at RT. This was followed by overnight incubation at +4°C with the appropriate primary antibody diluted in 0.5% BSA-TBS-T. After washing the nitrocellulose membranes with TBS-T, they were incubated with a suitable secondary antibody diluted in 0.5% BSA-TBS-T (dilution factor 1: 10,000-1: 30,000) for 1 h at RT. After the final washes with TBS-T, the signals were visualized with Odyssey infrared scanner (Li-Cor Biosciences Inc., Lincoln, NB). The membranes were stripped using Tris-buffered saline containing 0.1% Tween (TBS-T) and 0.5% (w/v) BSA for 1 h at RT after which enzyme expression was normalized against the expression of β-actin or β-tubulin with otherwise identical incubation conditions. Immunoblots were visualized by Odyssey and quantified using ImageJ, freely available Java-based image analysis software (https://imagej.nih.gov/ij/).

4.2.7 Enzyme activity assays (II & III)

Fluorescent based glycerol assays for enzyme activity determination

Enzyme activity in fluorescence-based glycerol assay was determined in a 96-well plate format (*II & III*). Briefly, MAGs were first hydrolyzed by the hydrolases e.g. MAGL, ABHD6 or ABHD12 to free FA and glycerol. The liberated glycerol was then converted by three enzymes glycerol-1-phosphatase (G-1-P), glycerol kinase (GK) and glycerol phosphate oxidase (GPO) to produce H_2O_2, which in the presence of horseradish peroxidase (HRP) converts Amplifu TM Red into the fluorescent product, resorufin (Fig. 6). The fluorescence was monitored kinetically with a Tecan Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland) using excitation and emission wavelengths of 530 and 590 nm, respectively.

This method was used to test substrate preference (*II & III)* for endocannabinoid hydrolases and ABHD11 by kinetically monitoring glycerol production individually for each of the

HEK293 cell lysates over-expressing the enzyme of interest. Each enzyme was incubated individually (0.3 μg of protein) in the assay mixes (50 mM Tris-HCl, pH 7.40; 5 mM MgCl2; 1 mM EDTA, 100 mM NaCl with 0.5 % w/v BSA and 10 mM ATP) containing the substrate and the coupling enzymes (HRP 200 U/ml; GPO 200 U/ml; GK (200 U/ml). Several substrates including mono-, di-, or triglycerides or LPA (25 μM final concentration) with varying acyl chain lengths and saturations, (detailed in Tables 4 and 10) were tested. For each tested substrate, assay blanks without enzyme, cellular background (HEK293/Mock cell lysates), and a glycerol quality control sample (520 pmol) were included to monitor assay performance. Fluorescence of the assay blank was subtracted before calculating the final results. The amount of glycerol in each well was calculated based on a glycerol standard (520 pmol).

Functional paranitrophenol assay (III)
Paranitrophenol (pNP) assay was adapted and modified from Muccioli et al. (2008) and Kakugawa et al. (2015). Briefly, enzyme preparations were first diluted followed by incubation with chromogenic substrates containing pNP (Table 10.).

Table 10. Chromogenic substrates

Name	Description	Source	Original publication
pNPC2	4-Nitrophenyl acetate	Sigma	III
pNPC4	4-Nitrophenyl butyrate	Sigma	III
pNPC8	4-Nitrophenyl octanoate	Sigma	III
pNPC10	4-Nitrophenyl decanoate	Sigma	III
pNCP12	4-Nitrophenyl dodecanoate	Sigma	III
pNAA	p-Nitroacetanilide	Sigma	III
pNPP	p-Nitrophenyl phosphate	Sigma	III
pNPCC	O-(4-Nitrophenylphophoryl)choline	Santa Cruz	III
pNPS	Potassium 4-Nitrophenyl sulfate	Sigma	III

The absorbance of the liberated pNP moiety (λ 405 nm) after the hydrolysis of the chromogenic substrate was kinetically monitored using a Tecan Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland). The amount of pNP in the wells was calculated based on a pNP standard.

Acyl-CoA hydrolase assay (III)
The Acyl-CoA hydrolase assay was used to study whether the ABHD11 could hydrolyze acyl-CoAs. The protocol was adapted and modified from a previous publication (Hunt et al., 2006). Briefly, lysates of HEK293 cells and ABHD11-HEK293 cells (2 μg/well) were incubated in the assay buffer (50 mM Tris-HCl, pH 7.4; 5 mM MgCl2; 100 mM NaCl, 1 mM EDTA. The assay buffer contained additionally 0.1% (w/v) fatty acid-free BSA together with either oleoyl-CoA or arachidonoyl-CoA at final concentrations of 25, 50 or 100 μM. The assay was performed in a 96-well-plate with a total volume of 200 μl/well. Hydrolysis of the acyl-CoA species liberated CoA-SH which reacted with DTNB (5,5′-dithiobis -2-nitrobenzoic acid, 500 μM final concentration). The reaction generated 2-nitro-5-mercaptobenzoic acid (NMBA) whose absorbance was colorimetrically monitored at RT for 30 min (λ 410 nm). NMBA standard that was prepared by combining 10 nmol GSH + 100 nmol DTNB per well was used to calculate the amount of NMBA in wells.

4.2.8 Competitive activity-based protein profiling (ABPP) (I-III)

ABPP was performed using the serine hydrolase specific (Simon and Cravatt 2010) fluorophosphonate probe, TAMRA-FP following the outlines of a previously published methodology (Blankman et al. 2007). Shortly, 3.75-25 μg (SH triplet) or 100 μg protein in

total volume of 25 µl of either cell lysates or tissue homogenates were preincubated with 0.5 µl of the indicated inhibitors and the concentration or a vehicle (DMSO) for 1 h at RT. Then, serine hydrolases were labeled with 0.5 µl of 100 µM TAMRA-FP for 1 h at RT and the reaction was stopped by adding 2 × SDS-loading buffer. Samples together with molecular weight standards were separated by SDS-PAGE (10% gels; 82 x 82 mm or 70 x200 mm) and visualized by in-gel fluorescent gel scanning with Fujifilm FLA-3000 laser fluorescence scanner (Fujifi lm, Tokyo, Japan) (λ_{ex} 532 nm; λ_{em} 580 nm).

4.2.9 Seahorse (III)

In order to determine potential mitochondrial linkage of ABHD11, the Seahorse XF24 extracellular flux analyser (Seahorse Bioscience Inc) was used to determine the cellular oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) according to the manufacturer's instructions. Between 20000 – 50000 LNCaP cells per well were seeded in a Seahorse XF 24-well assay and incubated overnight at + 37°C and 5 % CO_2 in DMEM with 10 % of FBS. The cells were treated with 10-100 nM of JZP-245 or 100 nM of FAAH inhibitor URB597 inhibitors for 30 min - 20h, after which the medium was washed and replaced with prewarmed running medium (XF-medium (Seahorse Bioscience) supplemented with 1 mM sodium pyruvate and 25 mM glucose, pH 7.4) and then incubated for 60 min in an incubator at 37 °C without CO_2. After recording the basal level of OCR and ECAR, the cells were subjected to a mitochondrial stress using 1 µM oligomycin, 1.5 µM FCCP and 1 µM antimycin A. Cells were lysed after measurements and protein content was determined using BCA Assay (Pierce).

5 Main Results and Discussion

5.1. ALTERNATIVE SERINE HYDROLASES REGULATE 2-AG HYDROLYSIS IN MAGL DEFICIENT MICE BRAIN SECTIONS (I)

Endocannabinoids are lipid neuromodulators; their inactivation, which is mainly performed by FAAH and MAGL, terminates endocannabinoid signaling. Both pharmacological (Lichtman et al. 2004) and genetic (Cravatt et al. 2001) inhibition of FAAH increases the levels of the endogenous substrate, AEA. This leads to various behavioral effects, such as analgesia and a reduction of inflammation, depression and anxiety, mediated by both CB receptors (Cravatt et al. 2001; Lichtman et al. 2004) without affecting the functionality or the number of CB1/2 receptors. On the contrary, the increase in 2-AG levels caused by chronic pharmacological inhibition of MAGL affected CB1 receptor signaling in mice, but evoked no CB1-dependent behavioral effects (Chanda et al. 2010; Schlosburg et al. 2010).

Several research groups (Chanda et al. 2010; Taschler et al. 2011; Zhong et al. 2011) have generated MAGL-KO mice. These mice have revealed the major role of MAGL as a controller of MAG levels, especially in the brain (Chanda et al. 2010; Schlosburg et al. 2010; Zhong et al. 2011), but also in liver and adipose tissue (Taschler et al. 2011). In the first study (I), we utilized functional autoradiography to investigate the possible alterations of CB1 receptor activation in global MAGL-KO mice brain sections. This study provided a direct evidence of reduced regional CB1 receptor activity in several brain regions in MAGL-KO mice brain sections. The results were consistent with those reported after chronic pharmacological blockade of MAGL (Schlosburg et al., 2010).

The LC/MS/MS analysis of 2-AG levels from MAGL-KO mice showed elevated 2-AG levels compared to WT mice brain sections. As observed here and in previous studies (Chanda et al. 2010; Schlosburg et al. 2010), the excess amounts of 2-AG resulted in tonic activation of CB1 receptors, MAGL-KO mice lacked the CB1 receptor-mediated so-called cannabimimetic effects (Chanda et al. 2010). In our experiments, MAGL-KO mice showed a reduced CB1 receptor activity in brain which is presumably a compensatory response to the excessive levels of 2-AG. The desensitization of CB1 receptors most likely accounts for the lack of CB1-mediated effects which is opposite to FAAH-KO mice that have an unaltered amount of CB1 receptors when compared to WT mice (Cravatt et al. 2001; Falenski et al. 2010) and normal CB1 receptor function (Schlosburg et al. 2010). Hence, these mice display CB1 and CB2 receptor-mediated behavioral effects. Several possibilities may explain these differences. One possibility could be in the efficacy of these endocannabinoids: 2-AG acts as a full agonist at the CB1 receptors whereas AEA is a partial agonist (Hillard 2000) which could presumably lead to different responses in the presence of excessive levels of endocannabinoids. Another possibility could be attributable to the different anatomical distribution of these enzymes (Schlosburg et al. 2010) i.e. MAGL acts in presynaptic neurones while FAAH functions mainly postsynaptically.

MAFP is a broadly acting serine hydrolase inhibitor (Hoover et al. 2008); it is known to block virtually all 2-AG hydrolase activity in brain tissue (Saario et al. 2004). Interestingly, treating MAGL-KO brain sections with MAFP increased 2-AG levels in MAGL-KO mice brain sections. The observed increase in 2-AG levels in MAGL-KO brain sections implies that there are additional serine hydrolases capable of regulating 2-AG levels. In view of this finding, the potential compensatory alterations of serine hydrolases in MAGL-KO mice

brain were explored using ABPP. The serine hydrolase pattern of MAGL-KO in mouse brain and WT mice brain was compared but, with the exception of MAGL, no changes were observed in the activity of the enzyme between WT and MAGL-KO mice brain. Several groups have explored the up-regulation of enzymes capable of hydrolyzing 2-AG in MAGL-KO mice. Nonetheless, no evidence of up-regulation of ABHD6, ABHD12 (Taschler et al. 2011; Zhong et al. 2011) or HSL (Taschler et al. 2011) has been found; nor have any alterations been detected in the activity of FAAH or of ABHD6 (Chanda et al. 2010; Pan et al. 2011; Schlosburg et al. 2010) which is in line with our result.

Taken together, the results of this study support the hypothesis that excess levels of 2-AG caused by inhibition of MAGL evoke tolerance and compensatory effects, such as desensitization of CB1 receptors. The increase in the 2-AG levels caused by MAFP in MAGL-KO mice brain sections indicates that alternative hydrolases regulate the hydrolysis of the endocannabinoid 2-AG in MAGL-KO mice brain sections. Because of the unaltered expression or activation levels, it has been postulated that putative additional 2-AG hydrolases such as FAAH, HSL, ABHD6 and ABHD12 (Blankman et al. 2007) and possibly some other unknown enzymes must be involved in controlling the elevated levels of 2-AG caused by the MAGL deficiency. Thus, it would be beneficial to learn more about these enzymes and to explore endocannabinoid hydrolases in more detail but for these tasks, we need specific enzyme activity assays.

5.2 FLUORESCENCE-BASED HYDROLASE ACTIVITY ASSAY FOR ENDOCANNABINOID HYDROLASES (II)

Study II focused on developing and optimizing a sensitive fluorescent enzyme activity assay utilizing the HTS platform. The assay was developed for characterizing two postulated endocannabinoid hydrolases (ABHD6 and ABHD12) in comparison to the well-established 2-AG hydrolyzing MAGL. The principle of this assay is described in Fig. 6.

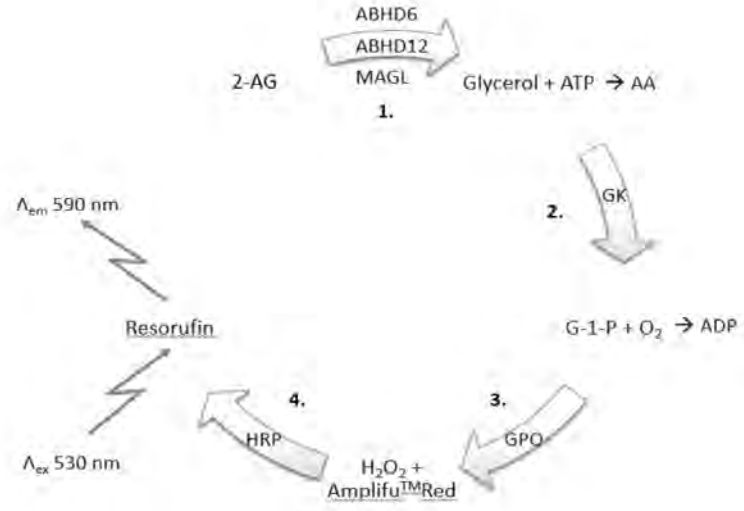


Figure 6. Principle of the fluorescence-based glycerol assay. In the fluorescence-based glycerol assay, the enzyme activity (1) was determined by measuring the glycerol liberated from the hydrolysis of glycerolipid substrates. The glycerol production was coupled via a three-step enzymatic cascade (2-4) to hydrogen peroxide (H_2O_2) dependent generation of resorufin whose fluorescence (λ_{ex} 530; λ_{em} 590 nm) was kinetically monitored using a fluorescent plate reader.

At first, the linearity of the fluorescence-based glycerol assay was evaluated; it was noted that the assay detects glycerol linearly in the concentration range 0-520 pmol/well at all measured time points (0-90 min). Next, the protein- and time-dependent (0.3 μg/well of protein) glycerol response was tested using the HEK293 cell lysates individually overexpressing ABHD6, ABHD12 or MAGL. These variables were linear at all tested protein concentrations and at all time-points. The repeatability was ensured by monitoring the substrate consumption in every experiment. To ensure that there was a sufficient substrate concentration as well as a linear velocity of the reaction, experiments were conducted in conditions where less than 10 % of the substrate (2-AG) was consumed. Finally, the optimal protein concentration was tested; the optimal signal-to-noise ratio was determined to be as low as 0.3 μg of total protein/well, showing to be an extremely sensitive assay.

5.2.1 Kinetic measurements

After the optimal assay conditions were established, the assay was used to determine the kinetic parameters of ABHD6 and ABHD12. MAGL was used as a control and 2-AG as the substrate. The results of kinetic values obtained from these studies are listed in Table 13. together with the kinetic parameters found in the literature. It must be noted that the kinetic parameters determined here were obtained using cellular lysates whereas the parameters gathered from the literature have been mostly determined using purified enzymes. Even though the lysate background activity is minimized, some cellular background activity was present, compromising the accuracy of the K_m determination. Despite this, the kinetic parameters obtained with MAGL using 2-AG as a substrate fell in the range of reported K_m values (Table 13.). In the parallel incubation, the K_m values for ABHD6 and ABHD12 were ~160 μM and ~120 μM, respectively. It was noticed that the K_m value obtained for ABHD6 (~160 μM) was higher compared to the K_m value of MAGL (110 μM), indicating that under these particular conditions, ABHD6 displayed lower affinity for 2-AG. The same observation was also reported with 1,3-oleyl glycerol (1(3)-OG) (Thomas et al. 2013), implying that ABHD6 could have a lower affinity towards MAG in general, which is in accordance with the belief that MAGL is the predominant MAG hydrolase. Recently, ABHD6 has been reported to hydrolyze BMP (bis(monoacylglycerol)phosphate) (Pripasnig et al. 2015). In the same study, Pripasnig et al. (2015) reported K_m values around 1 mM for three substrates i.e. BMP, MAG and LPG.

5.2.2 ABHD6 and ABHD12 act as genuine MAG lipases

MAGL is mostly responsible for hydrolyzing MAG in many tissues. Blankman et al. (2007) showed that ABHD6 and ABHD12 can also hydrolyze 2-AG. In order to assess whether these enzymes hydrolyze other related substrates, the substrate profiles of ABHD6, ABHD12 and MAGL were determined using the optimized fluorescence-based glycerol assay. The substrate preferences were tested using a panel of natural MAG, DAG, TAG or fatty acid amide substrates with varying acyl chain lengths and saturations (Table 11.). All three enzymes showed only minimal activity towards DAG, TAG (Fig. 7) or fatty acid amides. Based on the substrate profiling, the three hydrolases acted as genuine MAG lipases whereas ABHD6 and MAGL preferred medium-chain saturated MAGs. Interestingly, with the tested substrates, MAGL did not show any preferences between 2-isomer or 1(3)-isomer whereas ABHD6 clearly preferred the 1(3)-isomer (Fig. 7). Screening demonstrated a similar substrate preference profile for ABHD6 and MAGL (Fig. 7) which could indicate related functions. The role of ABHD6 as an omnivorous MAG hydrolase was in support of the results published by Thomas et al. (2013) who reported that ABHD6 could hydrolyze several lipid substrates *in vitro*, including lysophospholipids. Lately, there has

been accumulating evidence favoring the role of ABHD6 as a MAG lipase (Alhouayek et al. 2013; Thomas et al. 2013; Zhao et al. 2014; Zhao et al. 2016), participating in glycerophospholipid metabolism (Thomas et al. 2013). It has also been reported that ABHD6 hydrolyzes BMP (Pribasnig et al. 2015), which is highly expressed in intraluminal vesicles of late endosomes. In that study, the authors suggested that ABHD6 controlled BMP hydrolysis and could be a part of the late endosomal/lysosomal sorting machinery.

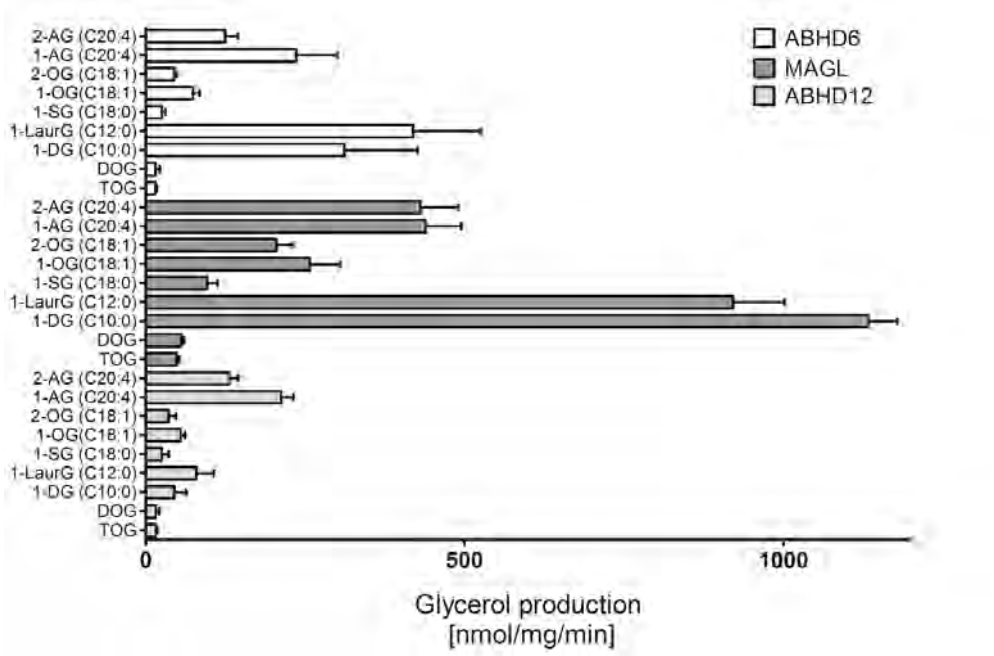


Figure 7. Substrate profile of human endocannabinoid hydrolases. ABHD6 and MAGL seem to have a similar substrate profile whereas ABHD12 has a distinct profile compared to ABHD6.

Unlike ABHD6 and MAGL, ABHD12 preferred 1/2-AG as a substrate (Fig. 7), more precisely its isomer 1(3)-AG and similar to ABHD6, also ABHD12 preferred 1(3)-isomers of the tested substrates. The role of ABHD12 controlling endocannabinoid signaling seems to be controversial. ABHD12 is known to degrade 2-AG (Blankman et al. 2007), and based on this feature, ABHD12 has often been associated with the ECS. Interestingly, ABHD12-KO mice have normal 2-AG levels in brain (Blankman et al. 2013) indicating an alternative *in vivo* role for ABHD12. Nonetheless, in a recent publication, an increase in the extracellular 2-AG levels was observed in microglia but not in neurons or astrocytes of the ABHD12-KO mice which indicates that ABHD12 may control extracellular levels of 2-AG (Viader et al. 2016). More studies are needed before any conclusions can be drawn about the role of ABHD12 in ECS.

Very little is known about the inscrutable enzyme ABHD12. It seems likely that ABHD12 has other functions in addition to its connection to ECS. Despite its distinct role compared to ABHD6, Pribasnig et al. (2015) reported that ABHD12 also hydrolyzed BMP *in vitro* (Pribasnig et al. 2015). It is known that ABHD12 is not present in liver (Lord et al. 2013), an organ where ABHD6 appears to have a significant role; instead ABHD is abundantly expressed in the brain, specifically in microglia. It is also abundantly present in related cell types, such as macrophages and osteoclasts (Fiskerstrand et al. 2010; Lord et al. 2013), where it may be responsible for hydrolyzing BMP (Pribasnig et al. 2015). The ABHD12-KO mice brain displayed about a 50 % reduction of BMP hydrolase activity, perhaps evidence

that ABHD12 has a role as a BMP hydrolase in the brain (Pribasnig et al. 2015). In addition to BMP, the ABHD12-KO models have pointed to a role for ABHD12 as an LPS lipase in the mammalian CNS (Blankman et al. 2013; Kamat et al. 2015).

Table 11. Substrates tested in the fluorescent-based glycerol assay and activity of ABHD6, ABHD12 and MAGL (% of 2-AG activity). The activity of 2-AG has been set to 100 %.

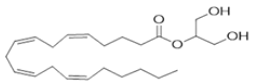
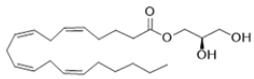
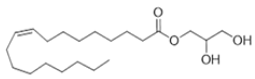
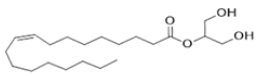
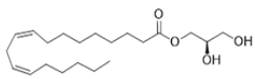
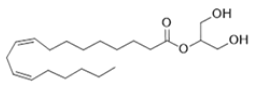
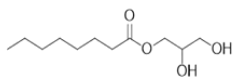
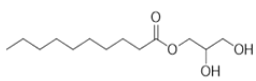
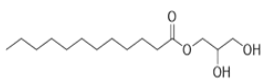
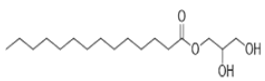
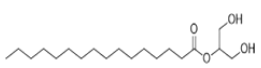
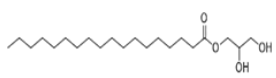
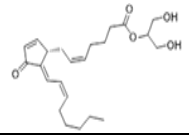
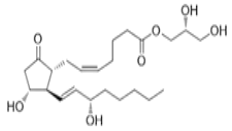
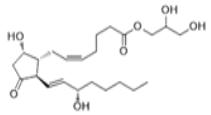
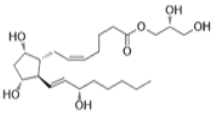
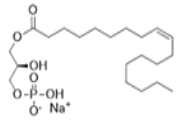
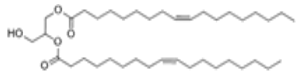
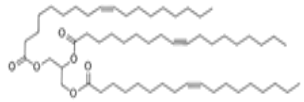
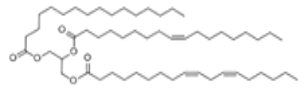
Substrate	Structure	ABHD6	ABHD12	MAGL
Monoacylglycerols (MAG)				
2-AG		100	100	100
1-AG		196 ± 27	165 ± 9	101 ± 13
1-OG		60 ± 14	39 ± 13	60 ± 18
2-OG		35 ± 11	25 ± 33	47 ± 13
1-LG		83 ± 9	56 ± 11	76 ± 16
2-LG		60 ± 22	43 ± 22	74 ± 8
1-CG		224 ± 35	12 ± 35	175 ± 7
1-DG		274 ± 37	32 ± 40	269 ± 4
1-LaurG		370 ± 25	61 ± 34	218 ± 9
1-MG		242 ± 21	95 ± 37	118 ± 10
2-PG		53 ± 19	40 ± 34	42 ± 12
1-SG		20 ± 23	19 ± 43	22 ± 17

Table 11. Substrates tested in the fluorescent-based glycerol assay and activity of ABHD6, ABHD12 and MAGL (% of 2-AG activity) (*Continue)*

Substrate	Structure	ABHD6	ABHD12	MAGL
		Prostaglandins		
15-deoxy-PGJ2-2GE		64 ± 34	14 ± 17	270 ± 23
PGE2-1GE		40 ± 32	31 ± 12	138 ± 5
PGD2-1GE		164 ± 17	14 ± 16	163 ± 5
PGF2α-1GE		155 ± 9	13 ± 21	155 ± 9
		Lysophosphatic acid		
LPA		21 ± 70	12 ± 82	8 ± 20
		Diacylglycerol (DAG)		
DOG		8 ± 40	8 ± 30	12 ± 9
		Triacylglycerol (TAG)		
TOG		8 ± 17	8 ± 17	10 ± 9
POLG		9 ± 21	8 ± 24	10 ± 11

5.2.3 Inhibitor profile of ABHD6 and ABHD12 using fluorescence-based glycerol assay (II)

In study II, we also provided an initial inhibitor profile for ABHD6 and ABHD12 using the fluorescence-based glycerol activity assay. Among the tested compounds, the broadly acting serine hydrolase inhibitor, MAFP (Fig. 8) was the most potent inhibitor of both enzymes with IC_{50} values of ~20 and ~90 nM for ABHD6 and ABHD12, respectively. This was not surprising since MAFP is the fluorophosphonate analog of 2-AG. The broadly acting lipase inhibitor THL also showed nanomolar potency for both enzymes. MAFP which has a 20 carbon acyl chain containing four double bonds (C20:4 acyl chain) inhibited

ABHD6 and ABHD12 within the same concentration range, whereas IDFP (C12:0 acyl chain) and HDSF (C16:0 acyl chain) showed >20-fold higher potency for ABHD6 over ABHD12. This result is consistent with the substrate profile where ABHD12 preferred MAG which has a long acyl chain (C20:4), whereas ABHD6 accepted shorter acyl chains as well. As expected, with the exception of RHC-80126, all of the tested non-selective inhibitors MAFP, IDFP, HDSF and THL inhibited both ABHD6 and ABHD12. One lipase inhibitor, RHC-80126, inhibited ABHD6 but not ABHD12, which is in line with a previous report (Hoover et al. 2008). Finally, the ABHD6 selective inhibitor WWL70 (W. Li et al. 2007) was shown to inhibit ABHD6 with almost identical potency (IC_{50}, 85 nM) compared to THL (IC_{50}, 50 nM). The IC_{50} value of WWL70 is nearly identical to the previously reported value (IC_{50}, 70 nM) (W. Li et al. 2007). In addition to WWL70, there are only a few selective ABHD6 inhibitors in the literature (Bachovchin et al. 2010; Hsu et al. 2012; Hsu et al. 2013), including inhibitors developed in our laboratory, JZP-430 (J. Z. Patel et al. 2015) and JZP-169 (J. Z. Patel, van Bruchem et al. 2015). ABHD12 has proved to be a challenging target for inhibitor development. The most potent ABHD12 inhibitors were the broadly acting THL and MAFP. We have also described some selective reversible inhibitors that are betulin derivatives (Parkkari et al. 2014). ABHD12 has a wide tissue distribution (Lord et al. 2013) and distinct from MAGL and ABHD6; ABHD12 also has a different subcellular location (Blankman et al. 2007; Viader et al. 2016). This points to a distinctive physiological role, which is in line with the data found for substrate preferences and supports the theory that ABHD12 possesses an alternative role from ABHD6, for example as a LPS hydrolase (Blankman et al. 2013; Kamat et al. 2015).

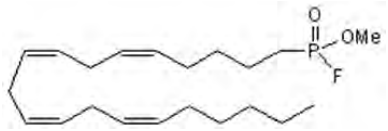


Figure 8. Structure of broadly acting serine hydrolase inhibitor MAFP.

In conclusion, in study II, we adapted and optimized a sensitive fluorescence-based glycerol assay method for characterizing the putative endocannabinoid hydrolases ABHD6 and ABHD12. The assay was shown to be linear in relation to substrate concentration, protein amount and time-dependence of the hydrolysis of 2-AG. The devised assay was capable of measuring reliably the activation of endocannabinoid hydrolases. As a result, we identified ABHD6, ABHD12 and MAGL hydrolases as genuine MAG lipases. Finally, we determined intial inhibitor profiles for ABHD6 and ABHD12. The inhibitor profiling provided structure-activity data which revealed striking differences between ABHD6 and ABHD12. Data from this study was used to design potent and selective inhibitors for ABHD6 (J. Z. Patel et al. 2015; J. Z. Patel, van Bruchem et al. 2015) and to discover novel inhibitors for ABHD12 (Parkkari et al. 2014).

5.3 CHARACTERIZATION OF ABHD11 (III)

5.3.1 Inhibitor screening using activity-based protein profiling (III)

Organophosphates are known to target metabolic serine hydrolases including ABHD11, LYPLA2 and arylformamidase (AFMID) (Medina-Cleghorn et al. 2014; Nomura et al. 2008; Nomura and Casida 2011; Quistad et al. 2006). Nonetheless, only very few selective inhibitors have been reported for ABHD11 and LYPLA2. For ABHD11, the most promising inhibitors are the urea-based AA44-2 (Adibekian et al. 2011), ML226 (Adibekian, Martin et al. 2010) and ML211 (Adibekian, Hsu et al. 2010) that is also believed to inhibit lysophospholipases LYPLA1 and LYPLA2 (also known as acyl-protein thioesterases 1/2;

APT1/APT2). In addition to ML211, β-lactone palmostatin B has been reported to inhibit LYPLA2 (Dekker et al. 2010; Rusch et al. 2011). So far, no selective inhibitors for AFMID participating in kynurenine metabolism (Pabarcus and Casida 2002) have been reported.

Study III had two aims; to characterize ABHD11 in comparison with LYPLA2 and AFMID by developing an enzyme activity assay and to optimize ABPP for screening inhibitors. The ABPP method was chosen for screening inhibitors due to its ability to recognize nearly every serine hydrolase in virtually any kind of biological material (Simon and Cravatt 2010). The power of ABPP lays in the probe TAMRA-FP that binds and labels active serine hydrolases. Labeled serine hydrolases are then separated by size using SDS-PAGE and further visualized by in-gel fluorescent gel scanning. Competitive ABPP, where the biological material is first treated with an inhibitor followed by incubation with the probe, has been a powerful tool for discovering inhibitors towards serine hydrolase family members in various biological materials (Bachovchin and Cravatt 2012). ABPP has been used previously in screening inhibitor libraries and at least two inhibitors, AS115 (Chiang et al. 2006) and JZL184 (Long, Li et al. 2009), for KIAA1363 and MAGL respectively, have been discovered in ABPP screening campaigns. Unfortunately, screening inhibitors for a certain enzyme using the full-scale ABPP method is time-consuming.

In our study, HEK293 cell lysates over-expressing ABHD11, LYPLA2 and AFMID were pooled (called hereafter the SH-triplet) against which inhibitors were screened using small-scale ABPP (82x82 mm). Using the SH-triplet, ~200 in-house and commercial compounds were screened at a 10 μM concentration after which the IC_{50} value for compounds selectively inhibiting one of these three enzymes could be determined. Three inhibitors (JZP-228, JZP-245 and JZP-249) were discovered to potently inhibit ABHD11 with IC_{50}-values of 2-3 nM. In addition, three compounds (JZP-110, JZP-427 and JZP-421) were found to target AFMID at submicromolar concentrations. The potency calculations by ImageJ were based on the intensity of the particular protein band. The amount of protein loaded per lane in the SH-triplet overexpressing the HEK lysate was a mere 3.75 μg, allowing a robust detection of the triplet signal with a negligible cellular background. While the incubation time of the inhibitors and TAMRA-FP probe was same as in the full-scale ABPP, the processing time in the gel was significantly shorter, making the method more efficient.

Although the use of the FP probe capable of labeling many serine hydrolases is an advantage, hydrolases with the same size may overlap even after separation on protein gels. Nevertheless, for example when compared to Western Blot that detects a certain protein based on the epitope of the protein, by definition, ABPP detects only active enzymes. Furthermore, ABPP detects only enzymes that form a covalent intermediate with their substrate. This covalent bond is an essential part of the method. However, since the total enzyme activity measurement is an endpoint measurement using a covalent inhibitor, we are not able to observe subtle changes in the K_m therefore it is necessary to determine the kinetic values of an enzyme and thus an enzyme activity assay is needed alongside the ABPP.

5.3.2 Enzyme activity assay for ABHD11 (III)

No endogenous substrate for ABHD11 has been identified so far. Therefore, we tested the enzymatic activity of ABHD11 alongside LYPLA2 and AFMID against a panel of MAG and prostaglandin glycerol esters using the fluorescence-based glycerol activity assay developed in study II. None of these three enzymes displayed any detectable MAG activity and no DAG lipase activity was observed when tested using a modified glycerol assay adapted from van der Stelt et al. (2015) indicating that these enzymes do not act as MAG or DAG lipases. It was decided to proceed by adapting two colorimetric substrate profiling

methods with the aim to screen enzyme activity in a broad manner using artificial chromogenic substrates to reveal possible phosphatase, sulphatase, lipase, amidase/protease and esterase activities (Table 12.). The assays were based on the release of pNP (paranitrophenol) (Kakugawa et al. 2015; Muccioli et al. 2008). While the activity towards other substrates did not differ from the HEK293 cell background activity, all three enzymes (LYPLA2 with minor activity), preferred acyl-pNP with short acyl chain 4-nitrophenyl butyrate, a typical chromogenic substrate for lipases (Table 12.). The possible role for ABHD11 as a lipase was recently supported by a study that reported a significant increase in phospholipid accumulation after disruption of the plant homolog, AtABHD11 (Vijayakumar et al. 2016). Interestingly, the overexpression of AtABHD11 in *E. coli* reduced phospholipid levels, further highlighting the possible function of ABHD11 as a lipase.

Table 12. Tested chromogenic substrates and activity of ABHD11, LYPLA2 and AFMID

Substrate	ABHD11	LYPLA2	AFMID
pNPC2	+	+	++
pNPC4	++	+	++
pNPC8	-	-	-
pNPC10	-	-	-
pNCP12	-	-	-
pNAA	-	-	-
pNPP	-	-	-
pNPCC	-	-	-
pNPS	-	-	-

pNP=paranitrophenyl; pNAA=p-nitroacetanilide (amidase/protease substrate); pNPP=pNP-phosphate (phosphatase substrate); pNPPC=pNP-phosphorylcholine (phospholipase C substrate); pNPS=pNP-sulfate (sulfatase substrate)

ABHD proteins are closely related, yet only a few crystal structures are available for this family. From the available template structures, ABHD11 had 55 % sequence homology with YbfF which is an esterase present in *Escheria coli.* Interestingly, YbfF has been shown to hydrolyze pNPC4 (Park et al. 2008). YbfF has also been reported to hydrolyze long-chain acyl-CoAs (Kuznetsova et al. 2005). Therefore, we decided to investigate whether ABHD11 could hydrolyze acyl-CoAs and to this end, the NMBA (2-nitro-5-mercaptobenzoic acid) assay was adapted (Hunt et al. 2006). Two acyl-CoA species (oleyl-CoA and arachidonoyl-CoA) were tested in parallel in the HEK293 cell lysate and in HEK293 cells over-expressing ABHD11 but the activity in HEK293 over-expressing ABHD11 did not differ from the background.

Table 13. Kinetic parameters of MAGL, ABHD6, ABHD11 and ABHD12 from our enzyme activity assays compared to several values from the literature. It must be noted that direct comparison cannot be performed, due to the difference between the sources of the enzymes

Substrate	Preparation	Km (μM)	Vmax (nmol/min/mg)	Reference
		MAGL		
2-AG	Cell lysate[¤]	110 ± 15	120 ± 10	(Navia-Paldanius et al. 2012)
1(3)-OG	Purified MAGL from erythrocytes	270	NA	(Somma-Delpero et al. 1995)
1(3)-OG	Purified MAGL	1200	89317	(Thomas et al. 2013)
1(3)-OG	Purified MAGL from rat adipose tissue	200	NA	(Tornqvist and Belfrage 1976)
1-OG	Sf9 homogenate	300	NA	(Karlsson et al. 2000)

2-OG	Purified MAGL from erythrocytes	490	NA	(Somma-Delpero et al. 1995)
2-OG	Purified MAGL from rat adipotissue	200	NA	(Tornqvist and Belfrage 1976)
		ABHD6		
2-AG	Cell lysate[¤]	159 ± 28	45 ± 5	(Navia-Paldanius et al. 2012)
1(3)-OG	Purified ABHD6[*]	1900	7977	(Thomas et al. 2013)
1-OG[#]	Purified ABHD6[*]	980 ± 210	13433 ± 1083	(Pribasnig et al. 2015)
LPG	Purified ABHD6[*]	750	1553	(Thomas et al. 2013)
LPG	Purified ABHD6[*]	1100 ± 330	1483000 ± 183	(Pribasnig et al. 2015)
BMP	Purified ABHD6[*]	980 ± 7	5800 ± 167	(Pribasnig et al. 2015)
		ABHD11		
pNPC4	Cell lysate[¤]	250 ±110	21 ± 1[§]	(Navia-Paldanius et al. 2016)
		ABHD12		
2-AG	Cell lysate[¤]	117 ± 14	42 ± 3	(Navia-Paldanius et al. 2012)

NA= not applicable; [¤]HEK293 cell lysates over-expressing the protein of interest; [*]Murine enzyme; [#]Racemic 1-OG; [§]Unpublished data

Even though the endogenous substrate for ABHD11 still awaits to be identified, the finding of a suitable substrate-based assay for ABHD11 with reasonable activity towards chromogenic 4-nitrophenyl butyrate (pNPC4) allowed us to determine values of K_m and V_{max} for ABHD11 (Table 13.). These kinetic parameters were determined using the substrate pNPC4 with concentration range 0-500 μM.

The chromogenic substrate-based assay was further used to explore the inhibition of ABHD11 using the three most potent ABHD11-inhibitors (JZP-228, JZP-245 and JZP-249) identified with the ABPP approach. The IC_{50}-values were 4-10 nM, which were in line with our results from the ABPP experiments (Fig. 9) with the SH-triplet indicating that our small-scale ABPP is suitable for screening a small inhibitor library.

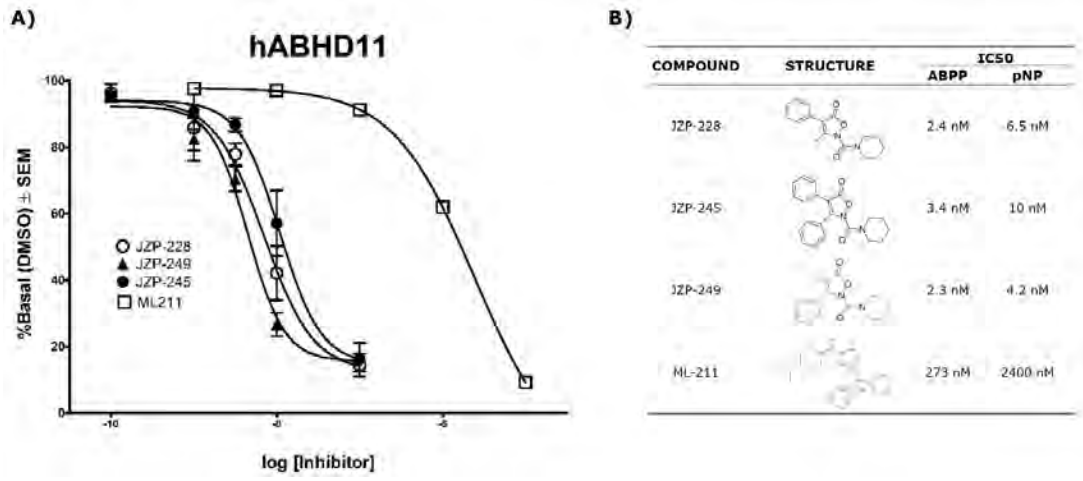


Figure 9. Inhibition of hABHD11-dependent pNPC4 hydrolysis by the JZP-compounds in comparison of ML211 (A). IC50 values from pNP and ABPP falls within the same range (B).

5.3.3 Selectivity of JZP-245

The selectivity of ABHD11 inhibitors was determined by ABPP using various cell and mice tissue proteomes. JZP-228 had an *in vitro* off-target (Fig. 10) that was identified as FAAH. With respect to the three ABHD11 inhibitors (JZP-228, JZP-245 and JZP-249), JZP-245 selectively inhibited ABHD11 *in vitro*. Based on the proteomic studies, ABHD11 has been predicted to be a mitochondrial enzyme (Lefort et al. 2009) therefore it was decided to treat the mitochondrial cell fraction of various mice tissues with JZP-245. As a result, JZP-245 sensitive enrichment of ABHD11 was observed in selected mice tissues, evidence for active ABHD11 enzyme expression in mitochondria. It is notable that in these proteomes, especially in brown fat and kidney, JZP-245 was determined to have an off-target that was identified as FAAH.

Before continuing to *in situ* experiments, the non-cytotoxicity of JZP-245 was confirmed using the CytoTox-Glo™ Cytotoxicity Assay with prostate cancer cell line LNCaP that abundantly expresses active ABHD11 (Nomura et al. 2011). JZP-245 showed no cytotoxicity at the measured concentrations (1-100 nM). The potency and selectivity of JZP-245 was tested *in situ* using three different prostate cancer cell lines (LNCaP, VCaP and PC3), all expressing active ABHD11 according to the ABPP assay. After *in situ* treatment, inhibition was visualized using ABPP. As a result, it was evident that JZP-245 was able to cross the cell membrane and inhibit ABHD11 in all three cell lines within 10 minutes at low nanomolar potency. However, JZP-245 inhibited another serine hydrolase identified as FAAH in LNCaP and VCaP cells. On the contrary, PC3 cells lacking FAAH (Nomura et al. 2011), showed no off-targets.

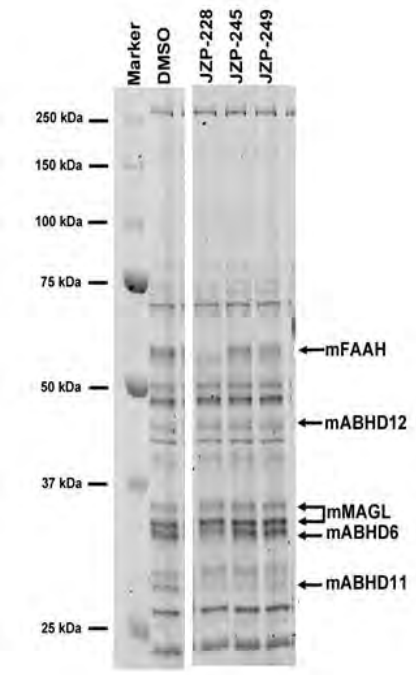


Figure 10. Selectivity of JZP-228, JZP-245 and JZP-249 in mouse brain membrane. JZP-228 has an off-target defined to be mFAAH (m=mouse).

Due to its putative role as a mitochondrial enzyme together with the hypothesis that ABHD11 could hydrolyze substrates with a short acyl chain and possibly participate in the electron transport chain in the mitochondria, it was examined whether inhibition of the ABHD11 had any effect on mitochondrial respiratory function. This was assessed using the

Seahorse analyzer. Cells were treated in parallel with either the ABHD11 inhibitor JZP-245 (10-100 nM) or the FAAH inhibitor, URB597 (100 nM). In these conditions, no significant changes in the OCR or ECAR were observed, indicating that neither FAAH nor ABHD11 were exerting any effects on mitochondrial respiratory function in LNCaP cells. However, before drawing any final conclusions, more optimized experiments using different conditions should be performed.

To summarize, three potent inhibitors for ABHD11 were discovered using ABPP by screening of our in-house library. The comparable potencies of ABPP and the chromogenic enzyme activity assay support the suitability of ABPP for inhibitor screening. The non-cytotoxic inhibitor, JZP-245 was shown to cross the cell membrane and inhibit ABHD11 with the same potency *in vitro* and *in situ* acting as a dual inhibitor targeting both ABHD11 and FAAH. By applying several enzyme activity assays and chromogenic substrates, it was possible to exclude the possible function of ABHD11 as a MAG or DAG esterase, lipase/esterase, phosphatase, sulfatase, phospholipase C, amidase/protease, phosphodiesterase or as acyl-CoA transferase. Even though the endogenous substrates and the physiological role of ABHD11 remains unknown, it was possible to devise a colorimetric enzyme activity assay for ABHD11 using an artificial chromogenic substrate for lipase (pNPC4).

6 Conclusions

At the beginning of this decade, generation of MAGL-KO mice clarified *in vivo* roles of MAGL in the ECS (Chanda et al., 2010; Schlosburg et al., 2010; Taschler et al., 2011). Both genetic and pharmacological inactivation of MAGL have been shown to increase the MAG levels. A lack of MAGL clearly increased 2-AG levels in mouse brain and this altered the expression and activity of CB1 receptors (Chanda et al., 2010; Schlosburg et al., 2010; Zhong et al., 2011) ultimately leading to functional and behavioral tolerance. Our autoradiography data from brain regions of MAGL-KO mice showed desensitization of CB1 receptors, further support for these findings. Interestingly, the lack of MAGL did not alter the CB1 receptor-mediated behavioral and physiological effect, so called tetrad (analgesia, hypomotility, catalepsy and hypothermia); alterations in the tetrad of behavior have been suggested to be a consequence of desensitization and downregulation of CB1 receptors (Chanda et al., 2010).

Our results also indicated that there were possible compensatory effects in MAGL-KO mice, which is a common phenomenon in KO animals. For example, Taschler et al. (2011) have shown that in WAT, HSL partly compensated for the lack of MAGL and the increased levels of MAGs. However, no evidence for compensatory changes in brain serine hydrolase activities was found in our experiments which is in agreement with other studies (Chanda et al. 2010; Pan et al. 2011; Schlosburg et al. 2010; Taschler et al. 2011; Zhong et al. 2011). Furthermore, inactivation of FAAH, ABHD6 and ABHD12 has not been shown to affect the bulk levels of 2-AG in brain. However, our results indicate that additional MAFP sensitive serine hydrolases are capable of degrading 2-AG. It has been suggested that the hydrolysis of 2-AG might depend on the relative contribution of 2-AG hydrolyzing enzymes that regulate 2-AG signaling as well as on subcellular origin and in cell type-specific expression level (Marrs et al., 2010; Taschler et al., 2011) which could explain the discrepancies.

In our studies with MAGL-KO mouse brain sections, we observed no significant desensitization of CB1 receptors in the molecular layer of the cerebellum, a brain region that plays a role in locomotor coordination. Interestingly, acute pharmacological treatment with a MAGL inhibitor has caused hypomotility (Long et al., 2009a). In contrast, a MAGL deficiency does not seem to interfere with the locomotor activity (Chanda et al., 2010; Taschler et al., 2011), indicating that MAGL-KO mice might have some other compensatory system or way of adapting to the elevated 2-AG levels. Several questions arise concerning the brain regional activation of CB1 receptors. Why does desensitization of CB1 receptors not occur in the molecular layer of the cerebellum? Why do alternative compensatory/adaptation occur especially in cerebellum? One explanation could be in the evolutionary importance of ensuring locomotor activity. For example, we observed desensitization in hippocampus, a brain region involved in memory. Mice with chronic agonist exposure are exceptionally sensitive to desensitization and downregulation of CB1 receptors in the hippocampus (Breivogel et al., 1999; McKinney et al., 2008). The ECS is known to regulate memory and the activation of CB1 receptor is known to participate in memory formation and consolidation (Busquets-Garcia et al., 2011; Clarke et al., 2008; Wise et al., 2009).

Until now, the methods used to study endocannabinoid hydrolases (Bisogno et al. 2003; Blankman et al. 2007; Saario et al. 2004) enabled measurement of one enzyme at a time, making

the investigation of these enzymes both expensive and time consuming. The fluorescence-based glycerol assay developed in this study enabled screening of a large panel of glycerophospholipid-derived substrates against different ABHD enzymes. The method is useful for investigating the MAG hydrolases generating fatty acids and glycerol.

At the beginning of the decade, not much was known about the ABHD hydrolases. In 2007, Blankman et al. reported that two novel enzymes, ABHD6 and ABHD12, could hydrolyze 2-AG and suggested that they could participate in the termination of endocannabinoid signaling together with MAGL. However, although the blockade of ABHD6 and ABHD12 did not affect the bulk 2-AG levels in the brain, more recently several publications have reported evidence indicating that ABHD6 and ABHD12 could act as the main 2-AG hydrolases in cells lacking MAGL (Marrs et al. 2010; Marrs et al., 2010b; Alhouayek et al.,2013). Study II demonstrated that ABHD6, ABHD12 and MAGL are all genuine MAG lipases. The similar substrate profile of ABHD6 and MAGL might indicate similar roles in the cell. ABHD6 has been found to localize to postsynaptic membranes whereas MAGL is expressed mainly in pre-synaptic areas (Marrs et al., 2010). This could indicate that while MAGL is responsible for ending the endocannabinoid signaling in the pre-synaptic neurons, ABHD6 could inactivate the excess 2-AG in post-synaptic regions (Fig. 4). In addition to 2-AG hydrolysis, MAGL is the main enzyme capable of degrading MAG into AA and glycerol via lipolysis. Interestingly, ABHD6 can hydrolyze MAG intracellularly (Blankman, 2007) and several reports have provided convincing evidence that ABHD6 and MAGL share similar functions but perform them in distinctive cellular locations (Thomas et al. 2013; Zhao et al. 2014; Zhao et al. 2015). ABHD6 seems to have a similar role as MAGL being a genuine MAG lipase and having a role as a 2-AG hydrolase in tissues lacking MAGL. The putative role of ABHD6 in regulating fuel homeostasis makes ABHD6 an extremely interesting enzyme and ensures that intensive research will continue around this enzyme.

Due to the challenges of developing enzyme activity assays, chemoproteomic probes have proved to be a powerful tool. The strength of chemoproteomic methods is their capability to explore protein families using a small molecule probe. ABPP has been used previously to discover and optimize inhibitors for serine hydrolases (Bachovchin and Cravatt 2012). Although the method is challenging to apply in an HTS format, we were able to expedite this procedure by using small-scale ABPP. Moreover, instead of exploring inhibitors against serine hydrolases in biological materials, inhibitors were screened for selected enzymes by pooling quantitatively equal amounts of over expressed enzymes. This approach was found to be successful and led to the discovery of three potent inhibitors for ABHD11.

Study III revealed the presence of active ABHD11 in many tissues including brain as well as an enrichment of active ABHD11 in the mitochondrial cell fraction, which is consistent with previous studies (Baggelaar et al. 2017; Forner et al. 2006; Lefort et al. 2009). Inhibition of ABHD11 showed no effect in mitochondrial respiratoy reactions, leaving unclear its physiological function in that organelle. We found a chromogenic substrate paranitrophenyl for ABHD11. Paranitrophenyl is a chromogenic substrate for lipases which we interpret as evidence favoring the putative role of ABHD11 to function as a lipase. For example, overexpression of YGR031W, which is the yeast homolog of ABHD11, was shown to decrease cellular TAG levels (Arya et al. 2017). Additionally, ABHD11 is known to have a lipase motif (Lord et al. 2013) further support for the hypothesis that ABHD11 functions as a lipase. Interestingly, ABHD11 has been detected in many cancers (Wiedl et al. 2011). Keeping in mind that cancer cells are capable of recruiting enzymes including lipases (Nomura and Cravatt 2013)

along with the suggested role ABHD11 as a lipase means that it is possible that ABHD11 could also be recruited by cancer cells.

In the post-genomic era, our challenge is to find physiological functions for the thousands of newly predicted proteins. Today, we have access to proteomic methods capable of analyzing large numbers of proteins (Chandramouli and Qian 2009). Nonetheless, most proteomic as well as genetic methods measure protein abundance and therefore provide only an indirect estimate of protein activity and no indication of the physiological function. Therefore, more sophisticated methods are needed. To partially meet this need, we devised an enzyme activity assay for the endocannabinoid hydrolases. The fluorescence-based glycerol assay has been further refined and optimized to measure the activity of ABHD16A (Savinainen et al., 2014). The usefulness of this fluorescence-based method has been reported also by Miller et al. (2016) who identified ABHD2 as a new player capable of hydrolyzing 2-AG. Additionally, the method was further modified for DAGL activity measurements (van der Wel et al. 2015). Finally, we adapted several chromogenic methods, and these proved to be suitable for assaying some poorly characterized enzymes and in this way, we discovered three potent inhibitors which can be used in further studies investigating ABHD11.

FINAL CONCLUSIONS:

These following conclusions have emerged from this study:

I. The first aim of this study was to explore regional regional CB1 receptor-mediated signaling in MAGL-KO mice brain. This aim was fulfilled and as a result we concluded that other hydrolases in addition to MAGL regulate 2-AG levels in brains of MAGL-KO mice.

II. The second aim was to characterize ABHD6 and ABHD12 by determining their substrate and inhibitor preferences in comparison with those of MAGL. To achieve this aim, we developed a novel and sensitive fluorescence-based method for measuring endocannabinoid hydrolase activity for various MAGs and prostaglandin glycerol esters. By using this method, we were able to conclude that *in vitro* three endocannabinoid hydrolases, ABHD6, ABHD12 and MAGL, are all genuine MAG lipases.

III. The third aim was to characterize ABHD11 and to apply activity-based approaches to discover inhibitors for this poorly-characterized enzyme. We adapted and optimized substrate-based activity assays for ABHD11 which were then further exploited in the discovery of novel inhibitors. By using this method, we discovered that ABHD11 readily utilized para-nitrophenyl butyrate (pNPC4), which we interpret as evidence that ABHD11 possesses lipase/esterase-type activity which could be exploited in the discovery of inhibitory compounds. Additionally, we discovered three potent inhibitors which can be used to further characterize ABHD11.

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