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Niina Aaltonen

Functional Autoradiography as a Pharmacological Approach for Studying G Protein-Coupled Lipid Receptor Signalling

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ABSTRACT

Lipids have recently been recognized as an important class of signalling molecules both in the central nervous system and the periphery. Bioactive lipids are produced by multistep enzymatic pathways from their membrane phospholipid precursors. After they exert their effect by activating their specific receptors, they are rapidly enzymatically degraded.

Lysophospholipids and endocannabinoids (eCBs) represent two groups of bioactive lipids. Lysophosphatidic acid (LPA) is a structurally simple lysophospholipid that mainly mediates its actions through six G protein-coupled receptors (GPCRs) ($LPA_{1–6}$). Endocannabinoids, such as anandamide and 2-arachidonoylglycerol (2-AG), are the body's natural agonists for the two GPCRs (CB_1 and CB_2) that also recognize Δ^9-tetrahydrocannabinol, the psychoactive component present in marijuana. Both LPA and eCBs are involved in the development and function of many organ systems as well as in the pathology of several serious diseases, such as atherosclerosis and cancer.

The main objectives of this study were to devise and optimize the methodology used in studying lipid-GPCR signalling and to characterize the enzymatic pathways responsible for lipid messenger synthesis and degradation. The principal method used in the current study was guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) autoradiography which is applied in a novel way to examine the enzymatic pathways that synthesize and degrade signalling lipids in brain sections.

In the first part of the study, the [^{35}S]GTPγS autoradiography method was characterized by mapping rat brain regions with prominent [^{35}S]GTPγS binding under basal conditions. A liquid chromatography-tandem mass spectrometric method was developed to permit the quantitative determination of LPA species from brain tissue samples. Further studies revealed that the enzymatic systems synthesizing and metabolizing lipid mediators were well preserved in rodent brain cryosections. When LPA/2-AG degradation was pharmacologically blocked, brain sections generated endogenous lipids which were able to activate their cognate GPCRs during the autoradiography incubations. It was concluded that lipid phosphate phosphatases (LPPs) degrade the signalling pool of LPA in brain sections but in addition to LPPs, there seems to be alternative phosphatases present in the brain that degrade LPA at the whole brain level. The CB_1 receptor-dependent G_i protein activity remained unaltered in several brain regions of diacylglycerol lipase (DAGL) deficient mice when compared to wild-type mice. Alternative enzymes in addition to DAGLs seem to be responsible for synthesizing 2-AG in brain sections. It appears that there are separate enzymes in the brain that synthesize/degrade the signalling and non-signalling lipid pools. Especially when combined with sensitive analytical methods, [^{35}S]GTPγS autoradiography represents a valuable tool for studying the life cycle of bioactive lipids.

National Library of Medicine Classification: QU 85.6, QU 93
Medical Subject Headings: Lipids; Receptors; Lysophosphatidic Acid; Lysophospholipids; Endocannabinoids; Guanosine 5′-O-(3-Thiotriphosphate)/metabolism; Enzyme inhibitors/pharmacology; Autoradiography; Tandem Mass Spectrometry; Brain/metabolism; Brain Mapping/methods; Animals

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TIIVISTELMÄ

Bioaktiiviset lipidit kuuluvat hormonien kaltaisiin välittäjäaineisiin keskushermostossa sekä muualla elimistössä. Bioaktiiviset lipidit tuotetaan monivaiheisten entsymaattisten reittien kautta lähtöaineinaan solukalvon fosfolipidit ja reseptorivälitteisen vaikutuksensa jälkeen ne hajotetaan entsymaattisesti.

Lysofosfolipidit ja endokannabinoidit muodostavat kaksi bioaktiivisten lipidien ryhmää. Lysofosfolipideihin kuuluva lysofosfatidihappo (LPA) välittää vaikutuksensa pääasiassa kuuden G-proteiinikytkentäisen reseptorin (LPA_{1-6}) kautta. Endokannabinoidit, kuten anandamidi ja 2-arakidonyyliglyseroli (2-AG), ovat elimistön kannabinoidireseptorien (CB_1 ja CB_2) luonnollisia ligandeja. Kannabinoidireseptorit välittävät myös kannabiksen psykoaktiivisia vaikutuksia. Sekä LPA että endokannabinoidit ovat osallisina monissa elimistön toiminnoissa ja häiriintynyt LPA:n ja endokannabinoidien signalointi liitetään useisiin sairauksiin, kuten ateroskleroosiin ja syöpään.

Bioaktiivisten lipidien toiminnan ymmärtämiseksi tarvitaan menetelmiä, joilla voidaan seurata niin lipidien synteesiä, reseptorivälitteistä signalointia kuin entsymaattista hajotustakin. Tässä väitöskirjatyössä kehitettiin ja optimoitiin menetelmiä lipidien elinkaaren tutkimiseen sekä selvennettiin entsymaattisia reittejä, jotka tuottavat ja hajottavat bioaktiivisia lipidejä aivokudoksessa. Pääasiallisena menetelmänä käytettiin guanosiini-5'-*O*-(3-[^{35}S]thio)-trifosfaatti ([^{35}S]GTPγS)-autoradiografiaa, jolla voidaan tutkia G-proteiinivälitteistä viestintää kudosleikkeissä. Tutkimuksessa menetelmää sovellettiin uudella tavalla entsyymitoiminnan tutkimiseen.

Tutkimuksessa [^{35}S]GTPγS-autoradiografiamenetelmää karakterisoitiin paikantamalla menetelmälle ominainen taustasitoutuminen rotan aivoleikkeissä. Lisäksi kehitettiin nestekromatografia-tandem-massaspektrometrimenetelmä LPA:n määrittämiseksi aivokudoksesta. Tutkimuksessa havaittiin, että estämällä LPA:a/2-AG:a hajottavien entsyymien toimintaa farmakologisilla entsyymi-inhibiittoreilla, endogeeninen lipidi kumuloituu aivoleikkeisiin saaden aikaan reseptoriaktivaation, joka voidaan havaita [^{35}S]GTPγS-autoradiografian avulla. Tutkimuksen perusteella lipidifosfaattifosfataasit hajottavat aivoleikkeissä reseptorivälitteisesti signaloivaa LPA:a, mutta aivokudoksessa toimii myös muita LPA:a hajottavia fosfataaseja. Diasyyliglyserolilipaasi (DAGL)-poistogeenisten hiirten aivoleikkeissä ei useimmilla aivoalueilla havaittu muutoksia CB_1-reseptorivälitteisessä G_i-proteiiniaktiivisuudessa verrattuna villityypin hiiriin. Lisäksi pääteltiin, että DAGL-riippumaton entsymaattinen aktiivisuus synnyttää 2-AG:a aivoleikkeissä. Aivoissa vaikuttaa olevan erillisiä entsymaattisia reittejä signaloivan ja ei-signaloivan lipidijoukon synteesiin/hajotukseen. Erityisesti yhdistettynä analyyttisiin menetelmiin, [^{35}S]GTPγS-autoradiografia tarjoaa mahdollisuuden seurata bioaktiivisten lipidien koko elinkaarta kudosleikkeissä.

Luokitus: QU 85.6, QU 93
Yleinen suomalainen asiasanasto: lipidit; reseptorit; lysofosfolipidit; lysofosfatidihappo; endokannabinoidit; entsyymit; inhibiittorit; funktionaalinen autoradiografia; aivot; koe-eläimet

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Kuopio, October 2013

Niina Aaltonen

List of the original publications

This dissertation is based on the following original publications:

I Aaltonen N, Palomäki VAB, Lecklin A, Laitinen JT. Neuroanatomical mapping of juvenile rat brain regions with prominent basal signal in [^{35}S]GTPγS autoradiography. *J Chem Neuroanat 35: 233-241, 2008.*

II Aaltonen N, Laitinen JT, Lehtonen M. Quantification of lysophosphatidic acids in rat brain tissue by liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr B 878: 1145-1152, 2010.*

III Aaltonen N, Lehtonen M, Varonen K, Arrufat Goterris G, Laitinen JT. Lipid phosphate phosphatase inhibitors locally amplify lysophosphatidic acid LPA_1 receptor signalling in rat brain cryosections without affecting global LPA degradation. *BMC Pharmacology 12:7, 2012.*

IV Aaltonen N, Riera Ribas C, Lehtonen M, Savinainen JR, Laitinen JT: Brain regional cannabinoid CB_1 receptor signalling and alternative enzymatic pathways for 2-arachidonoylglycerol generation in brain sections of diacylglycerol lipase deficient mice. *Eur J Pharm Sci 51:87-95, 2014.*

The publications were adapted with the permissions of the copyright owners. In addition, unpublished results are presented in Chapter 7.3.1.

Contents

Abbreviations

2-AG	2-arachidonoylglycerol
AA	arachidonic acid
ABHD	α/β-hydrolase domain-containing protein
AC	adenylate cyclase
ADP	adenosine diphosphate
AEA	*N*-arachidonoylethanolamide a.k.a anandamide
AGS	activator of G protein signalling
AlF_x^-	aluminium fluoride
ATP	adenosine triphosphate
ATX	autotaxin
BSA	bovine serum albumin
C1P	ceramide 1-phosphate
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COX	cyclooxygenase
DAG	diacylglycerol
DAGK	diacylglycerol kinase
DAGL	diacylglycerol lipase
Δ^9-THC	Δ^9-tetrahydrocannabinol
DFOM	deferoxamine mesylate
DMR	dynamic mass redistribution
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
DTT	dithiotreitol
eCB	endocannabinoid
Edg	endothelial differentiation gene
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
FAAH	fatty acid amide hydrolase
G protein	guanine nucleotide-binding protein
GABA	gamma-aminobutyric acid
GC	gas chromatography
GDP	guanosine diphosphate
GIP	GPCR interacting protein
GP	glycerol phosphate
GPAT	glycerophosphate acyltransferase
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
GTPγS	guanosine-5′-*O*-(3-thio)-triphosphate
[^{35}S]GTPγS	guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate
HPLC	high-performance liquid chromatography
IP_3	inositol-1,4,5-trisphosphate
IS	internal standard
KO	knockout

LC/MS	liquid chromatography/mass spectrometry
LC/MS/MS	liquid chromatography/ tandem mass spectrometry
LLOQ	lower limit of quantification
LOX	lipoxygenase
LPAAT	lysophosphatidic acid acyltransferase
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPI	lysophosphatidylinositol
LPL	lysophospholipase
LPP	lipid phosphate phosphatase
LPS	lysophosphatidylserine
MAFP	methylarachidonoylfluoro-phosphonate
MAG	monoacylglycerol
MAGK	monoacylglycerol kinase
MAGL	monoacylglycerol lipase
MRM	multiple reaction monitoring
MS	mass spectrometry
NaF	sodium fluoride
NAM	*N*-arachidonoylmaleimide
NAPE	*N*-acylphosphatidyl-ethanolamine
NArPE	*N*-arachidonoylphosphatidyl-ethanolamine
NEM	*N*-ethylmaleimide
NTE	neuropathy target esterase
PA	phosphatidic acid
PAF	platelet activating factor
PAP	phosphatidate phosphatase
PC	phosphatidylcholine
PHARC	polyneuropathy, hearing loss, ataxia, retinis pigmentosa, and cataract
P_i	inorganic phosphate
PIP_2	phosphoinositol-4,5-bisphosphate
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PPARγ	peroxisome proliferator-activated receptor γ
PRG	plasticity-related gene
QC	quality control
RGS	regulator of G protein signalling
RSD	relative standard deviation
S1P	sphingosine 1-phosphate
SPC	sphingosylphosphorylcholine
TEA	triethylamine
TGF	transforming growth factor
THL	tetrahydrolipstatin
TRPV1	transient receptor potential vanilloid type-1 receptor
WT	wild-type

1 Introduction

Cells are surrounded by the plasma membrane which is made up of a protein-enriched phospholipid bilayer. The plasma membrane acts as a kind of skin protecting the cell from substances from the outside environment but it also allows messages from outside the cell to be mediated into the cell. Lipids have traditionally been seen as structural components of cellular membranes or cellular energy sources without any informational functions. During last decades, however, lipids have been recognised as being an important class of signalling molecules in both the central nervous system (CNS) and the periphery. Several groups of these bioactive lipids act through specific G protein-coupled receptors (GPCRs). Under normal physiological conditions, the lifetime of bioactive lipids is tightly regulated. Bioactive lipids are produced by multistep enzymatic pathways, which are initiated by the de-esterification of membrane phospholipids. After the bioactive lipids have exerted their action by activating their specific receptors, they are rapidly enzymatically degraded.

Lysophospholipids and endocannabinoids (eCBs) represent two important groups of bioactive lipids. Lysophospholipids can be divided into lysoglycerophospholipids and sphingoid lipids. Lysoglycerophospholipids are simple lipids having three structural features: a 3-carbon backbone (glycerol), a single aliphatic hydrocarbon chain and a polar headgroup. Lysophosphatidic acid (LPA) is one of the best studied lysoglycerophospholipid. LPA mainly mediates its actions through six GPCRs ($LPA_{1–6}$). LPA generally evokes hormone- and growth factor-like responses and these are believed to be involved in the development and function of neural and vascular systems as well as in the function of immune and reproductive systems. Endocannabinoids are the body's natural agonists for the two GPCRs (CB_1 and CB_2) that also recognize Δ^9-tetrahydrocannabinol (Δ^9-THC), the psychoactive component present in marijuana. The two most extensively studied eCBs are anandamide and 2-arachidonoylglycerol (2-AG). In the CNS, eCBs are involved in neurogenesis as well as in cognition, emotional functions, regulation of food intake, and pain sensation. In the periphery, eCBs mediate cardiovascular, immune, metabolic, and reproductive functions.

Since both LPA and eCB signalling systems are involved in the development and function of several organ systems, it is not surprising that their dysregulated function would be associated with a variety of human diseases. Since lipid receptors are widely distributed in the body, use of exogenous receptor agonists and antagonists might induce side effects in other sites than their target organs. Another way to affect lipid functions would be the pharmacological manipulation of enzymes that synthesize and degrade these lipids. By inhibiting the activity of the synthesizing/degrading enzymes, one would predict that it would be possible to manipulate the levels of endogenous ligands and subsequent GPCR activity.

Before one can understand lipid signalling and subsequently how this knowledge can be exploited for drug discovery purposes, it is essential to have suitable methods to monitor each step of the life cycle of bioactive lipids, including biosynthesis, receptor signalling and enzymatic degradation. Guanosine-5'-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) autoradiography has been classically used to detect agonist-driven activity of the receptor-G protein axis in tissue sections. In the present study, [^{35}S]GTPγS autoradiography has been applied in studies of enzymatic pathways that synthesize and degrade signalling lipids in rodent brain sections. In brain sections, the receptor G protein-axis as well as enzymatic systems remain functional in the right anatomical context. Especially when combined with sensitive analytical methods, [^{35}S]GTPγS autoradiography represents a valuable tool for studying the regulation of lipid signalling.

2 *Review of the Literature*

2.1 G PROTEIN-MEDIATED SIGNALLING

2.1.1 The family of G protein-coupled receptors

In order to function properly, cells require a machinery that will permit the passage messages through the plasma membrane. The family of membrane-bound G protein-coupled receptors (GPCRs) constitutes one of the largest families of proteins coded by the mammalian genome (Fredriksson et al. 2003). GPCRs mediate the signal of a diverse set of endogenous ligands such as neurotransmitters, hormones and peptides but detect also signals of external origin, acting as sensory receptors for odorants, taste molecules and photons of light (Pierce et al. 2002, Maudsley et al. 2005). The total number of human GPCRs is estimated to be close to one thousand; about half of them are chemosensory receptors and one third (~ 370) represent receptors for endogenous ligands (endoGPCRs) (Vassilatis et al. 2003). GPCRs are ubiquitously expressed throughout the body but the majority of human GPCRs and endoGPCRs are expressed in the brain (Vassilatis et al. 2003).

GPCRs consist of seven α-helical transmembrane domains forming three interhelical loops on both sides of the membrane, an extracellular N-terminus, and an intracellular C-terminus (Latek et al. 2012). Due to the structure formed by a polypeptide passing through the plasma membrane seven times, GPCRs are often called seven-transmembrane receptors. One common classification (so-called GRAFS system) divides GPCRs into five groups, i.e. glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin (Fredriksson et al. 2003, Bjarnadottir et al. 2006). The rhodopsin family is the largest family of GPCRs and it includes receptors for odorants and endogenous small ligands. On the bases on sequence similarity, the rhodopsin family can be further divided into four subclasses (α, β, γ and δ) (Fredriksson et al. 2003). The subclasses not only differ between their preferred ligand but also in location of their ligand binding domain (Bjarnadottir et al. 2006). The crystal structures of 16 members of the rhodopsin family have been resolved (Stevens et al. 2013); the pioneering reports described the rhodopsin (Palczewski et al. 2000) and β_2-adrenergic receptors (Rasmussen et al. 2007, Cherezov et al. 2007).

At least one third, according to some estimations nearly half, of the currently marketed pharmaceutical drugs target GPCRs but there is still potential for drug companies to target GPCR signalling. There are receptors that exhibit the heptahelical conformation, the hallmark of the GPCRs, but for which there is no known natural ligand; these are called orphan receptors. Among the rhodopsin GPCR family, about 67 receptors remain classified as orphans and "de-orphanizing" these receptors would be an important goal for drug discovery purposes (Civelli et al. 2013).

2.1.2 Signalling via G proteins

The binding of the ligand induces a conformational change in the receptor protein allowing it to interact with other proteins. One feature defining GPCRs is their ability to interact with heterotrimeric guanine nucleotide-binding proteins (G proteins). Small, monomeric G proteins also exist but they do not couple to GPCRs (Csepanyi-Kömi et al. 2012).

Heterotrimeric G proteins consist of three subunits, α, β and γ. A substantial number of mammalian genes encode G protein subunits (16 genes for α subunit, 5 genes for β subunit, 12 genes for γ subunit) (Oldham & Hamm 2008). In the resting state, the α subunit of the G

protein binds guanosine diphosphate (GDP), and the Gα-GDP is tightly associated with the Gβγ-complex. After agonist ligand binding, the receptor adopts a conformation which allows it to interact with a G protein, resulting in the exchange of GDP for guanosine triphosphate (GTP) in the α subunit. As a consequence, the GTP-bound Gα dissociates from the Gβγ complex, enabling both subunit complexes to regulate a variety of effectors (Oldham & Hamm 2008, Tuteja 2009). The system returns to its resting state due to the activity of guanosine triphosphatase (GTPase) which is an intrinsic part of the α subunit; this enzyme hydrolyzes GTP back to GDP. The guanine nucleotide exchange cycle is presented in Figure 1.

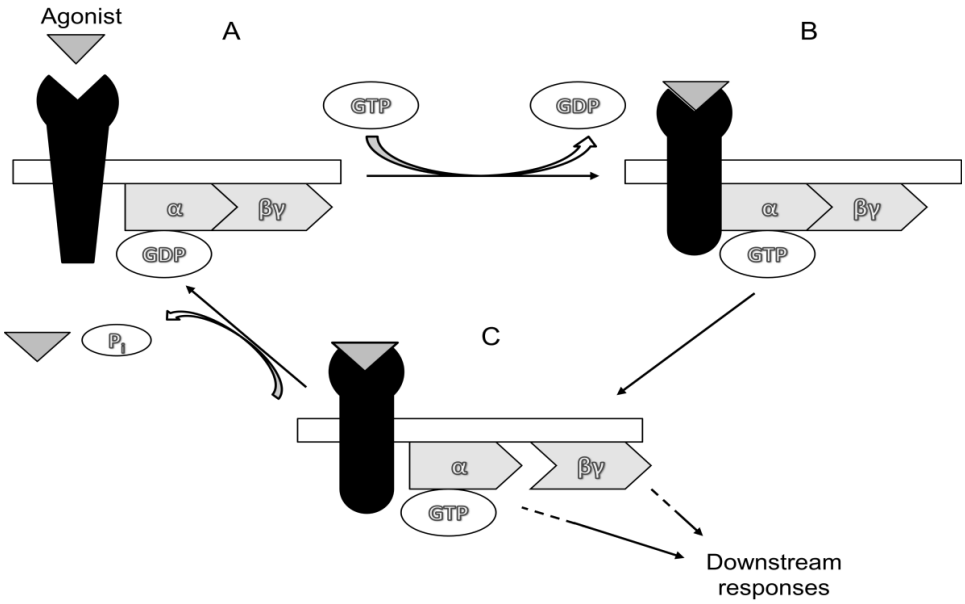


Figure 1. The guanine-nucleotide exchange cycle. (A) When agonist is not bound, the receptor is uncoupled from the G protein. (B) Binding of an agonist induces a conformational change in the receptor and this results in its coupling to the G protein. The G protein is activated and the bound GDP is exchanged for GTP. (C) The α subunit of the G protein dissociates from the βγ complex and both subunits interact with downstream signalling elements. The α subunit hydrolyses bound GTP back to GDP, agonist dissociates from the receptor and the system returns to the resting state (modified from Sovago et al. 2001).

Based on the amino acid sequence of the α subunits, G proteins can be divided into four subfamilies, G_i, G_s, G_q, and G_{12}, and the four Gα subfamilies can be further divided into subtypes (Figure 2) (Cabrera-Vera et al. 2003). In addition, five β subunits and twelve γ subunits have been identified (Malbon 2005). The majority of endoGPCRs couple to G_i type of G proteins (Wong 2003). The type of the α subunit determines the sunsequent downstream response (Malbon 2005). Classically, α_s activates and α_i inhibits adenylate cyclase. Adenylate cyclase converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) which serves as a second messenger that can activate protein kinase A and many other downstream effectors. The α_q class proteins activate phospholipase Cβ (PLCβ) that catalyzes the formation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) that regulate protein kinase C activity and intracellular calcium levels, respectively. The α_{12} class can stimulate Rho guanine nucleotide-exchange factors.

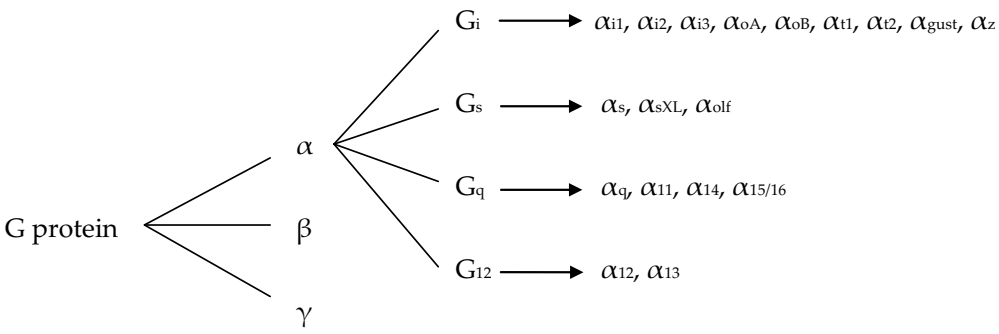


Figure 2. Illustration of subunits of heterotrimeric G proteins (based on Cabrera-Vera et al. 2003, Malbon 2005).

In addition to the heterotrimeric G proteins, GPCRs interact with other proteins, called GPCR interacting proteins (GIPs) (Bockaert et al. 2010). GIPs are believed to control GPCR subcellular localization and the fine-tuning of GPCR signalling. Desensitization is a mechanism to dampen GPCR signalling at the receptor level (Maudsley et al. 2005). This process starts with phosphorylation of either resting or agonist-stimulated receptors by kinases. β-Arrestins (type 1 and 2) are GIPs that bind to agonist-occupied, phosphorylated GPCRs (Shenoy et al. 2011, Shukla et al. 2011). β-Arrestins desensitize receptors by sterically preventing G protein coupling and also promote the internalization, endocytosis and recycling/degradation of GPCRs. In addition, β-arrestins can initiate signalling that is independent of G proteins via scaffolding signalling molecules in close proximity to an activated GPCR.

On the other hand, G proteins can be activated by other proteins distinct from GPCRs. These proteins, called activators of G protein signalling (AGS), can regulate heterotrimeric G protein signalling in the absence of GPCRs (Sato et al. 2006, Blumer et al. 2007). The third group of compounds, regulators of G protein signalling (RGS), can accelerate the GTPase activity of the G protein α-subunit and through this mechanism they can attenuate GPCR signalling (Neubig & Siderovski 2002). In summary, regulation of GPCR signalling is a complex process, in which several different proteins may be involved in addition to the classical cascade mediated by GPCR-heterotrimeric G protein axis.

2.1.3 Functional diversity of GPCR ligands

According to classical view, *agonist* ("key") is a molecule that binds to a receptor ("lock") and induces a conformational change in the receptor protein, which leads to stimulation of G protein activity. A more advanced receptor theory postulates that GPCRs exist in a dynamic equilibrium between inactive (R) and active (R*) states (Samama et al. 1993). According to this model, agonists shift the equilibrium toward the activated states. Agonists can be further divided into full and partial agonists; *full agonist* stabilizes the R* conformation and generates maximal response (full efficacy) whereas *partial agonists* have lower intrinsic efficacy, thus producing a sub-maximal response. Neutral *antagonists* bind to both R and R* and do not affect the basal equilibrium. They have no stimulating effect itself but they block agonists from binding. A constitutive receptor activity is a state where the receptors exist in their active conformation in the absence of any ligand (Lefkowitz et al. 1993). *Inverse agonists* bind preferentially to inactive states and decrease the level of constitutive activity (Samama et al. 1994). In nonconstitutively active systems, inverse agonists act as antagonists. So called *protean agonists* are ligands that act as partial agonists in some systems and as inverse agonists in others (Kenakin 2001).

Furthermore, the term *orthosteric ligand* refers to a ligand that binds to the natural ligand-binding site (orthosteric site) on the receptor and thus directly competes with the natural ligand for receptor binding (Kenakin & Miller 2010). In contrast, *allosteric ligands* are ligands that bind at a site different from the orthosteric binding site but they can still influence the functional properties of the receptor. *Bitopic ligands* have both orthosteric and allosteric properties.

The majority of the current drug molecules targeting GPCRs bind to an orthosteric site of the receptor. These drugs either activate receptor (agonists), block the binding of the natural agonist (neutral antagonists), or block constitutive receptor activity (inverse agonists). Another possible way in which a drug can influence GPCR function would be via so called allosteric modulation i.e. in that case the drug would either inhibit or potentiate an orthosteric ligand's binding affinity and/or modulate its signalling efficacy (Kenakin 2010). Allosteric modulators can also mediate receptor activation in their own right either via G proteins or in a G protein-independent manner (via β-arrestins). When compared to orthosteric ligands, allosteric modulators would provide a more selective effect e.g. they would act on only a certain receptor subtypes since they target unique regions of the receptor.

2.1.4 Lipids as ligands for GPCRs

Lipid-structured mediators can act in either an intercellular or intracellular manner. The intercellular lipid mediators include hormones and hormone-like signallig molecules that act via specific receptors, generally either via GPCRs or nuclear receptors (Shimizu 2009, Evans & Hutchinson 2010). Intracellular lipid mediators, instead, refer to the second messengers such as DAG and IP_3.

The first bioactive lipids that were recognized to signal via GPCRs were the cyclooxygenase (COX) products of arachidonic acid metabolism, the prostaglandins and thromboxane (Coleman et al. 1994). The prostaglandins and the COX enzymes are perhaps the most well-known and most widely utilized lipid targets; the classical COX-inhibitor, aspirin, has been on the market for more than 110 years. According to current knowledge, a number of bioactive lipids such as leukotrienes, prostanoids, platelet-activating factor, lysophospholipids, and endocannabinoids, act via GPCRs and regulate essential cellular functions and immune responses (Howlett 2005, Shimizu 2009) (Table 1). In addition, other lipids, such as bile acids and steroids as well as short and long chain fatty acids, have been reported to bind to GPCRs, but additional studies are still needed to confirm these lipid-GPCR interactions (Shimizu 2009).

After the cloning of the cannabinoid CB_1 receptor (Devane et al. 1988), several GPCRs for intercellular lipid mediators have been cloned. The classification of lipid GPCRs recognizes more than 30 receptors that belong to the rhodopsin family of GPCRs (Howlett 2005, Bäck et al. 2011, Ye et al. 2009, Brink et al. 2004, Woodward et al. 2011, Chun et al. 2010, Pertwee et al. 2010) (Table 1, Figure 3). A few specific families of lipid GPCRs have appeared; for example the endothelial differentiation gene (Edg) family consists of three receptors for lysophosphatidic acid ($LPA_{1–3}$) and five receptors for sphingosine 1-phosphate ($S1P_{1–5}$). Members of this family are 40% homologous with the cannabinoid receptors (CB_1 and CB_2) (Shimizu 2009). In addition to the identified lipid receptors, some of the GPCRs found in the phylogenetic tree still remain orphans (Figure 3).

Crystallography studies have revealed differences in the properties of GPCRs e.g. in the ligand binding pockets between different GPCR subfamilies, reflecting diversity of the endogenous ligands (Katritch et al. 2013, Rosen et al. 2013). Generally, the rhodopsin GPCR family bind their ligands from the extracellular milieu. At present, the crystal structure for one lipid GPCR, the $S1P_1$ receptor, has been resolved (Hanson et al. 2012). The structure of $S1P_1$ receptor reveals a unique configuration of the extracellular loops and the N terminus; the N terminus has a well-ordered α-helix on top of the receptor, which in conjuction with

the extracellular loops occludes access to the ligand binding pocket from the extracellular environment. Instead, there is a large gap between helices I and VII that provides direct lateral access of the ligand into the binding pocket from the lipid bilayer. Though the crystal structures of other lipid GPCRs have not been resolved, it is believed that also endogenous cannabinoids gain access to the cannabinoid receptors via the lipid bilayer (Hurst et al. 2010, Hurst et al. 2013).

Table 1. Lipid GPCRs, their endogenous ligands and primary biological functions (according to Howlett 2005, Bäck et al. 2011, Ye et al. 2009, Brink et al. 2004, Woodward et al. 2011, Chun et al. 2010, Pertwee et al. 2010).

Receptor	Endogenous ligands	Biological functions
Leukotriene and lipoxin		
$CysLT_1$, $CysLT_2$	Leucotrienes C_4, D_4 and E_4 (LTC_4, LTD_4 and LTE_4, respectively)	Inflammation, chemotaxis, immune regulation, smooth muscle contraction
BLT_1, BLT_2	Leucotriene B_4 (LTB_4)	
FPR_2/ALX	Lipoxin A_4 (LXA_4)	
Oxoeicosanoid		
5-oxo-ETE/OXE	5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), hydroperoxyeicosatetraenoic acid (5-HPETE), hydroxyeicosatetraenoic acid (5-HETE)	Chemotaxis
Prostanoids		
DP1, DP_2/$CRTH_2$	Prostaglandin D_2 (PGD_2)	Fever, pain, inflammation, vasodilatation
EP_{1-4}	Prostaglandin E_2 (PGE_2)	
FP	Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	
IP	Prostacyclin (PGI_2)	
TP	Thromboxane A_2 (TXA_2)	Platelet aggregation, vasoconstriction
Platelet-activating factor (PAF)		
PAF	PAF and PAF-like lipids	Inflammation, chemotaxis, platelet-activating mediator
Lysophospholipid		
LPA_{1-6}	Lysophosphatidic acid (LPA)	Cell proliferation, differentiation, migration, adhesion, morphogenesis
$S1P_{1-5}$	Sphingosine 1-phosphate (S1P)	
Cannabinoid		
CB_1, CB_2	2-arachidonoylglycerol (2-AG), anandamide	Brain function, immune regulation, analgesia

The life cycle of bioactive lipids can be divided into three main parts: synthesis from membrane phospholipids, receptor stimulation and rapid enzymatic hydrolysis. Traditionally, classical water-soluble neurotransmitters, hormones etc. are pre-synthesized and stored in vesicles before their release from the cell. Due to their hydrophobic nature, bioactive lipids are not stored in the vesicles but instead are produced locally only when needed, "on demand". After their biosynthesis and action on their specific receptors, bioactive lipids are usually degraded enzymatically. The synthesis, receptor activation and metabolism of lipid mediators are tightly regulated under normal physiological conditions, and enzyme and/or receptor dysfunction can lead to several disease states.

Pharmacological means to affect lipid GPCR function include not only exogenous receptor ligands/allosteric modulators but also compounds that target the enzymes that synthesize or degrade endogenous lipid ligands. Since lipids are synthesized on demand in

a site-dependent manner, pharmacological inhibition of the enzymes that degrade these lipids would be anticipated to lead to local accumulation of endogenous ligands and subsequent GPCR activation. This approach would make it possible to avoid the side-effects associated with exogenous agonists that target the receptors in all parts of the body. Conversely, inhibition of lipid synthesizing enzymes would decrease the levels of the endogenous agonist, and in this way, evoke a reduction in GPCR activity.

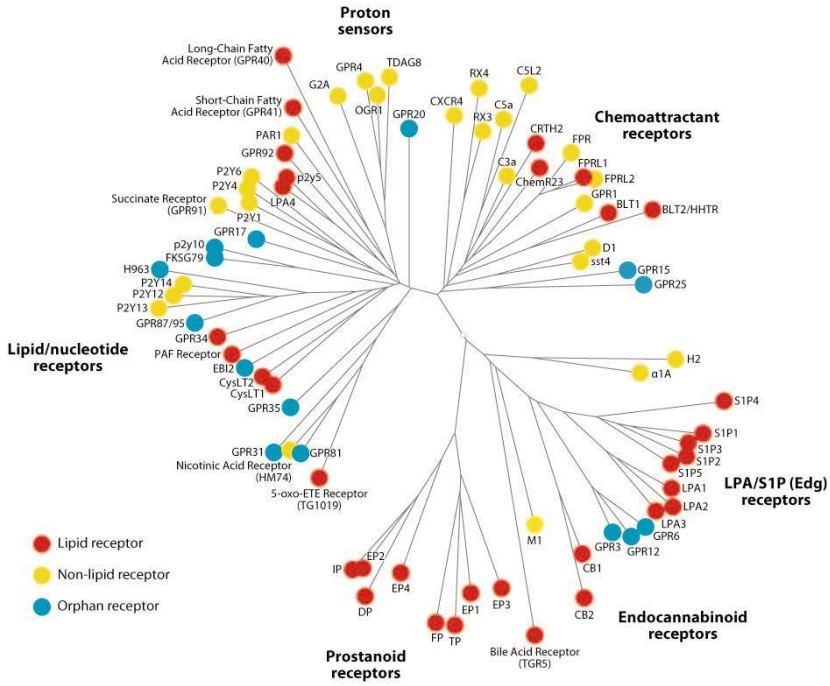


Figure 3. Phylogenetic tree of GPCRs including established lipid GPCRs and some orphan receptors (Reprinted from Shimizu 2009 with permission from Annual Reviews, Inc.).

2.2 OVERVIEW OF LYSOPHOSPHATIDIC ACID SIGNALLING

2.2.1 Definition of lysophospholipids

In the early 1900s, lysophospholipids were first recognized in a study investigating a snake venom that acted on lecithin (from Greek "lekithos" meaning egg yolk, later used as synonym for phosphatidylcholine (PC)) to produce lysolecithin, where the "lyso"-prefix referred to the hemolytic effect on red blood cells (Chun 2007). Lysophospholipids can be divided into lysoglycerophospholipids and sphingoid lipids. Lysoglycerophospholipids are enzymatically synthesized from membrane phospholipids and they display three structural features: a 3-carbon backbone, a single aliphatic hydrocarbon chain and a polar head group. A single carbon chain can vary in its length and saturation. Sphingoid lipids, instead, are synthetized by the sphingosine kinase-catalyzed phosphorylation of sphingosine (Pyne et al. 2009). Since lysophospholipids are rather simple in their structure they are able to interact with a diverse array of biomolecular targets, including both membrane and nuclear

receptors and enzymes (Parrill 2008). The two best characterized lysophospholipids are LPA and S1P (Figure 4). Other lysophospholipids include lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC), lysophosphatidylserine (LPS), lysophosphatidylethanolamine (LPE), and lysophosphatidylinositol (LPI) (Figure 4).

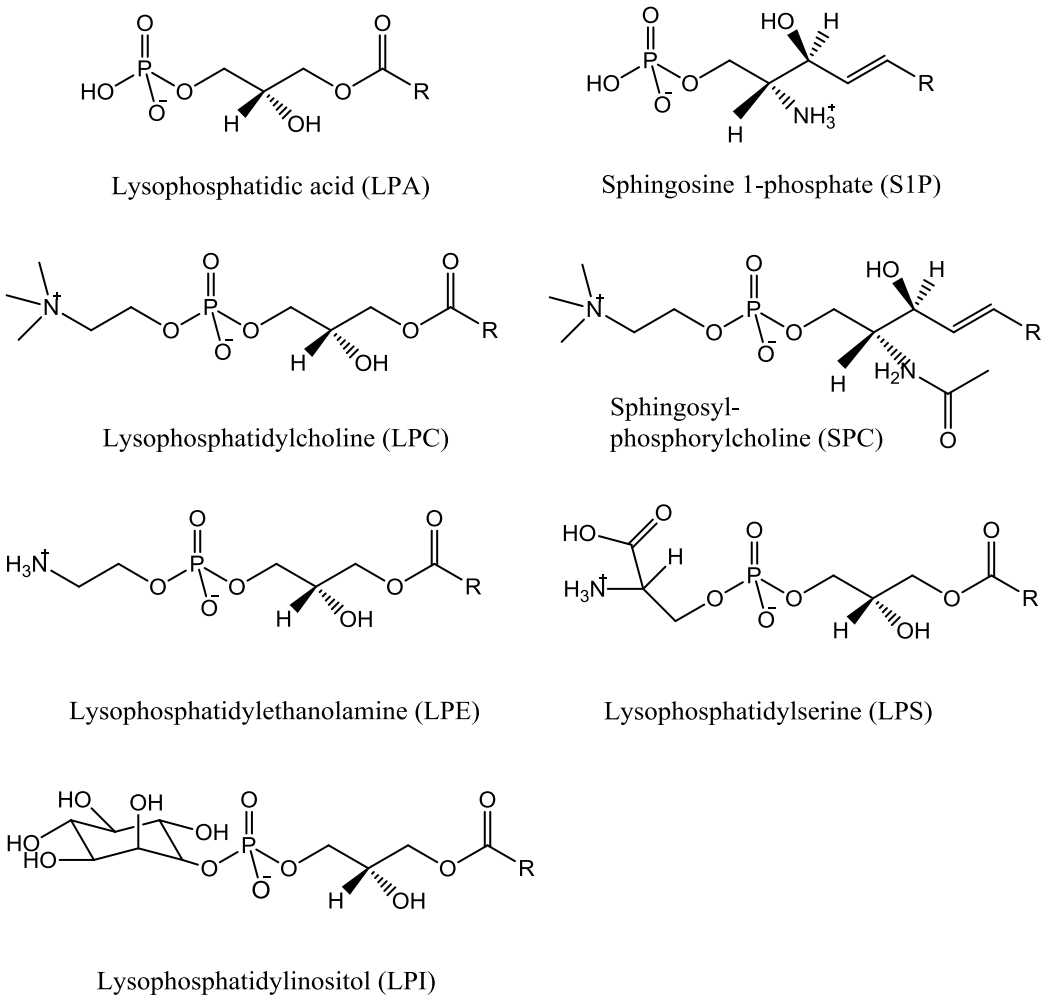


Figure 4. Chemical structures of lysophospholipids. For S1P and SPC, R = $(CH_2)_{12}CH_3$, for others, R = acyl with a variable chain length and unsaturation.

Lysophospholipids mediate the majority of their responses via specific GPCRs. There are currently 11 *bona fide* GPCRs identified for lysophospholipids ($LPA_{1–6}$ and $S1P_{1–5}$) and 10 receptor null-mice for lysophospholipid GPCRs have been described ($LPA_{1–5}$ and $S1P_{1–5}$) (Choi & Chun 2013). In addition to LPA and S1P receptors, there are potential receptors for other lysophospholipids among the large group of orphan GPCRs in the human genome. Orphan receptors G2A, GPR4, ORG1 and TDAG8 were first claimed to be activated by LPC, SPC and/or psychosine, but according to the current view, these receptors act as proton-sensing receptors and are not directly activated by lysophospholipids (Seuwen et al. 2006). Relatively strong evidence has been provided for the designation of GPR55 as an LPI receptor (Oka et al. 2007, Pertwee et al. 2010, Pineiro & Falasca 2012).

Lysophospholipid GPCRs are widely expressed both in the brain and the periphery. Lysophospholipids regulate a wide variety of cellular responses, being involved in the development of organ systems, such as nervous, vascular and reproductive systems, but they also enhance cancer growth and metastasis, inflammation and the development of atherosclerotic plaques (Mutoh et al. 2012). Their structure, wide expression pattern, and involvement in so many processes in the body make lysophospholipids attractive drug targets. The first drug on the market affecting lysophospholipid signalling is the immunosuppressive compound, fingolimod (FTY720, Gilenya®), that was approved by Food and Drug Administration in 2010 as the first oral therapy for multiple sclerosis (Brinkmann et al. 2010, Chun & Brinkmann 2011). In the body, fingolimod is phosphorylated by endogenous sphingosine kinases, resulting in the formation of the bioactive fingolimod-phosphate, which acts as an agonist for $S1P_1$ and $S1P_{3-5}$ receptors.

2.2.2 LPA and its physiological roles

Different molecular species of LPA exist *in vivo.* The acyl group of LPA can differ in length and its degree of unsaturation; there are saturated and mono- and poly-unsaturated variants of either *sn1* or *sn2* regioisomers. As a signalling molecule, the term LPA generally refers to 1-acyl-2-hydroxy-*sn*-3-phosphate (Figure 4).

It was long believed that the main source of LPA was blood. Early work indicated that LPA was present in serum and originated from activated platelets (Eichholtz et al. 1993). Currently it is known that in addition to blood, LPA can be found in other body fluids such as saliva (Sugiura et al. 2002), seminal plasma (Hama et al. 2002), and bronchoalveolar lavage fluid (Tager et al. 2008). The serum LPA is bound to albumin, gelsolin and other proteins which stabilize it in these hydrophilic environments and possibly protect it from rapid degradation (Tigyi & Miledi 1992, Goetzl et al. 2000). In addition to body fluids and platelets, several cell types, including adipocytes and ovarian cancer cells, can produce and release LPA (Mills et al. 2002, Federico et al. 2012).

LPA generally evokes hormone- and growth factor-like responses. Cells can respond in many different ways to LPA; LPA is most often associated with proliferative responses, but it also stimulates cell motility and migration, cytoskeletal reorganization, and process retraction (Moolenaar et al. 2004). Cellular migration plays central role in embryonic development and on the other hand, in the conversion of tumours so that they acquire an invasive and metastatic phenotype.

Significant amounts of LPA have been detected in the brain tissue (Sugiura et al. 1999, Nakane et al. 2002). In the brain, LPA has been identified in neural progenitors, primary neurons, oligodendrocytes, astrocytes, microglia, and brain endothelial cells, e.g. being involved in neurogenesis and myelination (Ye et al. 2002). In several types of primary neurons, LPA has been demonstrated to induce morphological changes, such as neurite retraction and growth cone collapse as well as to regulate migration, cell death/survival, synapse formation, and synaptic transmission (Ye et al. 2002, Pilpel & Segal 2006, Choi & Chun 2013).

2.2.3 LPA receptors

Early work indicated that LPA was a constituent of a mysterious smooth muscle-stimulating substance, Darmstoff (Vogt 1963). Further studies suggested that LPA could be involved in the regulation of blood pressure but the mechanism of this action was not clear (Sen et al. 1968, Tokumura et al. 1978). Later, signalling cascades mediated by LPA were shown to involve G proteins (van Corven et al. 1989). Currently, six LPA receptors belonging to the rhodopsin GPCR family have been identified (Table 2). The first LPA receptor was cloned in 1996 from the ventricular zone of the developing mouse cerebral cortex and originally named the ventricular zone gene-1 (Vzg-1) (Hecht et al. 1996). This

receptor, currently called LPA_1, belongs to the Edg family. In addition to LPA_1, two other Edg members (LPA_2, LPA_3) have been described (An et al. 1998, Bandoh et al. 1999). The $LPA_{1–3}$ receptors share 50–57% amino acid identity with each other. In addition to the Edg family, another non-Edg group of GPCRs has been claimed to act as LPA receptors ($LPA_{4–6}$) (Noguchi et al. 2003, Kotarsky et al. 2006, Lee et al. 2006, Pasternack et al. 2008, Lee et al. 2009). $LPA_{4–6}$ are closely related to the subfamily of P2Y purinergic receptors and share only 20–24% amino acid identity with $LPA_{1–3}$. Evidently, LPA receptors have evolved via two distinct lineages. Additional GPCRs for LPA have also been putatively described in the literature (Tabata et al. 2007, Murakami et al. 2008, Oka et al. 2010) but further studies will be needed to clarify if these receptors truly mediate the biological effects of LPA.

In addition to G protein-mediated pathways, LPA-stimulated GPCR activation can lead to activation of nuclear factor-κB pathway e.g. via β-arrestins (Sun & Yang 2010). LPA has also been demonstrated to activate the peroxisome proliferator-activated receptor γ (PPARγ) (McIntyre et al. 2003). The PPARγ acts as a transcription factor e.g. controlling genes that are involved in glucose and fatty acid metabolism and in adipocyte differentiation. LPA has been reported to displace the full agonist, the antidiabetic agent, rosiglitazone, from PPARγ (Parrill 2008). The relevance of LPA–PPARγ signalling still remains somewhat controversial.

Table 2. Confirmed GPCRs for LPA, their expression patterns and signalling pathways.

Receptor	Other names	Primary expression loci in mice	G protein coupling	Downstream responses	References
LPA_1	Edg2, Vzg-1	Brain, uterus, testis, lung, small intestine, heart, stomach, kidney, spleen, thymus, placenta, skeletal muscle	G_i, G_q, G_{12}	Inhibition of AC, activation of Ras, PI3K, PLC, Rho	Hecht et al. 1996, Choi et al. 2010
LPA_2	Edg4	Kidney, uterus, testis, lung, stomach, spleen, thymus, brain, heart	G_i, G_q, G_{12}	Inhibition of AC, activation of Ras, PI3K, PLC, Rho	An et al. 1998, Choi et al. 2010
LPA_3	Edg7	Testis, kidney, lung, small intestine, heart, stomach, spleen, brain, thymus	G_i, G_q	Inhibition of AC, activation of Ras, PI3K, PLC	Bandoh et al. 1999, Choi et al. 2010
LPA_4	GPR23, P2Y9	Heart, skin, thymus, ovary, developing brain, embryonic fibroblasts	G_i, G_q, G_{12}, G_s	Inhibition of AC activation of AC, Ras, PI3K, PLC, Rho	Noguchi et al. 2003, Lee et al. 2007, Choi et al. 2010
LPA_5	GPR92	Widely expressed, such as embryonic brain, small intestine, skin, spleen, stomach, thymus, lung, heart, liver, embryonic stem cells	G_q, G_{12}	Activation of PLC and Rho	Kotarsky et al. 2006, Lee et al. 2006, Choi et al. 2010
LPA_6	P2Y5	In humans: hair follicle	G_i, G_s, G_{12}	Inhibition of AC, activation of AC, Ras, PI3K, PLC, Rho	Lee et al. 2009, Pasternack et al. 2008

Abbreviations: AC, adenylate cyclase; PI3K, phosphatidylinositol-3-kinase; PLC, phospholipase C.

Each LPA GPCR displays a distinct ligand selectivity profile for the various LPA species, e.g. LPA_3 is more potently activated by an LPA with an acyl chain at the *sn*-2 position (Bandoh et al. 2000). Generally, LPAs with unsaturated fatty acids are more potent in activating LPA receptors than LPAs with saturated fatty acids (Bandoh et al. 2000, Fujiwara et al. 2005). LPA signalling is complex due to the large number of LPA species, receptors and signalling partners. Furthermore, different cells can express different receptors but some cells and tissues express several different LPA receptor subtypes (Moolenaar et al.

2004). The LPA-induced response largely depends on the cell in question, e.g. LPA increases the survival of certain cancer cells by protecting them from apoptosis (Meng et al. 2005), but on the other hand, it has been reported to promote apoptosis of hippocampal neurons (Ye et al. 2002).

All the six identified LPA receptors are expressed at varying levels in the different types of cells in the CNS, especially during embryonic development and/or postnatal life. The principal LPA receptor in the brain is LPA_1, and the major site of its expression is the white matter tracts of the developing nervous system (Choi et al. 2010). During embryonic stage, the LPA_1 receptor is highly expressed in the ventricular zone where the neural progenitor cells are located, playing a role in cortical development. During postnatal life, LPA_1 is located within oligodendrocytes, the myelinating cells of the CNS, as demonstrated by *in situ* hybridization (Weiner et al. 1998, Stankoff et al. 2002) and immunohistochemistry (Handford et al. 2001, Cervera et al. 2002). LPA_1 is also expressed in astrocytes, microglia, and neurons. In mice, LPA_1 expression peaks at 3–5 weeks after birth and diminishes thereafter (Contos & Chun 2001). In the healthy adult mouse nervous system, the LPA_1 receptor is weakly expressed but is upregulated following spinal cord or brain injury (Goldshmit et al. 2010).

Knockout (KO) mice for five LPA receptors ($LPA_{1–5}$) have been reported. In the LPA_1-KO, the brain development was disturbed as evidenced by a smaller brain size with reduced cortical width and cerebral wall thickness (Contos et al. 2000). Combined with a defect in the suckling behaviour due to impaired olfaction, these disabilities resulted in 50% neonatal lethality. In the LPA_1 receptor knockout mice, altered levels of neurotransmitters (serotonin, gamma-aminobutyric acid (GABA), glutamate) have been detected, indicating that LPA might have a role in modulating synaptic transmission (Harrison et al. 2003, Roberts et al. 2005). LPA_3-deficient female mice showed delayed embryo implantation, altered embryo spacing, and reduced litter size (Ye et al. 2005). Defects in prostaglandin levels were also observed indicating co-operation between the LPA_3 receptor and prostaglandin signalling in the embryo implantation. LPA_2 (Contos et al. 2002) and LPA_5 (Lin et al. 2012) knockout mice were born normally and displayed no phenotypic abnormalities. Conflicting data appear with LPA_4 knockouts; a normal phenotype was originally reported (Lee et al. 2008) but later, defects in blood vessel and lymphatic vessel formation have been observed (Sumida et al. 2010).

2.2.4 Biosynthesis of LPA

Biosynthesis of LPA occurs through multi-step enzymatic pathways. LPA is generated locally in specific tissues, both intracellularly and extracellularly. It is postulated that intracellular LPA mainly acts as an intermediate for phospholipid synthesis whereas extracellular LPA mediates signalling functions (Okudaira et al. 2010). Two main routes for LPA production have been postulated in the literature; in the first pathway, LPA is produced from lysophospholipids by autotaxin (ATX) and in the second pathway by deacylation of phosphatidic acid (PA).

LPA synthesis by autotaxin

Extracellularly, LPA is generated from lysoglycerophospholipids, such as LPC, by enzymatic removal of the polar headgroup (Figure 5). The principal enzyme for the generation of circulating LPA has been claimed to be ATX. ATX was originally detected as an autocrine motility factor isolated from the conditioned medium of cancer cells (Stracke et al. 1992). Ten years later, ATX was observed to act as a secreted lysophospholipase D (Umezu-Goto et al. 2002, Tokomura et al. 2002). Structurally ATX belongs to the nucleotide pyrophosphate/phosphodiesterase family of enzymes, which hydrolyse pyrophosphate or

phosphodiester bonds of several molecules, typically ATP and adenosine diphosphate (ADP). The main substrate for ATX is LPC, but ATX can also hydrolyse SPC to S1P (Clair et al. 2003). ATX does not hydrolyse compounds with a double acyl chain, e.g. PC, and therefore it is not identical in action to classical phospholipase D (PLD). The ATX–LPA axis shows feedback inhibition as the biosynthesis product LPA inhibits ATX activity (van Meeteren et al. 2005). ATX is activated by divalent cations such as Co^{2+}, Zn^{2+}, Mn^{2+}, and Ni^{2+} (Lee et al. 2001).

ATX is abundant in the blood and other biological fluids, and is also expressed in the brain as well as in the kidney and lymphoid organs (Okudaira et al. 2010). Several findings suggest that ATX is the main enzyme generating circulating LPA. It has been reported that plasma LPA levels correlate with the serum ATX activity (Hosogaya et al. 2008). Depletion of ATX completely prevents LPA production in the serum (Tanaka et al. 2006, Tsuda et al. 2006) whereas ATX overexpression in transgenic mice increases LPA plasma levels (Pamuklar et al. 2009). Heterozygous ATX-knockouts develop normally but have 50% lower plasma LPA concentrations when compared to the corresponding wild-type mice (Tanaka et al. 2006). Importantly, the homozygous ATX knockout in mice is embryonically lethal, due to the failure in the maturation of vessels in autotaxin-null embryos, indicating that the ATX–LPA axis is an essential component in the blood vessel formation during development (Tanaka et al. 2006). LPA receptor knockout animals show less severe phenotype than ATX knockout animals, which may reflect that ATX also acts on LPA-independent pathways or via yet unidentified LPA receptors. Crystal structures of ATX as well as the ATX–LPA complex have been resolved (Nishimasu et al. 2011, Hausmann et al. 2011). It has been postulated that in addition to generating LPA, ATX might behave as a lipid-carrier protein that transfers LPA directly to the LPA receptors (Nishimasu et al. 2011).

Other pathways for LPA generation

In the intracellular milieu or on the plasma membrane, LPA is mainly generated via a pathway in which PA is first generated from phospholipids or from DAG and then deacylated by phospholipase A_1/A_2 (PLA_1/PLA_2, respectively) (Figure 5). PLA_1 produces 2-acyl-lysophospholipids whereas PLA_2 produces 1-acyl-lysophospholipids and is also involved in the production of prostaglandins and the PAF (Aoki et al. 2008). Additional pathways for LPA generation include acylation of glycerol 3-phosphate by glycerophosphate acyltransferase (GPAT) or phosphorylation of monoacylglycerol (MAG) by monoacylglycerol kinase (MAGK) (Tigyi & Parrill 2003, Moolenaar et al. 2004, Bektas et al. 2005, Okudaira et al. 2010) (Figure 5).

The intracellular LPA synthesis occurs in the endoplasmic reticulum or in mitochondria. Intracellularly generated LPA likely serves as a precursor for glycerophospholipid synthesis rather than for signalling purposes. Intracellularly produced LPA might play a role in activating the nuclear receptor PPARγ (Zhang et al. 2004). The biosynthetic and degradative pathways for LPA have also been detected in the nuclear membrane and/or within the nucleus of cells (Baker & Chang 1999, Baker & Chang 2000). Nuclear localization may protect LPA from the otherwise rapid degradation.

There are several isozymes for both PLA_1 and PLA_2. Among PLA_1 isozymes, membrane-bound PA-selective $PLA_1\alpha$ and β (mPA-$PLA_1\alpha$ and β), also called LIPH and LIPI, respectively, hydrolyse PA producing 2-acyl-lysophosphatidylserine and 2-acyl-LPA, respectively (Aoki 2004, Aoki et al. 2008). Interestingly, evidence has been provided that mPA-$PLA_1\alpha$/LIPH is expressed in hair follicles. Deletion in the gene encoding mPA-$PLA_1\alpha$/LIPH cause hair growth defects in humans (Kazantseva et al. 2006). Defects in hair growth have also been detected when the gene encoding the LPA_6 receptor is disrupted (Pasternack et al. 2008, Shimomura et al. 2008). These observations and the co-localization of $PLA_1\alpha$/LIPH and the LPA_6 receptor in hair follicles indicate that LPA produced by mPA-

$PLA_1\alpha$/LIPH and signalling via the LPA_6 receptor plays a role in the regulation of hair growth (Inoue et al. 2011).

Pharmacological inhibition of LPA generation

At present, the development of pharmacological inhibitors of LPA-generating enzymes has focused on ATX. A few pharmacological inhibitors of ATX activity have been described including lipid analogs (e.g. cyclic phosphatidic acids), metal chelators (L-histidine, ethylenediaminetetraacetic acid (EDTA)) and nonlipid small molecules (Jankowski 2011). One of the first small molecular inhibitors is S32826 that inhibits ATX in nanomolar range, shows ATX inhibition in cellular models but is unstable *in vivo* (Ferry et al. 2008). More recently, thiazolidinediones with a boronic acid moiety (e.g. HA130 and HA155) (Albers et al. 2010, Albers et al. 2011) as well as compound PF-8380 (Gierse et al. 2010) were reported to inhibit ATX in nanomolar range and to reduce LPA levels *in vivo*.

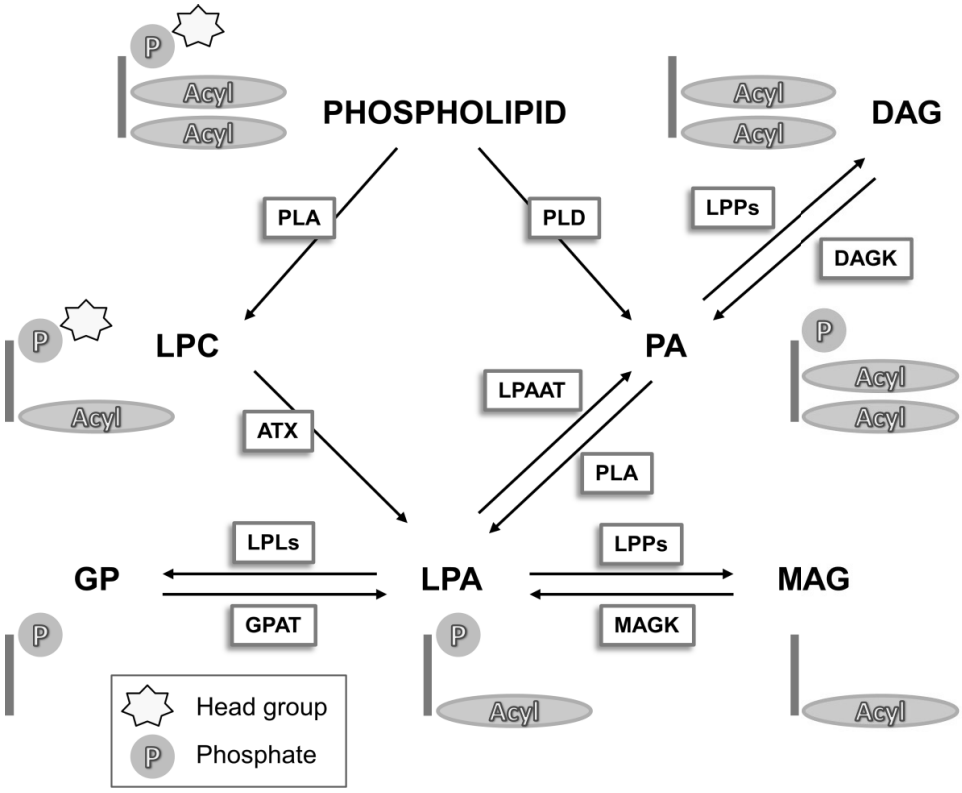


Figure 5. Enzymatic pathways for the biosynthesis and degradation of LPA. Abbreviations: ATX, autotaxin; DAG, diacylglycerol; DAGK, diacylglycerol kinase; GP, glycerol phosphate; GPAT, glycerophosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPL, lysophospholipase; LPP, lipid phosphate phosphatase; MAG, monoacylglycerol; MAGK, monoacylglycerol kinase; PA, phosphatidic acid; PLA, phospholipase A; PLD, phospholipase D (Based on Okudaira et al. 2010).

2.2.5 Enzymatic degradation of LPA

The lifetime of the signalling pool of LPA is thought to be controlled by enzymatic degradation. Although the degradation terminates LPA signalling as such, the degradation products can be used as precursors for other signalling lipids. The principal pathway for LPA degradation is believed to be enzymatic removal of the phosphate group producing MAG (Figure 5).

Dephosphorylation via lipid phosphate phosphatases

LPA is rapidly dephosphorylated by a family of integral membrane proteins known as lipid phosphate phosphatases (LPPs), originally named PAP2 (Pyne et al. 2004, Sigal et al. 2005, Brindley & Pilquil 2009). The PAP1 family of phosphatases (also known as lipins), instead, selectively dephosphorylate PA during lipid biosynthesis (Brindley et al. 2009). LPPs belong to a larger family of proteins that include sphingosine phosphate phosphatases, sphingomyelin synthases as well as other less well characterized proteins (Sigal et al. 2005). In addition to LPA, LPP substrates include PA, S1P, and ceramide 1-phosphate (C1P), producing diacylglycerol, sphingosine, and ceramide (Waggoner et al. 1996).

The LPP family comprises four members (LPP1, LPP2, LPP3 and the splice variant LPP1a) (Waggoner et al. 1996, Kai et al. 1997, Roberts et al. 1998). It has been demonstrated that LPP1 and LPP3 hydrolyse LPA and PA more effectively than C1P or S1P, whereas LPP2 most readily hydrolyses LPA and S1P (Pyne et al. 2004). LPP1 and LPP3 are widely expressed in human tissues whereas LPP2 expression is more restricted (Sigal et al. 2005). All the enzyme subtypes are expressed in the brain, with LPP1 and LPP3 being the most extensive (Kai et al. 1997, Hooks et al. 1998, Sigal 2005). At the subcellular level, LPPs have been localized to the endoplasmic reticulum and plasma membrane, and possibly to other endomembrane compartments, such as the Golgi apparatus. LPPs are characterized by six membrane spanning domains with three extracellular loops with the active site facing the extracellular side of the plasma membrane or the luminal side of intracellular membranes (Zhang et al. 2000b). The hallmarks of the LPP activity are that it does not require Mg^{2+} and it is resistant to the alkylating agent, *N*-ethylmaleimide (NEM) (Jamal et al. 1991).

There is evidence to indicate that LPP-catalysed degradation of LPA can regulate LPA signalling. For example, over-expression of LPP3 in ovarian cancer cells reduced tumour growth *in vitro* and *in vivo* (Tanyi et al. 2003a). LPP activity decreased LPA accumulation in platelets (Smyth et al. 2003) and in pre-adipocytes (Simon et al. 2002). Knockout studies of all the LPP subtypes have been described. A study with LPP1-KO mice indicated that LPP1 plays a role in regulating the degradation of circulating LPA *in vivo* (Tomsig et al. 2009). However, in that study there was no disruption of the LPP1 encoding gene in the brain, a phenomenom for which the authours could not supply a plausible explanation. In fact, LPP1 knockout mice showed a normal phenotype. In contrast, the knockout of LPP3 proved to be embryonically lethal (Escalante-Alcade et al. 2003). *In vitro* studies using cell lines lacking LPP3 link LPP3 to early neural development (Sanchez-Sanchez 2012). LPP2 knockout mice exhibit no obvious phenotypic defects (Zhang et al. 2000a).

Plasticity-related genes (PRGs) are a group of recently identified, brain-specific membrane proteins that were originally proposed to act as LPA phosphatases (Bräuer & Nitsch 2008, Strauss & Bräuer 2013). Among the family of PRGs, PRG-1 shares close homology to the LPPs, having three conserved integral membrane domains facing the extracellular side of the plasma membrane, a feature that enables LPPs to dephosphorylate their lipid substrates. However, the catalytic residues responsible for LPP activity are not fully conserved in PRGs (Bräuer & Nitsch 2008) suggesting that PRGs might not act as LPA phosphatases. Instead, PGR-1 was recently demonstrated to be active at the postsynaptic side of the excitatory glutamatergic synapse where it may mediate the uptake of bioactive lipids (Trimbuch et al. 2009). PRG-1 was found to effectively control the levels of LPA in the

synapse though its mechanism of action seems to be more transporter-like than dephosphorylating.

Other pathways for LPA degradation

In comparison to dephosphorylation, the other pathways for LPA metabolism are less well characterized. The other known pathways include the *de novo* formation of PA by lysophosphatidic acid acyltransferase (LPAAT) and the lysophospholipase-catalyzed hydrolysis of the acyl group to form glycerol phosphate (GP) (Tigyi & Parrill 2003) (Figure 5). The LPAAT enzymes transfer an acyl group from acyl-coenzyme A to LPA to form PA. Several members of the LPAAT family have been sequenced including both transmembrane and membrane associated proteins (Tigyi & Parrill 2003). A majority of the characterized lysophospholipases act on LPC. Neuropathy target esterase (NTE) is a membrane-bound serine hydrolase that is localized within neurons and it has been shown to act as a lysophospholipase, hydrolyzing LPC (Quistad et al. 2003, van Tienhoven et al. 2002, Vose et al. 2008). The NTE-related esterase was reported to hydrolyze both LPC and LPA (Kienesberger et al. 2008). However, very little is known about the role of these enzymes *in vivo*, especially the possibilities to act as regulators of LPA receptor signalling.

Pharmacological inhibition of LPA degradation

Thus far, only a few pharmacological inhibitors for LPA degradation have been described. General phosphatase inhibitors such as vanadate have been used to inhibit LPPs (English et al. 1999, Simon et al. 2002, Gaveglio et al. 2011). Increased LPA levels were observed in vanadate-treated adipocytes when compared to control cells (Simon et al. 2002). Propranolol, better known as a β-adrenoceptor blocking agent, only modestly inhibits LPPs (Roberts et al. 1998, Holinstat et al. 2007). Fluorophosphonate analogs of PA have been claimed to specifically inhibit LPP1 (Smyth et al. 2003).

2.2.6 LPA signalling as a therapeutic target

Direct activation/inhibition of LPA receptors

LPA signalling regulates several functions in the body, and it has been reported to be involved in the pathology of several common diseases (Table 3). LPA plays both positive and negative roles in different disease processes and depending on the expression of the different receptors in various cell types, LPA may exert either a protective or detrimental effect (Table 3). Currently, there are no drugs on the market targeting LPA signalling (Gotoh et al. 2012). The diversity of LPA signalling makes the LPA-targeting drug discovery challenging; there are several LPA species that activate several LPA receptor subtypes (and additional receptors will likely be identified in the future) that are widely distributed in tissues and act on overlapping targets. First, one needs to decide whether enhancing or reducing LPA activity is needed to achieve a therapeutic response, and tissue-selectivity should be obtained to avoid harmful side effects. Generally, the inhibition of LPA signalling might be beneficial in cancer and in atherosclerosis as well as in the treatment of neuropathic pain and obesity. In contrast, LPA agonism might be beneficial in schizophrenia and reproductive disorders.

Several LPA receptor agonists or antagonists have been reported, but most show only modest selectivity and a relative lack of *in vivo* validation (Tamaryua et al. 2004, Mutoh et al. 2012). Due to the large number of LPA receptors, it is difficult to achieve selectivity for a certain subtype; moreover, there are few cell types where only one LPA receptor subtype is endogenously expressed. Some promising results have been obtained with the $LPA_{1/3}$ receptor-selective antagonist, VPC-12249, which conferred protection against the renal

ischemia-reperfusion injury in a mouse model (Okusa et al. 2003). Another $LPA_{1/3}$ receptor-selective antagonist, Ki16425 (Ohta et al. 2003), was able to inhibit breast cancer cell proliferation and metastasis in mice (Boucharaba et al. 2006) and it also blocked nerve injury-induced neuropathic pain (Ma et al. 2009). Recently, compound AM095 was reported to be an orally bioavailable, LPA_1 receptor-selective antagonist (200-fold selectivity over LPA_1 versus LPA_3), showing anti-fibrotic properties in rodents. It is currently in preclinical development to treat lung and skin fibrosis (Swaney et al. 2011).

Table 3. Examples of alterations in LPA signalling during diseases.

Disease	**Examples of reported effects**
CNS diseases	
Alzheimer's disease	Hallmarks of Alzheimer's disease include extracellular accumulation of β-amyloid peptide and hyperphosphorylated Tau protein in brain (Minati et al. 2009). LPA induces increased production of β-amyloid in mouse neuroblastoma cells (Shi et al. 2013). Increased Tau protein phosphorylation has been detected in human neuroblastoma cells during LPA-induced neurite retraction (Sayas et al. 1999).
Nerve injury-induced conditions	LPA in the blood may enter the brain during CNS injury and evoke pathological events. Upregulation of LPA receptors has been observed in injured tissues and in reactive astrocytes in mice (Goldshmit et al. 2010) and post-mortem human brain (Frugier et al. 2011). The hemorrhagic brain injury results in the elevations of LPA levels in the cerebrospinal fluid in piglets (Tigyi et al. 1995).
Neuropathic pain	LPA plays a role in initiating neuropathic pain (Ueda 2011). LPA_5 receptor knockout mice are protected from neuropathic pain (Lin et al. 2012). LPA causes activation of peripheral nociceptor endings (Renbäck et al. 2000) and induces demyelination, an aspect of chronic neuropathic pain, through the ATX–LPA1 receptor axis in mice (Nagai et al. 2010).
Schizophrenia	LPA_1 knockout mice strains show a schizophrenia-like phenotype (Harrison et al. 2003, Roberts et al. 2005).
Other diseases	
Atherosclerosis	LPA promotes the development of intimal hyperplasia by inducing platelet activation and is found in the lipid-rich core of atherosclerotic plaques in humans and rodents (Siess et al. 1999, Smyth et al. 2008, Bot et al. 2013). In atherosclerotic lesions, LPA induces endothelial dysfunction, monocyte attraction and adhesion, low density lipoprotein uptake, and proinflammatory cytokine release (Schober & Siess 2012). On the other hand, LPA seems to play a protective role under ischemic conditions by protecting rat mesenchymal stem cells from hypoxia-induced apoptosis (Chen et al. 2008, Liu et al. 2009a).
Airway disease	In mouse models, exogenous LPA promotes inflammatory responses in lung epithelial cells and endogenous LPA levels in the bronchoalveolar lavage (BAL) increase in lung inflammatory diseases, such as asthma, fibrosis and acute lung injury (Zhao & Natarajan 2013).
Cancer	LPA accumulation has been identified in the ascites and blood of ovarian cancer patients (Xu et al. 1998, Yoon et al. 2003). LPA stimulates ovarian cancer cell proliferation and protects them from apoptosis (Xu et al. 1995, Meng et al. 2005, Ren et al. 2006). LPA regulates ovarian cancer cell motility and metastasis (Boucharaba et al. 2006). LPA is involved also in other cancers including breast, gastric, colon, prostate, pancreas, liver, and brain (glioma) (Lin et al. 2010). LPA has angiogenic properties in breast cancer (Boucharaba et al. 2009).
Fibrosis	LPA_1 receptor signalling has been implicated in the development of renal, pulmonary and liver fibrosis (Pradere et al. 2008). In patients with pulmonary fibrosis, LPA levels are elevated in BAL, and LPA_1 is highly expressed in lung fibroblasts (Tager et al. 2008).
Ischemic stroke	LPA levels in plasma are increased in patients with ischemic cerebrovascular disease (Li et al. 2008).

Continued

Table 3. Examples of alterations in LPA signalling during diseases. *Continued*

Disease	Examples of reported effects
Obesity	Reduction in brown adipose tissue may lead to the development of obesity; LPA decreases the differentiation of mouse brown adipocyte cells (Federico et al. 2012). LPA_1 receptor knockout protects from weight gain in mice on a high fat diet (Dusaulcy et al. 2009).
Reproductive disorders	LPA receptors are expressed in both the testis and ovary and other reproductive tissues (Ye 2008). In humans, LPA_4 expression is highest in the ovary in comparison with other tissues (Noguchi et al. 2003, Ye 2008). LPA_3 knockout mice suffer interference in timing and spacing of embryo implantation (Ye et al. 2005). The timing impairment is caused by the disruption of prostaglandin signalling. $LPA_{1/2/3}$ triple knockout mice show reduced sperm production and lowered mating activity, followed by age-related azoospermia (Ye 2008).
Rheumatoid arthritis	Significant amounts of LPA, autotaxin and its substrate LPC are present in the synovial fluid of patients with rheumatoid arthritis (RA). LPA enhances COX2 expression and prostaglandin E_2 production in human RA synovial cells; these actions are inhibited by Ki16425 (Nochi et al. 2008).
Wound healing	Topical LPA promotes dermal wound healing (Demoyer et al. 2000, Balazs et al. 2001). Following injury, fibroblasts proliferate and migrate to the wound site; thus modulation of the proliferation and migration of fibroblasts might be an effective strategy for regulating wound healing. LPA induces the proliferation of human fibroblasts (Jang et al. 2003). LPA enhances wound healing in aged rats by inducing DNA synthesis, proliferation and migration of senescent cells (Rhim et al. 2010).

Pharmacological inhibition of LPA biosynthesis or degradation

In addition to receptor agonists and antagonists, another possible way to affect LPA signalling is to develop compounds that affect LPA biosynthesis or degradation. Generally, the inhibition of ATX would be beneficial if one wishes to decrease LPA levels, whereas the inhibition of LPPs and other degradative enzymes would be anticipated to elevate LPA levels.

The ATX–LPA receptor signalling axis provides a promising opportunity especially for cancer therapy (Gotoh et al. 2012). Many malignant cancers, such as breast cancer, lymphoma and glioblastoma, express high ATX levels (Yang et al. 2002, Baumforth et al. 2005, Kishi et al. 2006). ATX has been indicated to enhance metastatic and invasive potential of tumour cells, which likely is caused by the role of ATX in blood vessel formation. ATX can promote tumour cell motility and metastasis through LPA production, and it is claimed to be among the 40 most up-regulated genes in highly metastatic cancers (Euer et al. 2002). In transgenic mice, increased expression of either ATX or $LPA_{1–3}$ receptors is sufficient to induce invasive and metastatic breast cancers (Liu et al. 2009b). In melanoma cells, ATX-stimulated motility was suppressed by Ki16425 (Hama et al. 2004).

In addition to cancer, increased ATX activity has been observed in several other diseases. ATX has been reported to be upregulated in the brains of Alzheimer's disease patients (Umemura et al. 2006). The ATX-synthesized LPA has been demonstrated to induce neuropathic pain (Nagai et al. 2010). In atherosclerotic lesions, low density lipoprotein oxidation generates LPC that is converted by ATX to LPA, which then recruits monocytes and triggers inflammation (Schober & Siess 2012). Increased lysoPLD activity has been observed in pregnant women in the third trimester of pregnancy and there is an extended increase in women expecting a preterm delivery (Tokumura et al. 2002). The ATX–LPA axis has been shown to be up-regulated in inflammatory conditions such as rheumatoid arthritis (Kehlen et al. 2001) and ATX levels are high in liver fibrosis (Nakagawa et al. 2011). Since both LPA and ATX are present in various biological fluids, they could also be used as biomarkers to predict the presence of certain cancers and other diseases.

In contrast to ATX, LPP activity has been demonstrated to be low in several cancer cells e.g. the LPP expression is decreased in the majority of ovarian cancers (Tanyi et al. 2003b), whereas over-expression of LPP3 in ovarian cancer cells reduces tumour growth *in vitro* and *in vivo* (Tanyi et al. 2003a). It has been proposed that the combined effect of low LPP expression and high ATX expression in tumours makes them hypersensitive to growth-stimulating factors (Samadi et al. 2011). LPA-independent actions of LPPs in cancer cells have also been reported. Unexpectedly, an increased LPP3 expression was observed in glioblastoma tumours and glioblastoma cell lines whereas LPP3-knockdown inhibited tumour growth (Chatterjee et al. 2011). These findings indicate that LPP3 can amplify the activities of β-catenin and cyclin-D1, known enhancers of cell cycle progression. On the other hand, LPP1 overexpression causes several abnormalities in mice including reduced body size and impaired spermatogenesis but there are no alteration in LPA levels in the blood (Yue et al. 2004). Recently, inactivation of the gene encoding LPP3 enhanced neointima formation in response to arterial injury *in vivo,* which was considered as evidence for a protective role for LPP3 in the atherotrombotic disease (Panchatcharam et al. 2013).

At present, there are relatively few reports describing pharmacological ATX inhibitors that are active *in vivo*. Intravenously injected compound HA130 reduces LPA levels in mice (Albers at al. 2010). Compound PF-8380 is able to block inflammation-induced LPA synthesis and reduces inflammatory hyperalgesia with the same efficacy as naproxen in rats (Gierse et al. 2010). The pharmacological inhibitors of LPPs reported so far include general phosphatase inhibitors and fluorophosphonate analogs, whose *in vivo* bioavailability is limited, and propranolol, that exerts significant other (detrimental) actions at the concentrations required to block LPPs. Much of work still needs to be done to develop specific and potent inhibitors capable of influencing LPA levels *in vivo*.

2.3 OVERVIEW OF THE ENDOCANNABINOID SYSTEM

The endocannabinoid (eCB) system comprises of two G protein-coupled cannabinoid receptors, their natural ligands (the eCBs), and the synthetic and degradative enzymes regulating the ligand levels. The eCB system regulates a wide variety of functions both in the CNS and periphery. In the CNS, eCBs are involved in synapse formation and neurogenesis as well as in cognition, memory, emotional functions, regulation of food intake, and pain sensation. In the periphery, eCBs mediate cardiovascular, immune, metabolic, and reproductive functions.

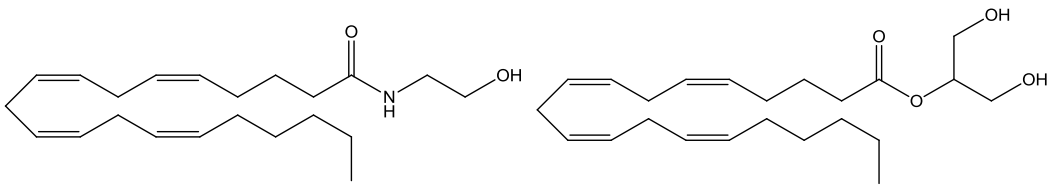
2.3.1 Cannabinoid receptors and their natural ligands

Cannabis has long been used for its hedonistic properties but also for the self-medication of several diseases and to ease pain. The *Cannabis sativa* plant has been demonstrated to contain around 70 compounds termed phytocannabinoids. The isolation of the main psychoactive compound, Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni & Mechoulam 1964), initiated the rational research in this field. Later, two GPCRs mediating cannabinoid responses were cloned and named CB_1 and CB_2 (Devane et al. 1988, Munro et al. 1993). The human CB_1 and CB_2 receptors share 44% overall amino acid identify (Munro et al. 1993). Both CB_1 and CB_2 are mainly coupled with G_i proteins. In addition to CB_1 and CB_2, other cannabinoid receptors have been proposed, such as the orphan receptor GPR55 (Ryberg et al. 2007, Lauckner et al. 2008). Since studies with GPR55 show contradictory results, GPR55 has not been generally accepted as a cannabinoid receptor (Pertwee et al. 2010, Henstridge et al. 2011). Instead, it has been suggested that LPI may be the specific ligand for GPR55 (Oka et al. 2007, Pertwee et al. 2010, Pineiro & Falasca 2012).

The CB_1 receptor is mainly expressed in the brain and spinal cord and it is one of the most abundant GPCRs in the brain (Herkenham et al. 1991). CB_1 is mainly responsible for the neurobehavioral effects of Δ^9-THC (Zimmer et al. 1999). In the rat brain, CB_1 is highly expressed in basal ganglia and moderately in the hippocampus, cerebellum and neocortex (Herkenham et al. 1991). In the brain, the majority of the CB_1 receptors have been shown to be localized presynaptically on both GABAergic interneurons and glutamatergic neurons, but more intensively on the former nerves (Katona et al. 1999, Hajos et al. 2000, Hajos et al. 2001, Katona et al. 2001). CB_1 is also expressed in the periphery, such as in the lungs, small intestine and reproductive organs (Sugiura & Waku 2002). A functional CB_1 receptor has also been found in the preimplantation embryo (Paria & Dey 2000). CB_1 receptor knockout mice are healthy and fertile but do suffer an elevated mortality rate and reduced locomotor activity (Zimmer et al. 1999). Aged CB_1 receptor knockout mice also show deterioration in cognitive functions (Albayram et al. 2011, Albayram et al. 2012).

The CB_2 receptor is mainly expressed in peripheral tissues, especially the immune system, including white blood cells in the spleen, tonsils, and thymus (Munro et al. 1993, Atwood & Mackie 2010), being involved in the regulation of immune responses. There is also evidence that neurons (Skaper et al. 1996, Van Sickle et al. 2005, Ashton et al. 2006, Gong et al. 2006) as well as microglia and astrocytes (Benito et al. 2003, Esposito et al. 2007) can express CB_2. CB_2 has been postulated to play a role in the brain especially during neuroinflammation and brain injury but its role in the healthy brain remains unclear (Atwood & Mackie 2010). The CB_2 receptor knockout mice are healthy and fertile (Buckley et al. 2000, Buckley 2008).

Cloning of the cannabinoid receptors initiated the search for their natural ligands, termed eCBs. The first endogenous cannabinoid identified was arachidonoylethanolamide (anandamide, AEA) (Devane et al. 1992). Later, 2-arachidonoylglycerol (2-AG) was identified in the canine gut (Mechoulam et al. 1995) and rat brain (Sugiura et al. 1995). At present, AEA and 2-AG (Figure 6) remain the best characterised and most widely studied endocannabinoids. There are other putative endocannabinoids, e.g. 2-arachidonoyl glyceryl ether (noladin ether) (Hanus et al. 2001), *O*-arachidonoyl ethanolamine (virodhamine) (Porter et al. 2002), and *N*-arachidonoyldopamine (Huang et al. 2002). It remains uncertain whether noladin ether is a true endocannabinoid; e.g. some research groups have not been able to detect this compound in the brains of several mammalian species (Oka et al. 2003). Exogenous cannabimimetic compounds include classical cannabinoids, non-classical cannabinoids, and aminoalkylindoles (Pertwee et al. 2010). Cannabidiol, instead, is a phytocannabinoid that has no agonist activity at cannabinoid receptors and lacks any psychotropic effects (Mechoulam & Parker 2013).



N-Arachidonoylethanolamide (AEA) 2-Arachidonoylglycerol (2-AG)

Figure 6. Chemical structures of the two principal endocannabinoids.

Both AEA and 2-AG have been detected in several brain regions as well as in the periphery. Endocannabinoid signalling plays a role in several important processes during neuronal development (Berghuis et al. 2007, Keimpema et al. 2010, Argaw et al. 2011) as well as in adult neurogenesis (Goncalves et al. 2008). In the brain, 2-AG concentrations are

approximately 200-fold higher than those of AEA (Bisogno et al. 1999). It has been noted that 2-AG is rapidly generated in rat brain after decapitation (Sugiura et al. 2001), a fact that needs to taken into account when determining post-mortem tissue 2-AG levels. 2-AG acts as a potent full agonist at CB_1 and CB_2 receptors whereas AEA acts as a partial agonist for both receptors (Gonsiorek et al. 2000, Savinainen et al. 2001, Sugiura 2009). In the CNS, 2-AG is believed to be the physiologically relevant ligand for the CB_1-mediated synaptic signalling (Hashimotodani et al. 2007) but AEA may also mediate certain forms of synaptic homeostasis and plasticity (Gerdeman et al. 2002, Kim & Alger 2010). The actions of AEA and 2-AG in the brain are local, partly due to the hydrophobic nature of these compounds and poor solubility in the hydrophilic extracellular tissue fluid. In addition to the partial agonistic properties for the CB receptors, AEA acts as a full agonist for the transient receptor potential vanilloid type-1 (TRPV1) receptor that is a non-selective cation channel belonging to the larger family of TRP channels (Zygmunt et al. 1999).

2.3.2 Biosynthesis and response of eCBs

Generally, endocannabinoids are synthesized on demand from plasma membrane phospholipid precursors and released from cells immediately after their production. The levels of eCB precursors depend on the expression and activity of the biosynthetic and degrading enzymes, and also on the diet and its content of arachidonic acid (AA) and its precursor linoleic acid (DiMarzo & Petrosino 2007, Katona & Freund 2012).

The principal eCB function is the modulation of synaptic activity through retrograde signalling. In postsynaptic neurons, eCB synthesis is triggered by an increase in intracellular calcium concentrations induced by cell depolarization or stimulation of G_q protein-coupled receptors (Kano et al. 2009). After their production, eCBs move across the synaptic cleft in a retrograde manner, activate presynaptic CB_1 receptors and suppress neurotransmitter release (Figure 7). Most often, the presynaptic CB_1 receptor activity suppresses GABAergic and glutamatergic transmission causing depolarization-induced suppression of inhibition (DSI) or depolarization-induced suppression of excitation (DSE). The ratio of DSI and DSE determines the net effect of this fine tuning of neurotransmission (Kano et al. 2009, Piomelli 2003). It should be noted that neurotransmission is often a heterosynaptic process. The mechanism by which the lipophilic eCBs move through the extracellular space from the postsynaptic side to the presynaptic side is not known.

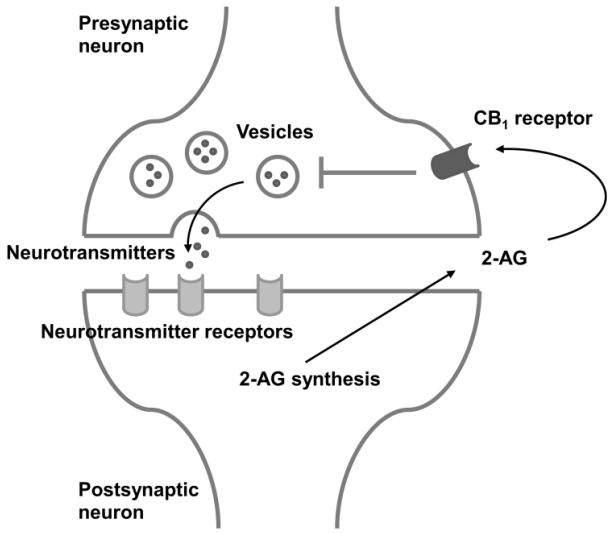


Figure 7. Illustration of retrograde eCB signalling (modified from Sugiura 2009).

Although retrograde signalling is believed to be the principal mechanism of eCB action, alternative ways of signalling have been proposed. It has been postulated that eCBs may signal in an autocrine manner or alternatively via astrocytes to modulate synaptic function (Castillo et al. 2012). In addition to the cannabinoid receptors, there is evidence indicating that AEA-activated TRPV1 channels may participate in eCB signalling (De Petrocellis & Di Marzo 2010). In addition to their signalling functions, eCBs have been claimed to exert supplemental effects. A detectable amount of 2-AG is present in a non-stimulated brain without permanent activation of CB_1 receptors, indicating that not all of the 2-AG present is used for cannabinoid signalling (Tanimura et al. 2010, Gao et al. 2010). It has been speculated that a pool of 2-AG might be presynthesised and kept waiting to be released after an appropriate stimulus (Min et al. 2010a, Alger & Kim 2011, Di Marzo 2011). Since 2-AG acts at the crossroads of multiple biochemical pathways in lipid metabolism, it is likely that a proportion of 2-AG generated in the brain serves as a lipid intermediate in these pathways (Piomelli 2003). Although the signalling pool of 2-AG is apparently circulating between post- and presynaptic neurons, another pool might be present in other cell types, such as astrocytes and microglia, and not be involved in retrograde signalling.

Biosynthesis of AEA

At least four different synthetic mechanisms for AEA have been proposed (Di Marzo 2011). The most important pathway for AEA generation is thought to be the release from *N*-arachidonoylphosphatidylethanolamine (NArPE), which is first formed from phosphatidylcholine and phosphatidylethanolamine, by *N*-acyltransferase. *N*-acylphosphatidylethanolamine-specific PLD (NAPE-PLD) converts NArPE into anandamide (and PA). In the brain, NAPE-PLD has been demonstrated to be localized in different strucrures, e.g. hippocampus and cerebellum (Egertova et al. 2008, Cristino 2008). Other routes for AEA generation from NArPE have been proposed such as conversion by the sequential actions of the α/β-hydrolase domain-containing protein 4 and glycerophosphodiesterase-1 or by the soluble PLA_2 followed by conversion into anandamide by a lyso-PLD (Di Marzo 2011). In addition, generation by sequential actions of a PLC and a phosphatase has been proposed.

Biosynthesis of 2-AG

The primary precursor for 2-AG is believed to be DAG generated from phosphatidylinositols by the action of PLCβ or from PA by phosphatases (Sugiura et al. 2006, Bisogno 2008). DAG is further hydrolyzed by the *sn*-1-specific DAG lipase (DAGL) to generate 2-AG (Oudin et al. 2011, Reisenberg et al. 2012) (Figure 8).

Two isoforms of the Ca^{2+}-sensitive, *sn*-1-selective DAGLs generating 2-AG have been cloned and named as DAGLα and DAGLβ (Bisogno et al. 2003). DAGLα and -β have arisen from a gene duplication event and they are very closely related. The main difference between the enzymes is the presence of a C-terminal tail in DAGLα that is absent from DAGLβ. The two isoforms are expressed differentially in different cells; DAGLα is highly expressed in the CNS when compared to other tissues whereas DAGLβ possesses a broader but somewhat lower expression pattern (Bisogno et al. 2003). In the adult brain, DAGLs are expressed in the same brain regions as the CB_1 receptor, DAGLα more intensively than DAGLβ (Bisogno et al. 2003, Yoshida et al. 2006). DAGLα is concentrated in the heads of dendritic spines located postsynaptically opposite to presynaptic glutamatergic axons expressing CB_1 in several brain regions, such as the hippocampus (Katona et al. 2006), striatum (Uchigashima et al. 2007), and prefrontal cortex (Lafourcade et al. 2007). However, in the developing mouse brain DAGLα and DAGLβ are expressed in a specific and restricted manner in the same axonal tracts and in the same growth cones as CB_1 (Berghuis et al. 2007) indicative of a loss of DAGLs from axons during development.

There is convincing evidence that DAGLα is the major enzyme involved in the biosynthesis of 2-AG used in retrograde synaptic signalling. Overexpression of DAGLα results in a significant increase in basal 2-AG levels whereas knockdown of DAGLα by RNA interference can reduce 2-AG levels (Jung et al. 2007). It was observed that the retrograde synaptic suppression was absent in several brain regions of DAGLα-knockout mice whereas it remained intact in the brains of DAGLβ-knockout mice (Tanimura et al. 2010, Gao et al. 2010, Yoshino et al. 2011). Furthermore, there were up to 80% reductions in 2-AG levels in DAGLα-KO mice brain whereas in the brains of DAGLβ-KO mice, the 2-AG levels remained unaltered (Tanimura et al. 2010, Yoshino et al. 2011), or were reduced up to 50% (Gao et al. 2010). With the exception of a slight decrease in body weight, the phenotypes of the DAGLα-KO and DAGLβ-KO animals were found to be normal when compared to wild-type (WT) animals (Gao et al. 2010). The role of DAGLβ seems to be more important in peripheral tissues; it was demonstrated that DAGLβ inactivation lowers 2-AG levels in mouse peritoneal macrophages, indicating that DAGLβ is involved in the regulation of inflammatory responses in macrophages (Hsu et al. 2012).

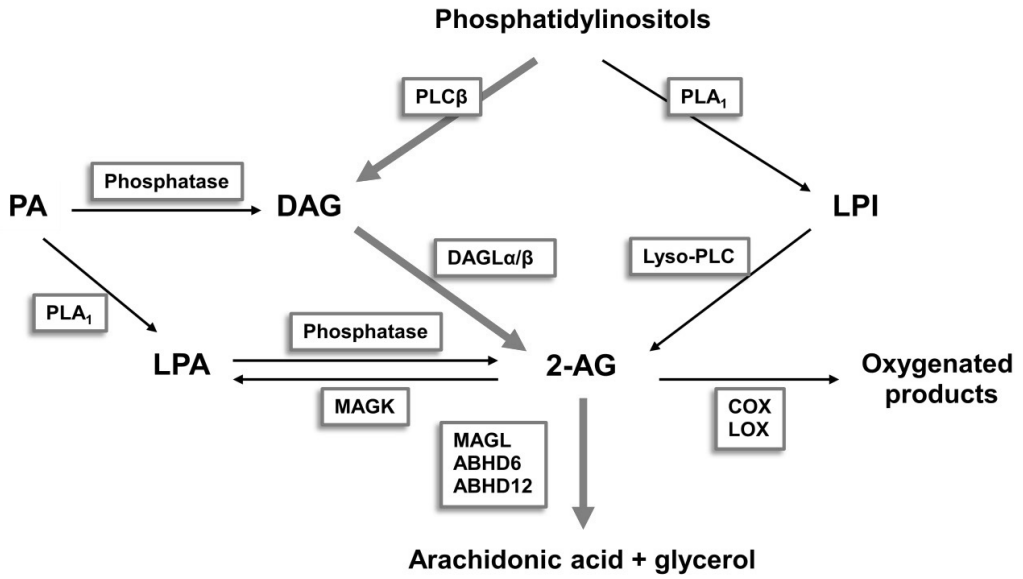


Figure 8. Enzymatic pathways for 2-AG biosynthesis and degradation. The principal pathway is presented with thick arrow. Abbreviations: 2-AG, 2-arachidonoylglycerol; ABHD, α/β-hydrolase domain-containing protein; COX, cyclooxygenase; DAG, diacylglycerol; DAGL, diacylglycerol lipase; LOX, lipoxygenase; LPA , lysophosphatidic acid; LPI, lysophosphatidyl inositol; MAGL, monoacylglycerol lipase; MAGK, monoacylglycerol kinase; PA, phosphatidic acid; PLA, phospholipase A; PLC, phospholipase C.

Although DAGLα evidently generates the majority of 2-AG for retrograde synaptic signalling, evidence has been provided to show that DAGLα is not involved in the hippocampal DSI (Min et al. 2010b). It is noteworthy that in some brain regions, highly enriched in CB_1 receptors (such as the substantia nigra), DAGLα is only sparsely expressed, as evidenced by the weak labelling with the DAGLα antibodies in immunohistochemical studies (Uchigashima et al. 2007, Kano et al. 2009, Tanimura et al. 2010). This is considered as support for the existence of alternative biochemical routes that could generate CB_1 receptor-activating eCBs in those brain regions with sparse DAGLα expression. In addition

to neurons, it has been demonstrated that mouse microglia (Walter et al. 2003, Witting et al. 2004, Carrier et al. 2004) as well as astrocytes (Walter et al. 2004) have the capacity to produce 2-AG. Additional pathways for 2-AG generation include the release from *sn*-2-arachidonic acid-containing phospholipids by sequential actions of phospholipase A_1 (PLA_1) and lyso-PLC or by phosphatase-mediated conversion from 2-arachidonoyl-LPA (Nakane et al. 2002, Sugiura et al. 2006, Bisogno 2008) (Figure 8).

Pharmacological inhibition of eCB biosynthesis

Only a limited number of pharmacological inhibitors for eCB-generating enzymes are currently available. At present, the search has focused on inhibitors of DAGLs. In experimental settings, tetrahydrolipstatin (THL, also known as orlistat) and RHC80267 have been used to inhibit DAGLs (Bisogno et al. 2003). These inhibitors, however, are poorly selective for DAGLs and they have additional enzyme targets. More recently, some fluorophosphonate compounds, such as O-3841, O-3640, O-5596, and O-7460 (Bisogno et al. 2006, Bisogno et al. 2009, Bisogno et al. 2012) as well as the THL-analog OMDM-188 (Ortar et al. 2008) have been reported as being selective DAGL inhibitors but none of these inhibitors can discriminate between the α and β isoforms. Recently, some triazole urea compounds (KT109, KT172) were reported to selectively inhibit DAGLβ (Hsu et al. 2012).

2.3.3 Enzymatic degradation of eCBs

Soon after their generation, eCBs are initially removed from the extracellular space by cellular uptake and then degraded by hydrolytic enzymes (Blankman & Cravatt 2013). The hydrolysis of eCBs generally terminates their function but the hydrolysis products can also be used as precursors for other signalling molecules.

The mechanism by which eCBs are moved across the plasma membrane has remained elusive. It is not clear if eCBs are taken up by cells via a plasma membrane transporter, by endocytosis or simply by passive diffusion. Furthermore, it is unclear whether AEA and 2-AG have the same or distinct uptake mechanisms. Several AEA carrier proteins have been proposed, such as fatty acid binding proteins, heat shock protein 70, albumin and the fatty acid amide hydrolase (FAAH)-like AEA transporter (Fowler 2012a), but more studies will be needed to elucidate the involvement of these proteins in eCB uptake.

AEA degradation

The primary enzyme catalyzing the hydrolysis of AEA is FAAH (Deutsch & Chin 1993). FAAH belongs to the family of serine hydrolases, and it hydrolyzes AEA into AA and ethanolamine (Long & Cravatt 2011). FAAH has been well characterized and its crystal structure has been resolved (McKinney & Cravatt 2005). FAAH is abundantly expressed in the brain; it is located intracellularly mainly in postsynaptic neurons (Egertova et al. 1998, Gulyas et al. 2004).

Especially in the periphery, additional enzymes capable of hydrolyzing AEA have also been described, e.g. *N*-acylethanolamine-hydrolyzing acid amidase (Tsuboi et al. 2007) and FAAH-2. This latter enzyme is expressed in primates and some related mammals, but not in lower placental mammals including the mouse and rat (Wei et al. 2006). AEA is also metabolized by COX-2 to prostaglandin ethanolamides (Ross et al. 2002, Kozak et al. 2002a) and by lipoxygenase (LOX) into 12- and 15-hydroxy AEA (Ueda et al. 1995, Edgemont et al. 1998). Some cytochrome P450 species can metabolize AEA to the corresponding epoxides and hydroxy metabolites in liver microsomes (Bornheim et al. 1995, Snider et al. 2008).

2-AG degradation

2-AG is enzymatically hydrolyzed into AA and glycerol (Figure 8). The first enzyme implicated as a 2-AG hydrolase was monoacylglycerol lipase (MAGL) (Dinh et al. 2002, Dinh et al. 2004, Saario et al. 2004). MAGL is a serine hydrolase that catalyses the hydrolysis of monoacylglycerols to their corresponding fatty acids. Originally the gene encoding MAGL was cloned from mouse adipose tissue, but MAGL has also been detected in other peripheral tissues such as the kidney, ovary, testis and heart (Karlsson et al. 1997). In the brain, MAGL is localized in the presynaptic terminals that also express CB_1 receptors (Dinh et al. 2002, Gulyas et al. 2004). Studies have suggested MAGL as being the main hydrolase terminating 2-AG actions in the brain, accounting for ~85% of the total 2-AG hydrolysis (Blankman et al. 2007). The crystal structure of MAGL has been resolved by two independent laboratories; the enzyme has shown to be a dimer, existing in both a soluble form and associated with cell membranes (Bertrand et al. 2010, Labar et al. 2010).

In addition to MAGL, two novel α/β-hydrolase domain-containing proteins, ABHD6 and ABHD12, have been found to be able to hydrolyze the brain 2-AG and, together with MAGL, these three serine hydrolases account for ~99% of the brain 2-AG hydrolase activity (Blankman et al. 2007). Both ABHD6 and ABHD12 are integral membrane proteins that are expressed in several brain regions. The active site of ABHD6 is believed to face the cell interior whereas the active site of ABHD12 faces the lumen/extracellular space (Blankman et al. 2007). The three 2-AG hydrolases hydrolyse only monoacylglycerols (not di- or triacylglycerols or LPA) having different substrate and isomer preferences (Navia-Paldanius et al. 2012). The three serine hydrolases display different subcellular localization, suggesting that they could access and hydrolyze distinct pools of 2-AG. When compared to MAGL and ABHD6, ABHD12 is expressed at higher levels in microglia (Fiskerstrand et al. 2010).

Under certain conditions, FAAH has been shown to metabolize 2-AG (DiMarzo et al. 1998, Goparaju et al. 1998). COX-2 and LOX are capable of metabolizing 2-AG to glyceryl prostaglandins (Kozak et al. 2000) and to hydroxyeicosatetraenoic acids glyceryl esters (Kozak et al. 2002b), respectively (Figure 8). In addition, NTE has been reported be able to hydrolyze 2-AG (van Tienhoven et al. 2002). In some cells, including human monocytic leukaemia cells, non-specific esterases such as human carboxylesterases 1 and 2 can catalyze 2-AG hydrolysis (Rouzer & Marnett 2011). Thus the contribution of a particular enzyme to 2-AG metabolism largely depends on the cell in question.

Pharmacological inhibition of eCB degradation

The primary enzymes responsible for AEA and 2-AG degradation are considered to be FAAH and MAGL, respectively, and the major efforts on inhibitor development have been focused on these two targets.

Since the initial research in the cannabinoid field focused on AEA, studies on the pharmacological inhibition of eCB degradation began with FAAH. Several inhibitors of FAAH have been described representing both reversible and irreversible mechanisms. Irreversible inhibitors include sulfonyl fluorides such as phenylmethylsulfonyl fluoride, hexadecylsulfonyl fluoride, methylarachidonoylfluorophosphonate (MAFP) as well as the carbamate compound URB597 (Deutsch et al. 1997a, Deutsch et al. 1997b, Kathuria et al. 2003). The reversible inhibitors include trifluoromethyl ketones such as arachidonoyl trifluoromethyl ketone (Koutek et al. 1994). All of these compounds, however, possess limited selectivity for FAAH. More recently, some piperidine/piperazine ureas, PF-750, PF-3845, and PF-04457845 have been claimed to be selective for FAAH over other serine hydrolases (Ahn et al. 2007, Ahn et al. 2009, Johnson et al. 2011).

The search for MAGL inhibitors began soon after that of FAAH inhibitors. Non-specific mercuric compounds, fatty acid trifluoromethylketones, sulfhydryl reagents and disulphide compounds, and organophosphorus compounds, such as MAFP, irreversibly

inhibit MAGL (Fowler 2012b). In the brain, virtually all 2-AG hydrolase activity is sensitive to MAFP (Saario et al. 2004). The non-selectivity of these compounds led to attempts to develop inhibitors that would selectively target MAGL. The carbamate compound URB602 was first reported to be a selective pharmacological inhibitor of MAGL (Hohmann et al. 2005), but further studies called into question its selectivity (Vandevoorde et al. 2007). The sulfhydryl reagent *N*-arachidonoylmaleimide (NAM) is a potent, irreversible and rather selective inhibitor of MAGL, blocking ~85% of the brain 2-AG hydrolase activity (Saario et al. 2005, Blankman et al. 2007). The carbamate compound JZL184 has been reported to be a specific inhibitor of MAGL (Long et al. 2009), but its low potency may limit its use (Savinainen et al. 2010, Aaltonen et al. 2013). Recently, compounds ML30 (Morera et al. 2012), KML29 (Chang et al. 2012) and JJKK-048 (Aaltonen et al. 2013) were reported as being selective MAGL inhibitors and of these, JJKK-048 seems to be the most potent and MAGL-selective inhibitor currently available.

2.3.4 Endocannabinoid system as a target for drug discovery

Direct activation or inhibition of the eCB system

Cannabis has been used for its medicinal properties for millennia, long before its mechanism of action was understood. To date, alterations in the endocannabinoid system have been reported in several different diseases in both the CNS and periphery (Pacher et al. 2006, Pacher & Kunos 2013) (Table 4). In the CNS, eCBs are generally considered to be neuroprotective (Sánchez & García-Merino 2012). One hallmark of the neurodegenerative diseases is inflammation and eCBs may relieve symptoms by possessing anti-inflammatory properties. In neurodegenerative diseases, there is often down-regulation of CB_1 receptors but up-regulation of CB_2 receptors. The neuroprotective effect is thought to be mediated through the activation of CB_1 receptors, and the inhibition of inflammatory microglial responses through the activation of CB_2 receptors. In cancer, the role of eCBs is still controversial, but generally eCBs suppress cancer progression (Hermanson & Marnett 2011). Other well known effects of eCBs are their antinociceptive properties (Karst et al. 2010) as well as their ability to increase appetite (Matias & Di Marzo 2007).

The alterations of the eCB system can be either protective or deleterious depending on the cell or tissue type (Table 4), and it must be carefully determined whether the activation or blocking eCB system would be desired to achieve the therapeutic response (Di Marzo 2008). In some diseases, the current knowledge is still contradictory, as both both positive and negative changes in the eCB system have been reported. On the other hand, the levels of AEA and 2-AG might be regulated oppositely in the same disease. The endocannabinoid system has been a target of an intensive drug discovery but the challenge has been to obtain a selective effect in each organ or tissue and to avoid harmful side-effects especially in those brain regions controlling mood and cognitive functions.

Currently, Δ^9-THC (dronabinol) and its synthetic analogue, nabilone, which directly activate cannabinoid receptors, are used for the treatment of nausea and vomiting induced by radiotherapy or chemotherapy, and for preventing the wasting syndrome, such as encountered in patients with acquired immunodeficiency syndrome (Pacher & Kunos 2013). Sativex®, a preparation consisting of a mixture of cannabidiol and Δ^9-THC, is licenced for the use in the multiple sclerosis-related spasticity and pain in several countries (Karst et al. 2010). The first selective CB_1 receptor antagonist/inverse agonist rimonabant was briefly on the market for the treatment of obesity in several European countries during 2006–2008 but it had to be withdrawn mainly due to its psychiatric side effects, including increased anxiety, depression and suicidal intentions (Idelevich et al. 2009). In addition to brain-penentrant CB_1 antagonists, peripherally restricted antagonists for CB_1 receptors are under development for the treatment of metabolic disorders (Silvestri & Di Marzo 2012).

Peripherally restricted agonists for CB_1 receptors would be useful for pain treatment since the cannabinoid-induced analgesia has been proven largely to be mediated via CB_1 receptors in peripheral nociceptive afferents (Agarwal et al. 2007). In addition to orthosteric ligands, allosteric modulators for cannabinoid receptors (e.g. ORG27569, PSNCBAM-1) have been reported (Ahn et al. 2013, Cawston et al. 2013). These compounds have been reported to be able to modulate the actions of orthosteric ligands as well as to mediate G protein-independent signalling via the β-arrestins.

Table 4. Examples of alterations in the eCB system during diseases and/or potential therapeutic usefulness of exogenous and endogenous cannabinoids.

Disease	Examples of reported effects
Neurodegenerative diseases	
Alzheimer's disease (AD)	CB_1 receptors are down-regulated in neurons whereas CB_2 receptors are up-regulated particularly in microglia in AD patients (Ramirez et al. 2005, Benito et al. 2003). Elevation in eCB levels provides neuroprotection against β-amyloid peptide toxicity in rats (van der Stelt et al. 2006).
Amyotropic lateral sclerosis (ALS)	At present, only anti-glutamatergic agents are used in delaying the disease progression. Cannabinoids exert anti-glutamatergic and anti-inflammatory actions through activation of the CB_1 and CB_2 receptors, respectively, and may have therapeutic potential in the treatment of ALS (Bilsland & Greensmith 2008).
Huntington's disease (HD)	CB_1 receptor down-regulation has been detected in the human globus pallidus and substantia nigra whereas CB_2 receptors are up-regulated in the striatum (Glass et al. 2000, Palazuelos et al. 2009).
Multiple sclerosis (MS)	MS is an inflammatory, demyelinating disease. Both CB_1 and CB_2 receptors are expressed in cells (such as microglia and macrophages) that are located in human MS plaques (Benito et al. 2007). Cannabinoid agonists have remyelinating properties in mice (Arevalo-Martin et al. 2003). Sativex® is licensed for the treatment of MS related spasticity and neuropathic pain in several countries.
Parkinson's disease (PD)	Role of the eCB system in the modulation of the dopaminergic system links eCBs to PD, in which dopaminergic neurons are destroyed. So far conflicting data exist; both increased and decreased striatal eCB levels have been reported (Scotter et al. 2010). In a rat model, increased cannabinoid tone has been observed in globus pallidus (DiMarzo et al. 2000).
Mental disorders	
Anxiety and depression	Low doses of cannabinoid agonists are anxiolytic in animal models (Zanettini et al. 2011).
Schizophrenia	Increased levels of eCBs have been detected in the cerebrospinal fluid (Giuffrida et al. 2004) and the blood (De Marchi et al. 2003) of schizophrenic patients. Increased levels of 2-AG but decreased levels of AEA have been detected in postmortem brains of schizophrenic patients (Muguruza et al. 2013). Region-specific changes in the density of CB_1 receptors in brain have been observed in schizophrenic patients (Dean et al. 2001, Zavitsanou et al. 2004, Newell et al. 2006, Dalton et al. 2011).
Control of food intake	
Anorexia	Exogenous and endogenous cannabinoids increase food intake (Matias & Di Marzo 2007, Li et al. 2011). eCBs may mediate the rewarding effect of self-starvation in anorexia nervosa patients (Monteleone et al. 2005).
Obesity	Hyperactivity of the eCB system in obesity; high eCB levels are found in the blood and visceral adipose tissue of obese and hyperglycaemic patients. CB_1 receptor blockade suppresses food intake (Matias & Di Marzo 2007, Li et al. 2011).

Continued

Table 4. Examples of alterations in the eCB system during diseases and/or potential therapeutic usefulness of exogenous and endogenous cannabinoids. *Continued*

Disease	Examples of reported effects
Other diseases	
Cancer	Generally, cannabinoids and endocannabinoids inhibit tumour growth by inducing apoptosis and inhibiting proliferation and angiogenesis (Hermanson & Marnett 2011). Elevated levels of AEA and 2-AG have been reported in several types of tumours. An opposite regulation of AEA and 2-AG levels has been observed; e.g. AEA levels decrease and 2-AG levels increase in the human glioma tissue (Wu et al. 2012). The role of CB_1 and CB_2 receptor expression in relation to disease prognosis depends on the cancer type, e.g. a high expression of CB_1 receptors in prostate cancer tumour samples is associated with disease severity and poor prognosis (Chung et al. 2009).
Cardiovascular diseases	Increased AEA and 2-AG levels have been detected in coronary artery disease patients. CB_1 antagonists have been reported to be antiatherosclerotic and to reduce several metabolic cardiovascular risk factors (Pacher & Steffens 2009).
Drug addiction and alcoholism	Interconnected role of CB_1 and opiate receptors; cannabinoids reduce opioid withdrawal symptoms in rodent models (Fattore et al. 2005). The eCB system is involved in the reinforcing properties of ethanol; in rodents, chronic consumption of ethanol increases eCB synthesis and reduces CB_1 expression in brain regions associated with addiction pathways (Pava & Woodward 2012).
Epilepsy	Exogenous cannabinoids have been reported to exert both pro- and anti-convulsive activities in animal models and humans, depending on the model. Endocannabinoids seem to be anti-convulsive via CB_1 receptors in mice (Lutz 2004).
Glaucoma and retinopathy	Cannabinoids decrease intraocular pressure in humans (Tomida et al. 2004).
Nausea	Cannabinoids have anti-emetic properties and are clinically used as anti-emetics (Todaro 2012).
Osteoporosis	CB_2 receptor deficient mice experience accelerated age-related bone loss (Ofek et al. 2006).
Pain	Cannabinoids relieve acute and chronic pain, especially neuropathic pain; evident both in animal models and human studies (Karst et al. 2010).
Rheumatoid arthritis (RA)	Elevated levels of AEA and 2-AG have been detected in the synovial fluid of RA patients (Richardson et al. 2008).

Pharmacological inhibition of eCB biosynthesis or degradation

In addition to targeting CB receptors by exogenous ligands and allosteric modulators, eCB-synthesizing and -degrading enzymes represent attractive targets for drug development (Di Marzo 2009). Inhibition of eCB degradation by an enzyme inhibitor would increase eCB levels and lead to prolonged activation of CB receptors but only at sites where eCB was originally synthesized. In this way, the pharmacological effect woud be more localized when compared to exogenous agonists that target CB receptors in all parts of the body. It is thought that treatment with enzyme inhibitors would less likely cause psychoactive effects than CB receptor agonists. Conversely, one would predict that pharmacological inhibitors of eCB-generating enzymes would be useful if one wished to block eCB signalling and to treat pathological conditions that involved overactivity of eCB system. The postulated eCB transporters would also provide targets for drug development but further studies will be required to demonstrate the actual existence of these proteins.

In order to obtain a specific effect in a certain tissue, it is important that the inhibitor has selectivity and does not block other enzymes. Only a limited number of selective pharmacological inhibitors for eCB-synthesizing enzymes are currently available. The primary enzymes responsible for AEA and 2-AG degradation are considered to be FAAH and MAGL, respectively, and the major efforts on inhibitor development have been focused on these two enzymes. Since AEA and 2-AG may be regulated in opposite ways in certain

diseases and dual FAAH/MAGL blocking may result in unwanted cannabis-like effects (including weight gain and impairments in cognition and motor control), the selectivity over AEA and 2-AG degrading enzymes is a desired goal in inhibitor development. However, due to their chemical properties, e.g. poor stability or poor permeability through biological membranes, many of the inhibitors reported in the literature are not suitable for *in vivo* administration. Furthermore, the *in vivo* data for some recently reported inhibitors are publicly not available. Examples of the currently reported *in vivo* effects of pharmacological inhibitors of eCB-synthesizing and -degrading enzymes in rodents are listed in Table 5.

It is noteworthy that continuous treatment of mice with a high dose of JZL184, as well as the genetic deletion of MAGL, have been reported to result in 2-AG overflow and subsequent CB_1 receptor desensitization and functional antagonism of the eCB system (Chanda et al. 2010, Sclosburg et al. 2010, Taschler et al. 2011). However, this effect was avoided by using lower doses of JZL184 (Busquets-Garcia et al. 2011). Receptor desensitization represents a potential problem that needs to be overcome when aiming at developing drugs inhibiting 2-AG breakdown.

Table 5. Selective inhibitors of eCB-synthesizing and -degrading enzymes and examples of their *in vivo* effects in rodents.

Enzyme	Inhibitor	*In vivo* effects in rodents	References
Synthesizing enzymes			
DAGLα/β	O-3841, O-3640	Not suitable *in vivo (*lack of stability and poor permeability through the plasma membrane)	Bisogno et al. 2006
	O-5596	Reduction in food intake	Bisogno et al. 2009
	O-7460	Reduction in food intake	Bisogno et al. 2012
	OMDM188	Not determined	Ortar et al. 2008
DAGLβ	KT109, KT172	Not determined	Hsu et al. 2012
Degrading enzymes			
FAAH	URB597	Reduction of inflammatory pain, anxiolysis	Kathuria et al. 2003, Naidu et al. 2010
	PF-750	Not determined	Ahn et al. 2007
	PF-3845	Reduction in inflammatory pain	Ahn et al. 2009, Booker et al. 2012
	PF-04457845	Reduction in acute inflammatory and chronic noninflammatory pain	Ahn et al. 2011
MAGL	URB602	Enhancement of stress-induced analgesia, reduction of inflammatory pain, neuroprotection in neonatal ischemic brain injury	Hohmann et al. 2005, Guindon et al. 2007, Comelli et al. 2007, Carloni et al. 2012
	NAM	Potentiation of CB_1 receptor-mediated behavioural effects of 2-AG	Burston et al. 2008
	JZL184	Acute analgesia, reduction of inflammatory pain, anti-emesis, anxiolysis	Long et al. 2009, Guindon et al. 2011, Kinsey et al. 2011, Sticht et al. 2012, Ghost et al. 2013
	ML30	Not determined	Morera et al. 2012
	KML29	Not determined	Chang et al. 2012
	JJKK-048	Not determined	Aaltonen et al. 2013
ABHD6	WWL70	Anti-inflammation and neuroprotection	Tchantchou & Zhang 2013
ABHD12	No specific inhibitor described so far		

Abbreviations: ABHD, α/β-hydrolase domain-containing protein; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAM, *N*-arachidonoylmaleimide.

Endocannabinoid-independent pathophysiological roles of serine hydrolases

There are recent findings demonstrating that the enzymes degrading eCBs are involved in the pathology of several diseases also in a manner independent of eCB signalling. Pathophysiological roles of MAGL have been reported in cancer and neurodegenerative diseases. MAGL is intensely expressed in aggressive human cancer cells and primary tumours (Nomura et al. 2010). MAGL contributes to the cancer pathogenicity by providing free fatty acids for the production of bioactive lipids such as LPA and prostaglandins. The blockade of MAGL impairs invasiveness and tumorigenicity of several cancer cells; this effect is mainly due to the reduction in the levels of these other bioactive lipids, and does not involve eCB. Recently it has been demonstrated, that in addition to terminating 2-AG signalling, MAGL releases AA to be used as a precursor for the pro-inflammatory prostaglandin synthesis in the brain (Nomura et al. 2011). The pharmacological or genetic inactivation of MAGL has attenuated neuroinflammation and exerted neuroprotective effects in a Parkinson's disease mouse model (Nomura et al. 2011) and in an Alzheimer's disease mouse model (Chen et al. 2012). These findings thus link the eCB and eicosanoid pathways together through MAGL. Thus, MAGL-inhibitors may exhibit antinociceptive and anti-inflammatory responses by the simultaneous enhancement of eCB levels and reduction of eicosanoid levels in the brain. It is believed that MAGL may play a role in the prostaglandin production in the brain but not in the gut, where the cytosolic PLA_2 predominantly releases AA for prostaglandin production. Therefore, MAGL inhibitors potentially would not exhibit the gastrointestinal toxicity commonly associated with COX1/COX2 inhibitors. In addition, ABHD6 has been reported to be expressed in tumours (Li et al. 2009, Max et al. 2009). Mutations in the gene encoding ABHD12 have been connected to the neurogenerative disease PHARC (polyneuropathy, hearing loss, ataxia, retinis pigmentosa, and cataract) (Fiskerstrand et al. 2010). A knockout of the gene encoding ABHD12 induces an age-dependent PHARC-like phenotype in mice but does not increase brain bulk levels of 2-AG or other MAGs (Blankman et al. 2013).

2.4 FUNCTIONAL AUTORADIOGRAPHY AS A METHOD TO STUDY LIPID GPCR SIGNALLING

2.4.1 General methods to study GPCR activity

Methods to study GPCR function can be divided into groups depending on which step of the GPCR signalling cascade they monitor (Zhang & Xie 2012). Classical receptor binding assays can be used to characterize the affinity of a compound in targeting its cognate GPCR. Methods that measure either G protein activation or G protein-mediated downstream events, such as second messenger generation, are defined as functional assays. In addition, some functional assays for G protein-independent signalling exist.

Traditionally, radioligand binding with receptor-containing membranes has been used to identify compounds that target GPCRs (or other receptors). Since the pioneering experiments (Lefkowitz et al. 1970), ^{3}H- and ^{125}I-labeled ligands have been used widely. Receptor binding assays can be applied to characterize the interaction between a receptor and its ligand but they provide little information about the downstream signalling events. Furthermore, the availability of labelled ligands may limit the use of the method. Functional assays measuring cellular levels of cAMP can be used in studies of GPCRs coupled to the $G\alpha_s$ or $G\alpha_i$ class of G proteins that activate or inhibit adenylyl cyclase, respectively (Hill et al 2010). The measurement of intracellular inositol phosphates or Ca^{2+} can be used for the assessment of PLC activity in relation to the activation of the $G\alpha_q$ class of G proteins (Liu et al. 2008, Emkey & Rankl 2009). Since GPCR activation alters the gene

transcription via the responsive elements for second messengers, cell-based reporter assays can be used for GPCR screening (Cheng et al. 2010). In reporter assays, the reporter gene constructs usually contain second messenger responsive elements upstream of a promoter, which regulates the expression of a selected reporter protein e.g. linked to a fluorescent protein.

Recently, new cell-based methods to study GPCRs have been established, such as a label-free dynamic mass redistribution (DMR) technology (Schröder et al. 2011). The DMR technology detects processes that occur when biomolecules change their localization within a cell, associated with mass relocation. The DMR technology has been applied to studies of GPCRs coupled to all four families of G proteins (Schröder et al. 2010). Another novel approach to study especially poorly characterized $G_{12/13}$ coupled receptors is a transforming growth factor-α (TGFα) shedding assay, in which GPCR activation causes ectodomain shedding of a membrane-bound proform of alkaline phosphatase-tagged TGFα and its release into a conditioned medium (Inoue et al. 2012). Originally, the ectodomain shedding of TGFα was observed to take place downstream of LPA_6 receptor signalling (Inoue et al. 2011).

G protein-independent functional assays include receptor internalization assays and β-arrestin recruitment assays (Haasen et al. 2006, Siehler 2008, Zhang & Xie 2012). These assays often exploit the combination of high-resolution fluorescence microscopy and automated image analysis for monitoring fluorophore-labelled biomolecules in cells. Either ligands or β-arrestins can be labelled with a fluorophore in order to detect the internalization of the receptor. Alternatively, specific antibodies can be directed to the N- or C-terminus of the GPCR and co-internalized with the receptor. These primary antibodies can be detected with fluorophore-labelled secondary antibodies. Non-imaging-based β-arrestin recruitment assays include bioluminescence resonance energy transfer, where the GPCR is tagged with a fluorescent protein tag whereas the β-arrestin is tagged with Renilla luciferase, or *vice verca*. After β-arrestin recruitment, the two tags come into close proximity and their interaction results in a detectable emission of light (Bertrand et al. 2002).

2.4.2 [^{35}S]GTPγS binding techniques

Functional [^{35}S]GTPγS binding techniques provide an opportunity to monitor the first step in the GPCR-G protein signalling cascade by measuring the guanine nucleotide exchange. After agonist binding, the GPCR will interact with a G-protein, GDP is released from the α subunit in the assay, it is replaced with a radiolabelled GTP analog. The substitution in the γ-phosphate renders [^{35}S]GTPγS resistant to hydrolysis, resulting in the accumulation of radioactivity and this can be detected (Weiland & Jakobs 1994) (Figure 9). Classically [^{35}S]GTPγS binding assays have been performed using membrane preparations (Lazareno 1997).

[^{35}S]GTPγS autoradiography

Receptor autoradiography generally refers to a method, in which either synthetic or natural ligands are labelled with radioactive isotopes, followed by their binding to specific receptors, their binding sites can be anatomically localized using a film or photoemulsion layer. The finding that receptor–G-protein complexes are still able to function in tissue sections (Zarbin et al. 1983) led to the development of the [^{35}S]GTPγS autoradiography method that provides an approach to detect agonist-driven activity at the receptor–G protein axis in tissue sections (Sim et al. 1995, Sovago et al. 2001, Laitinen 2004). In addition to providing information about the receptor's anatomical distribution, [^{35}S]GTPγS autoradiography detects only active receptors in their native cellular microenvironment, and is often referred to as functional autoradiography. One advantage of the technique over

the classical receptor autoradiography is that several receptors can be studied simultaneously using a single radiolabelled compound.

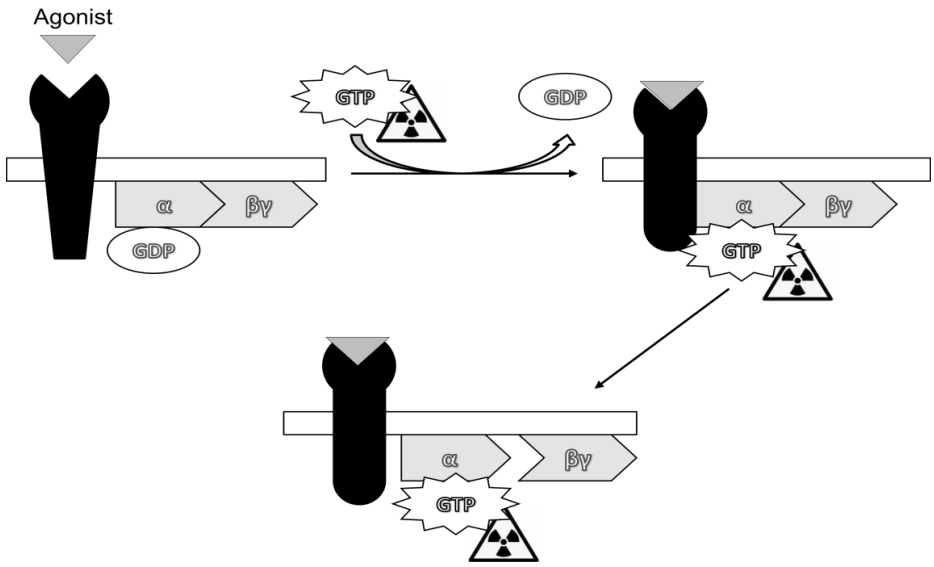


Figure 9. The principle of [^{35}S]GTPγS binding assay. The symbol of radioactivity refers to the ^{35}S of [^{35}S]GTPγS, a radiolabelled analog of GTP that is also resistant to hydrolysis and remains bound to the G protein α subunit resulting in the accumulation of radioactivity (modified from Sovago et al. 2001).

[^{35}S]GTPγS autoradiography was first introduced in 1994 to detect the light-stimulated G protein activity in the *Drosophila* compound eye (Yarfitz et al. 1994), and was applied to brain cryostat sections in 1995 (Sim et al. 1995). Thereafter, several examples of successful detection of many receptor systems in the brain have been published, including cholinergic, opioidergic, serotonergic, adrenergic, dopaminergic, histaminergic, GABAergic, cannabinoidergic, and lysophospholipid receptors (Sovago et al. 2001, Laitinen 2004). Altogether, approximately 20 distinct GPCRs have been successfully studied using the method, and the method has been applied to brain sections of different species, including man (Sovago et al. 2005). The method seems largely restricted to studies of the GPCR subtypes that signal through the G_i class of G proteins. This may be explained by the relative abundance of G_i in many cells over all other classes of heterotrimeric G proteins and by the relatively higher intrinsic rates of basal GDP–GTP exchange among the α subunits of this subclass (Laitinen 2004).

The general procedure of [^{35}S]GTPγS autoradiography is presented in Figure 10. It should be noted, that each assay protocol must be optimized for each particular receptor; e.g. assay buffer compositions and incubation times may vary. In order to detect agonist-stimulated responses, the method requires a large excess of GDP to inactivate G protein α subunits and to reduce basal [^{35}S]GTPγS binding (Sim et al. 1997, Sim-Selley & Childers 2002). In [^{35}S]GTPγS autoradiography, typically 1-2 mM of GDP is needed, in contrast to the membrane [^{35}S]GTPγS binding assay, where micromolar concentration of GDP (1-10 μM) is sufficient. This is partly explained by the higher protein content of the tissue sections compared to membrane preparations as well as by the high degradative capacity of brain sections. In brain sections, practically all cellular enzymes, including the nucleotide

hydrolases capable of hydrolyzing GDP, are potentially preserved. Due to the high degradative capacity of brain sections in the presence of a high GDP concentration, high concentrations of agonists are also needed. Sodium is required in the assay buffer to reduce basal binding by inhibiting spontaneously active receptors whereas magnesium is required to promote agonist-stimulated G protein activation (Sim et al. 1997, Sim-Selley & Childers 2002). The pH of the assay buffer must be appropriate to avoid tissue damage. Depending on the agonists used, other components can be included in the assay buffer. Bovine serum albumin (BSA, 0.1 - 0.5 %) needs to be included in the assay buffer to prevent lipophilic agonists, such as lysophospholipids and cannabinoids, from adsorbing to the tissue or containers. If one is examining peptide ligands, then protease inhibitors will be needed to prevent their degradation. The reducing agent dithiotreitol (DTT) can be included in the assay buffer to prevent non-specific binding of [^{35}S]GTPγS in tissue sections (Happe et al. 2001).

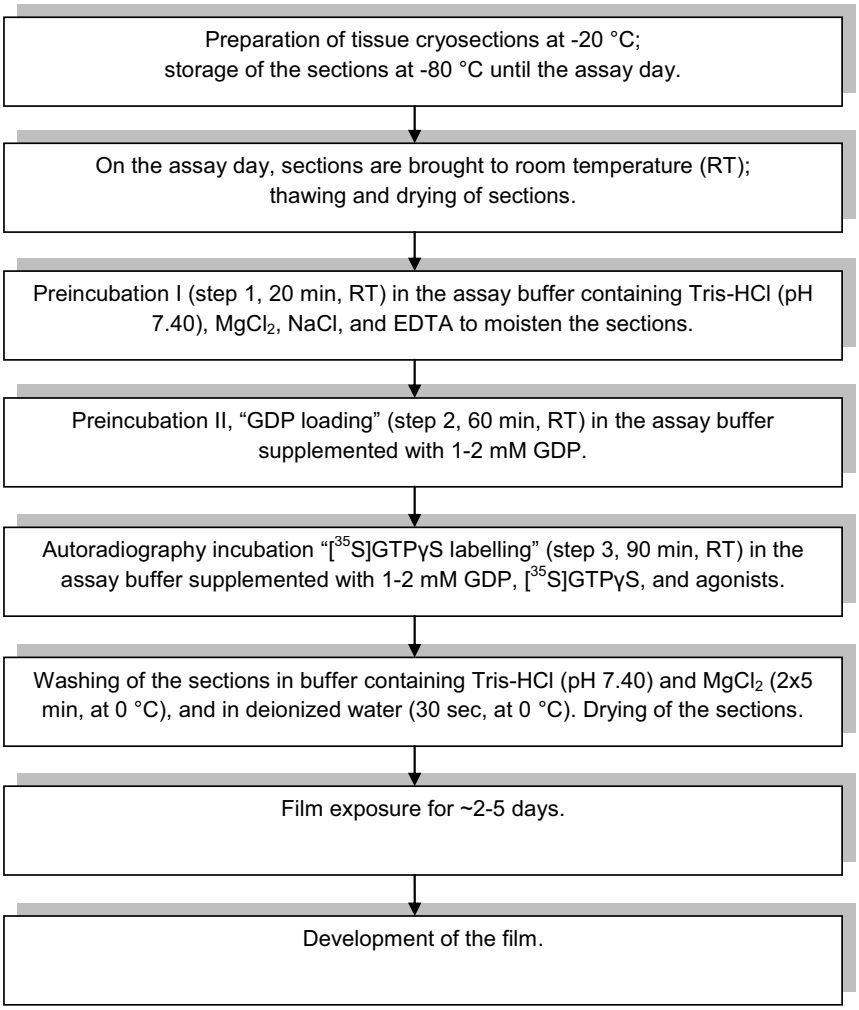


Figure 10. Typical procedure of the [^{35}S]GTPγS autoradiography method. Depending on the agonists used, the procedure might require some modification (modified from Sovago et al. 2001).

[^{35}S]GTPγS autoradiography has been proven to be suitable for studying brain lipid receptors, especially the G_i-coupled LPA_1, $S1P_1$, and cannabinoid CB_1 receptors. In [^{35}S]GTPγS autoradiography, the LPA-stimulated G protein activity is largely restricted to the developing white matter tracts in the brain of 4-week-old rats whereas S1P, as well as a $S1P_1$ receptor-selective agonist will evoke a robust signal throughout the gray matter areas both in the developing and adult rat brain (Waeber & Chiu 1999, Laitinen 2004, Sim-Selley et al. 2009) (Figure 11). Among other lysophospholipids – when modified assay conditions were used (4 °C temperature, 3 h incubation time, 0.1 mM GDP) – LPI induced [^{35}S]GTPγS binding response in the adult rat prefrontal cortex and hippocampus (Rojo et al. 2012). The cannabinoid CB_1 receptor-mediated [^{35}S]GTPγS binding response is prominent in several gray matter areas, including the cerebral cortex, hippocampus, caudate putamen, and the molecular layer of the cerebellum (Sim et al. 1996, Palomäki et al. 2007) (Figure 11), convergent with the mapping performed by the classical receptor autoradiography (Herkenham et al. 1991). In contrast to the abundantly expressed CB_1 and $S1P_1$ receptors, receptor densities for several classical GPCRs are low and [^{35}S]GTPγS responses therefore more restricted (Figure 11). Although CB_1 and $S1P_1$ receptors are widely expressed in the brain and exogenous agonists for these receptors produce robust responses, endogenous responses in the brain are local. For example, it is believed that the endogenous 2-AG signals within a 20-µm-distance from its site of origin (Castillo et al. 2012).

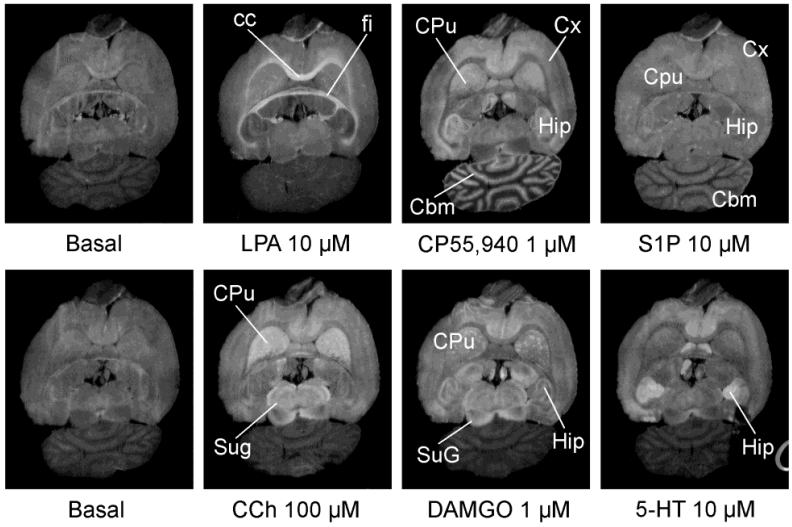


Figure 11. [^{35}S]GTPγS binding responses in horizontal brain sections of a 4-week-old rat evoked by lipid mediators (upper row) and classical neurotransmitters (lower row). Agonists used: LPA for LPA receptors, CP55,940 for cannabinoid receptors, S1P for S1P receptors, CCh (carbamylcholine) for muscarinic acetylcholine receptors, DAMGO for opioid receptors, and 5-HT for serotonin receptors. Abbreviations: Cbm, molecular layer of the cerebellum; cc, corpus callosum; CPu, caudate putamen; fi, fimbria of the hippocampus; Hip, hippocampus; SuG, superficial gray layer of the superior colliculus. Aaltonen 2008, unpublished.

One characteristic of the functional autoradiography is [^{35}S]GTPγS binding that is evident in several brain regions even without exogenously added agonists, i.e. activity under basal conditions. Endogenous receptor-activating ligands, such as neurotransmitters and neuromodulators, as well as their precursors may be retained in tissue sections or they may be formed during the incubations, contributing to the widespread and

heterogeneously distributed basal [^{35}S]GTPγS binding (Laitinen 1999, Palomäki & Laitinen 2006). A substantial portion of this basal labelling has been shown to derive from the endogenous formation of adenosine in brain sections during the incubations and consequent activation of the abundant and widely-distributed adenosine A_1 receptors (Laitinen & Jokinen 1998, Laitinen 1999, Moore et al. 2000). Therefore, one needs to deplete adenosine either with adenosine deaminase or selective blockade of the A_1 receptors with an antagonist (8-cyclopentyl-1,3-dipropylxanthine, DPCPX). Another source of basal binding in brain sections has been shown to be tonic LPA_1 receptor activity, especially in the developing white matter tracts (Palomäki & Laitinen 2006).

Recently, it was demonstrated that the comprehensive elimination of enzymatic hydrolysis of 2-AG in brain sections by irreversibly acting inhibitors leads to 2-AG accumulation and subsequent cannabinoid CB_1 receptor activation that can be successfully revealed using [^{35}S]GTPγS autoradiography (Palomäki et al. 2007). Evidently, brain sections preserve functional enzymatic machinery to generate endocannabinoids during the incubations. In addition to GPCR agonists and antagonists, enzyme activators and inhibitors thus represent promising novel approaches to study the life cycle of lipid modulators, i.e. a novel way of exploiting this technique.

3 Aims of the Study

The general objective of the study was to deepen the understanding of lipid GPCR signalling in the CNS. The study comprised of two main parts; the first part involved the development and optimization of the methodology used for studying lipid-GPCR signalling and the second part focused on the characterization of the enzymatic pathways responsible for the lipid messenger synthesis and degradation. The second part especially focused on the lipid phosphate phosphatases as the regulators of LPA–GPCR signalling and diacylglycerol lipases as the regulators of 2-AG–GPCR signalling.

The specific aims of this study were:

1. To characterize and optimize [^{35}S]GTPγS autoradiography method by mapping the rat brain regions with prominent [^{35}S]GTPγS binding in basal conditions.

2. To develop a LC/MS/MS method for the quantitative determination of LPA from brain tissue samples.

3. To reveal the enzymatic pathways regulating the levels of signalling LPA in rat brain sections, especially to clarify if pharmacological manipulation of LPA-synthesizing or -degradating pathways would affect the tonic LPA_1 receptor signal.

4. To investigate if comprehensive pharmacological blockade of 2-AG hydrolysis would result in the DAGL-mediated 2-AG accumulation in mouse brain sections and whether DAGL-deficiency would alter the functionality of CB_1 receptor-G_i axis.

4 General Experimental Procedures

4.1 MATERIALS

4.1.1 Chemicals (Chapters 5–8)

Chemicals used in the present study are presented in Table 6. All other chemicals were of the finest purity available.

Table 6. Chemicals used in the present study and their targets/functions.

Chemical	Target receptor/enzyme or other function	Origin
Receptor agonists		
DAMGO ([D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin)	Opioid	Tocris (Northpoint, UK)
CP55,940 ((−)-3-[2-Hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol)	Cannabinoid	Tocris (Northpoint, UK)
Neuropeptide Y	Neuropeptide Y	Tocris (Northpoint, UK)
Histamine	Histamine	Fluka (Buchs, Switzerland)
2-chloroadenosine	Adenosine	Sigma (St. Louis, MO, USA)
S1P (Sphingosine 1-phosphate)	S1P	Biomol (Plymouth Meeting, PA, USA)
(2S)-OMPT (1-oleoyl-2-methyl-*sn*-glycero-3-phosphothionate)	LPA_3	Avanti Polar Lipids (Alabaster, AL, USA)
16:0 LPA (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate)	LPA	Avanti Polar Lipids (Alabaster, AL, USA)
18:0 LPA (1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphate)	LPA	Avanti Polar Lipids (Alabaster, AL, USA)
20:4 LPA (1-arachidonoyl-2-hydroxy-*sn*-glycero-3-phosphate)	LPA	Avanti Polar Lipids (Alabaster, AL, USA)
18:1 LPA (1-oleyl-2-hydroxy-*sn*-glycero-3-phosphate)	LPA	Sigma (St. Louis, MO, USA)
AEA (Arachidonoylethanolamide, anandamide)	Cannabinoid	Cayman Chemical (Ann Arbor, MI, USA)
2-AG (2-arachidonoyl glycerol)	Cannabinoid	Cayman Chemical (Ann Arbor, MI, USA)
Receptor antagonists/ inverse agonists		
Naloxone	Opioid	Tocris (Northpoint, UK)
Thioperamide	Histamine H_3	RBI (Natick, MA, USA)
AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide)	Cannabinoid CB_1	Tocris (Northpoint, UK)
BIIE 0246 (*N*-[(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b*,*e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide)	Neuropeptide Y Y_2	Tocris (Northpoint, UK)

Continued

Table 6. Chemicals used in the present study and their targets/functions. *Continued*

Chemical	Target receptor/enzyme or other function	Origin
Receptor antagonists/ inverse agonists		
L-152,804 (5,5-Dimethyl-2-(2,3,4,9-tetrahydro-3,3-dimethyl-1oxo-1*H*-xanthen-9-yl)-1,3-cyclohexanedione)	Neuropeptide Y Y_5	Tocris (Northpoint, UK)
BIBP 3226 (*N*-[(1*R*)]-4-[(Aminoiminomethyl)amino-1-[[[(4hydroxyphenyl)methyl]amino]carbonyl]butyl-α-phenylbenzeneacetamide trifluoroacetate)	Neuropeptide Y Y_1	Bachem (Torrance, CA, USA)
DPCPX (8-cyclopentyl-1,3-dipropylxanthine)	Adenosine A_1	Sigma (St. Louis, MO, USA)
Ki16425 (3-(4-[4-([1-(2 chlorophenyl)ethoxy] carbonyl amino)-3-methyl-5-isoxazolyl]benzylsulfanyl propanoic acid))	$LPA_{1/3}$	Sigma (St. Louis, MO, USA)
VPC 23019 ((R)-phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester)	$S1P_{1/3}$	Avanti Polar Lipids (Alabaster, AL, USA)
Enzyme activators/inhibitors		
Propranolol	Non-specific LPP-inhibitor	Biomol (Plymouth Meeting, PA, USA)
Na_3VO_4	Non-specific LPP-inhibitor	Sigma (St. Louis, MO, USA)
THL (Tetrahydrolipstatin)	Non-specific lipase inhibitor	Sigma (St. Louis, MO, USA)
$AlCl_3$	Component of AlF_x^-	Merck (Darmstat, Germany)
NaF	Component of AlF_x^-	Merck (Darmstat, Germany)
MAFP (Methylarachidonoylfluorophosphonate)	Non-specific serine hydrolase inhibitor	Cayman Chemical (Ann Arbor, MI, USA)
JZL184 (4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5 yl(hydroxy)methyl)piperidine-1-carboxylate)	Specific MAGL inhibitor	Cayman Chemical (Ann Arbor, MI, USA)
PIP_2 (Phosphoinositol-4,5-bisphosphate)	PLD-activator	Avanti Polar Lipids (Alabaster, AL, USA)
Oleate	PLD-activator	Sigma (St. Louis, MO, USA)
Neomycin	PIP_2-depletor	Sigma (St. Louis, MO, USA)
1-butanol	Non-specific PLD inhibitor	Merck (Darmstat, Germany)
Compound 4k (5-fluoro-2-indolyl des-chlorohalopemide)	PLD-inhibitor	University of Eastern Finland, Kuopio, Finland
Other chemicals		
tert-butanol	Control compound for 1-butanol	Merck (Darmstat, Germany)
Nadolol	Control compound for propranolol	Sigma (St. Louis, MO, USA)
Glycerol 3-phosphate	LPA precursor	Sigma (St. Louis, MO, USA)
PA (Phosphatidic acid)	LPA precursor	Avanti Polar Lipids (Alabaster, AL, USA)
PC (Phosphatidylcholine)	LPA precursor	Sigma (St. Louis, MO, USA)
BSA (Bovine serum albumin, fatty acid-free)	Carrier protein	Sigma (St. Louis, MO, USA)
DTT (Dithiotreitol)	Reducing agent	Sigma (St. Louis, MO, USA)

Continued

Table 6. Chemicals used in the present study and their targets/functions. *Continued*

Chemical	Target receptor/enzyme or other function	Origin
Other chemicals		
NEM (N-ethylmaleimide)	Alkylating agent	Sigma (St. Louis, MO, USA)
GDP (Guanosine diphosphate)	Guanine nucleotide	Sigma (St. Louis, MO, USA)
GTPγS (Guanosine-5'-*O*-(3-thio)-triphosphate)	Guanine nucleotide used for determining non-specific binding	Sigma (St. Louis, MO, USA)
[^{35}S]GTPγS (Guanosine-5'-*O*-(3-[^{35}S]thio)-triphosphate)	Radioisotope-labelled guanine nucleotide	NEN Life Science Products Inc. (Boston, MA, USA)
DFOM (Deferoxamine mesylate)	Aluminium chelator	Sigma (St. Louis, MO, USA)
17:0 LPA (1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphate)	Internal standard	Avanti Polar Lipids (Alabaster, AL, USA)
AEA-d8 (Arachidonoylethanolamide-d8)	Internal standard	Cayman Chemical (Ann Arbor, MI, USA)
2-AG-d8 (2-arachidonoyl glycerol-d8)	Internal standard	Cayman Chemical (Ann Arbor, MI, USA)
Acetonitrile	Solvent	J.T. Baker (Deventer, The Netherlands)
Methanol	Solvent	J.T. Baker (Deventer, The Netherlands)
Chloroform	Solvent	Riedel-de Haen (Seelze, Germany)

4.1.2 Animals (Chapters 5–7)

4 week-old male Wistar rats were supplied by the National Laboratory Animal Centre (University of Eastern Finland, Kuopio, Finland). Approval for the harvesting of animal tissue was obtained from the local welfare officer of the University of Eastern Finland. The experiments did not involve any *in vivo* treatment. The animals were housed in groups of five to ten individuals per cage under standard laboratory conditions (12:12 h light–dark cycle, food and water *ad libitum*, 60% relative humidity). The rats were decapitated 7–9 h after lights on, and within the next 5 min, the whole brain was dissected out, dipped briefly in isopentane (chilled on dry ice) and stored at –80 °C. Horizontal, coronal or sagittal brain sections (20 μm thick) were cut according to Rat Brain Atlas (Paxinos & Watson 1998) at –19 °C to –21 °C using a Leica cryostat, thaw-mounted onto Superfrost®Plus slides (Menzel-Gläser, Germany), dried for 1–4 h at room temperature under a constant stream of air and stored thereafter at –80 °C.

4.2 METHODS

4.2.1 [^{35}S]GTPγS autoradiography (Chapters 5, 7, 8)

[^{35}S]GTPγS autoradiography was performed using a three-step (Chapters 7 and 8) or a two-step (Chapter 5) protocol. In the former procedure, the sections were processed in three sequential steps consisting of preincubation for 20 min (step 1), GDP-loading for 60 min (step 2) and [^{35}S]GTPγS labelling (step 3) for 90 min. In the two-step protocol, the GDP-loading step was omitted. All the steps in both protocols were performed at 20 °C using Tris-based buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$). In the three-step protocol, steps 2 and 3 included 2 mM GDP, whereas in the two-step protocol, 2 mM GDP was present only during the autoradiography step, which additionally included 1 mM DTT and 32–252 pM [^{35}S]GTPγS in both protocols. For selected slides, chemicals under interest and 0.1% BSA were included in the assay during the step 3, or if indicated, during the step 1 or step 2. In addition, some slides in each experiment were incubated in the presence of 10 μM GTPγS (step 3) to determine non-specific binding. After

the 90 min autoradiography step, the slides were rinsed twice (5 min each time) in ice-cold washing buffer (50 mM Tris-HCl, pH 7.4 and 5 mM $MgCl_2$), dipped for 30 s in ice-cold deionized water, air-dried and arranged into a cassette together with [^{14}C] standards (Amersham, Little Chalfont, Bucks, UK) and exposed against a radiosensitive film (BioMax MR™, Kodak Scientific Imaging Film) for 2–5 days. After exposure, the films were developed for 3–4 min at 4 °C with Kodak D-19 developer.

4.2.2 LC/MS/MS for LPA determination (Chapters 6, 7)

Extraction of LPA for mass spectrometric determination

Slides with two horizontal brain sections were incubated for 40 min in the presence of AlF_x (NaF 10 mM + $AlCl_3$ 50 μM) mimicking the [^{35}S]GTPγS autoradiography. The control slides were incubated similarly in the assay buffer. After a 40 min incubation, slides were rinsed twice (2 min each time) in ice-cold washing buffer, dipped for 30s in ice-cold deionized water and air-dried. The modified extraction method of Bligh and Dyer (1959) was applied for the isolation of LPA from the tissue matrix. One sample consisted of pooled tissue obtained from four slides. The brain tissue was scraped manually from the slides with a spatula using the mixture of 50 mM Tris-HCl, pH 7.40 and methanol with a ratio of 1:4 (v/v); this mixture also included an internal standard (17:0 LPA) used in the quantification. The tissue was transferred to a screw-capped Pyrex® borosilicate glass test tube. The mixture of 50 mM Tris-HCl, pH 7.40 and methanol (1:4, v/v) was added to the test tube to bring the volume up to 200 μl. Chloroform was added to yield a water/methanol/chloroform ratio of 1:4:2 (v/v/v) and the samples were shaken for 1 h with a vertical shaker (Heidolph Multi Reax, Heidolph Instruments GmbH & Co, Schwabach, Germany). To achieve the phase separation, 80 μl of chloroform and 80 μl of water were added. After vortexing for 1 min, the samples were centrifuged at 1800 x *g* for 15 min at room temperature. The upper aqueous layer was transferred to an HPLC sample vial.

Liquid chromatography-tandem mass spectrometry (LC/MS/MS)

The high-performance liquid chromatography (HPLC) system comprised of an Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Waldbronn, Germany) consisting of a solvent micro vacuum degasser, a binary pump, a thermostatted column compartment SL, and an autosampler SL. Ten microliter of sample solution were injected onto a reversed phase HPLC column (XBridge™ C8 2.1 × 50 mm, 2.5 μm) (Waters, Ireland) using gradient elution with 50 μM ammonium acetate + 1% triethylamine (TEA) (A) and 1% TEA in 90% methanol (B) as follows: 0–6.0 min 20% B → 90% B, 6.0–10.0 min 90% B, 10.0–10.1 min 90% B → 20% B, 10.0–15.0 min 20% B. The flow rate was 0.3 ml/min, column temperature was maintained at 40 ºC and the autosampler tray temperature was set to 10 °C.

The mass spectrometric analysis was carried out with an Agilent 6410 Triple Quadrupole LC/MS equipped with an electrospray ionization source (ESI; Agilent Technologies, Palo Alto, CA, USA). The following ionization conditions were used: ESI negative ion mode, drying gas (nitrogen) temperature 300 ºC, drying gas flow rate 8 l/min, nebulizer pressure 40 psi and capillary voltage 4000 V. Analyte detection was performed using multiple reaction monitoring (MRM) with the following transitions: *m/z* 409 → 153 for 16:0 LPA, *m/z* 437 → 153 for 18:0 LPA, *m/z* 435 → 153 for 18:1 LPA, *m/z* 457 → 153 for 20:4 LPA, and *m/z* 423 → 153 for 17:0 LPA. The fragmentor voltage was 160 V and collision energy 20 V, except 23 V for 17:0 LPA. Data were acquired by Agilent MassHunter Workstation Acquisition software (Agilent Technologies, Data Acquisition for Triple Quad., version B.01.03).

4.2.3 Statistical analysis (Chapters 5–8)

Autoradiography films were digitized using a HP scanjet 7400c scanner. For the quantitative data, optical densities of the autoradiograms were measured using ImageJ, a freely available java-based image analysis software system developed in the National Institutes of Health, USA (http://rsb.info.nih.gov/ij/). Optical densities were converted to nCi/g using nonlinear transformation based on the greyscale values of [^{14}C] standards. Quantitative data were calculated as nCi/g equivalents with non-specific binding subtracted from total binding.

In LC/MS/MS experiments, an internal standard was used for quantification, and peak area ratios of the analyte to the IS were calculated as a function of the concentration ratios of the analyte to the internal standard using Agilent MassHunter software (Quantitative Analysis Version B.01.03). For Figure 25, the protein content of brain sections was determined by the Pierce BCA Protein Assay Kit with BSA as the standard.

Unless otherwise stated, the statistical differences were determined using one-way ANOVA with Tukey's multiple comparison *post hoc* test with $^{***}p < 0.001$, $^{**}p < 0.01$, or $^{*}p < 0.05$ considered as statistically significant. All statistical data analyses were conducted using GraphPad Prism® (version 4.03 or 5.03) for Windows (San Diego, CA, USA).

5 [^{35}S]GTPγS Binding Under Basal Conditions[1]

Abstract: [^{35}S]GTPγS autoradiography represents a powerful functional approach to detect receptor-dependent G_i protein activity in anatomically defined brain structures. Inherent in this technique, however, is the notable basal signal evident in several brain regions in the absence of receptor stimulation by exogenously added agonist. In the rat brain, much of this basal labelling derives from tonic activation of adenosine A_1 and LPA_1 receptors in the grey and white matter regions, respectively. Despite the elimination of the two receptor activities, prominent basal [^{35}S]GTPγS labelling is still evident in discrete brain structures, possibly reflecting regional enrichment of G_i and/or constitutive receptor activity or the presence of still unknown endogenous ligands activating their orphan receptors. Here, the anatomical distribution of the enhanced basal signal was systematically mapped in brain sections of 4-week-old male Wistar rats. Regions with prominent basal [^{35}S]GTPγS labelling represented neuroanatomically distinct structures, in particular various thalamic and hypothalamic nuclei. For instance, the paraventricular thalamic nucleus, the bed nucleus of the stria terminalis and the subfornical organ were highly labelled, as were the periaqueductal gray and the nucleus of the solitary tract. Pre-treatment with *N*-ethylmaleimide, an alkylating agent preventing all known receptor-driven G protein activity in cryostat sections markedly decreased the basal binding in all examined regions. In preliminary screening, selective antagonists for various brain-enriched G_i-coupled receptors failed to suppress the basal signal in any of the studied regions.

[1] Adapted with permission of Elsevier from: **Aaltonen N, Palomäki VAB, Lecklin A, Laitinen JT. Neuroanatomical mapping ofjuvenile rat brain regions with prominent basal signal in [^{35}S]GTPγS autoradiography. J Chem Neuroanat 35: 233-241, 2008.**

5.1 INTRODUCTION

Inherent to the functional autoradiography is that there is a certain level of [^{35}S]GTPγS binding evident in several brain regions even without exogenously added agonists, i.e. activity under basal conditions. A substantial portion of this basal labelling has been shown to derive from the endogenous formation of adenosine in brain sections during the incubations and consequent activation of the abundant and widely-distributed adenosine A_1 receptors (Laitinen & Jokinen 1998, Laitinen 1999, Moore et al. 2000). Tonic adenosine A_1 receptor activity is prominent in the grey matter regions and is particularly evident in the molecular and granular cell layers of the cerebellum and in the cerebral cortex (Figure 12a). The adenosine signal can be efficiently blocked by the selective adenosine A_1 receptor antagonist DPCPX or by the adenosine degrading enzyme adenosine deaminase (Laitinen & Jokinen 1998, Laitinen 1999, Moore et al. 2000). Recently, another source of basal binding in brain sections was shown to be tonic LPA_1 receptor activity, especially evident in the developing white matter areas, e.g. the corpus callosum and the fimbria of the hippocampus, which in turn can be eliminated using a potent $LPA_{1/3}$ receptor-preferring antagonist Ki16425 (Palomäki & Laitinen 2006) (Figure 12a). However, even after elimination of the two above-mentioned signals, prominent basal labelling is still notable deep in the brain in several midline structures (Figure 12b). As a prelude to ongoing studies aiming at further characterization and optimization of [^{35}S]GTPγS autoradiography with brain sections, we have undertaken the first systematic anatomical mapping of the adenosine A_1 and LPA_1 receptor-independent basal [^{35}S]GTPγS signal in brain sections of 4-week-old male Wistar rats and identify the brain structures involved.

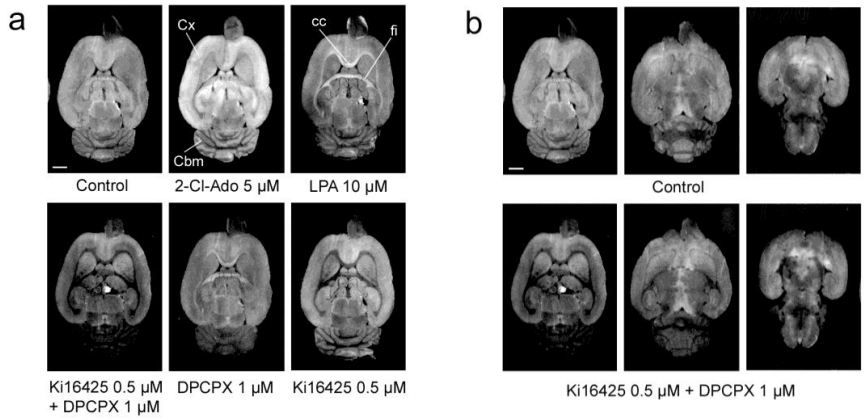


Figure 12. (a) The adenosine signal is prominent throughout the majority of grey matter areas, especially in the molecular cell layer of the cerebellum (Cbm) and in the cerebral cortex (Cx) whereas the LPA signal is evident in the developing white matter tracts, such as the corpus callosum (cc) and the fimbria of the hippocampus (fi). Treatment of rat brain sections with the adenosine A_1 receptor antagonist DPCPX and/or the $LPA_{1/3}$ receptor antagonist Ki16425 reduces [^{35}S]GTPγS binding compared to the control condition without any added ligands. (b) The effect of DPCPX and Ki16425 compared to the basal condition without added ligands in different levels of the rat brain. Note that even after elimination of tonic adenosine and LPA receptor signals, some prominent basal [^{35}S]GTPγS labelling is evident, especially in several midline structures. Scale bar = 2 mm.

5.2 MATERIALS AND METHODS

The materials and general methods are described in Chapter 4.

5.2.1 Tissue staining

To facilitate the identification of neuroanatomical structures, adjacent sections to those used in [^{35}S]GTPγS autoradiography (eight individual animals) were stained with Luxol Fast Blue and Cresyl Violet staining according to Geisler et al. (2002). This method allows simultaneous demonstration of cell bodies and myelinated fibre tracts in the mammalian brain.

5.3 RESULTS AND DISCUSSION

5.3.1 Optimization of the assay conditions

The three-step standard protocol for [^{35}S]GTPγS autoradiography comprises of a preincubation for 20 min, GDP loading for 60 min, and [^{35}S]GTPγS labelling for 90 min. For practical and economical reasons, we first tested whether the second step was necessary if one wished to visualize the basal labelling. These experiments indicated that [^{35}S]GTPγS binding throughout the brain sections was higher without the GDP loading step. For example, quantitative comparison of optical density values (mean ± SD, n=2) indicated that the two-step protocol resulted in 6.3 ± 2.1 % higher values for the cortex, 5.7 ± 2.0 % for caudate putamen and 4.8 ± 1.8 % for hippocampus. Nonetheless, the enhanced basal signal was clearly visible in the typical brain structures (data not shown). Additional experiments revealed that the inclusion of 1 mM DTT during the [^{35}S]GTPγS labelling step clarified the boundaries of the labelled structures to some extent (data not shown). Different assay buffer compositions were also investigated, especially in the light of findings demonstrating that the use of a glycylglycine-based assay buffer instead of the more common Tris-based buffer resulted in reduction of overall basal binding (Happe et al. 2001). In our experiments, however, basal binding was not significantly lower when a glycylglycine buffer was used instead of Tris buffer (data not shown). Based on these preliminary experiments, all subsequent studies were performed in Tris buffer using the two-step protocol consisting of preincubation for 30 min and [^{35}S]GTPγS labelling for 90 min. In addition, 1 mM DTT, 1 μM DPCPX and 0.5 μM Ki16425 were included in the [^{35}S]GTPγS labelling step.

5.3.2 Brain structures with enhanced basal [^{35}S]GTPγS labelling

In the brain sections of 4-week-old rats, the prominent regions with high basal [^{35}S]GTPγS binding were largely localized near the midline and often in the close vicinity of the cerebral ventricles, representing phylogenetically conserved brain structures, including various thalamic and hypothalamic nuclei (Table 7, Figure 13, Figure 14). We also compared autoradiographs of 4-week-old animals to those of adult (12-week-old) animals and found that the same structures showed enhanced labelling in both age-groups under basal conditions (data not shown).

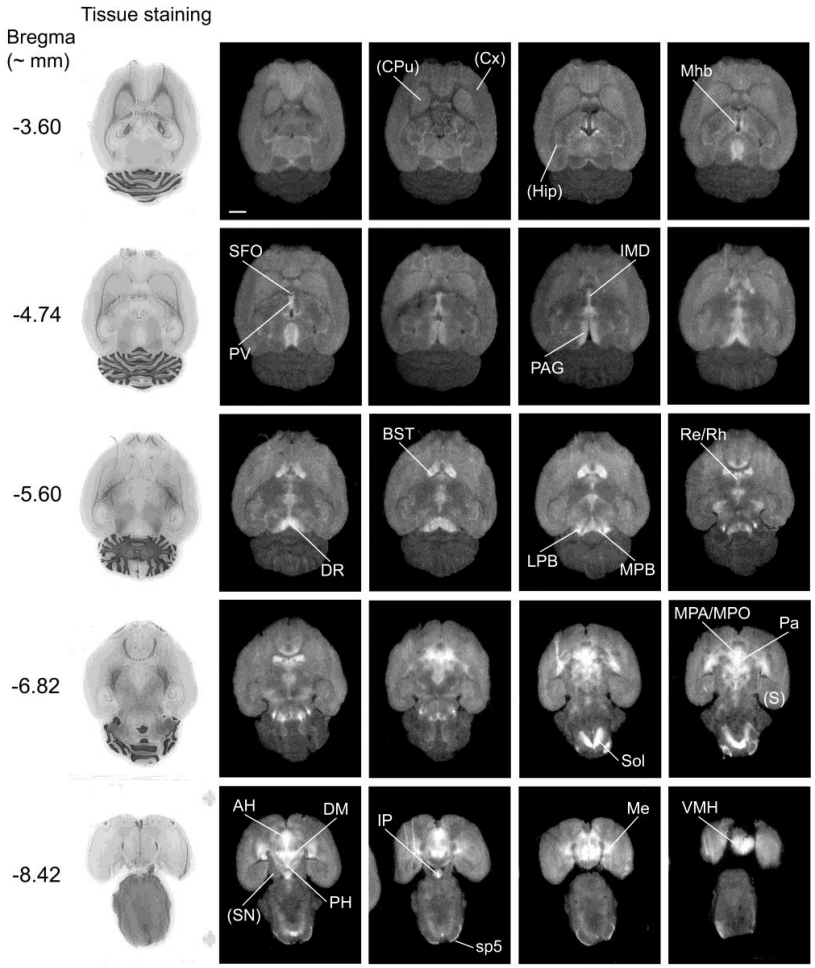


Figure 13. The prominent basal [^{35}S]GTPγS signal is heterogeneously distributed and localizes to the deep brain structures. Luxol Fast Blue and Cresyl Violet staining was performed on adjacent sections. The number shown in the left indicates the distance (mm) of the first section in the row from the Bregma. Each of the stained sections is adjacent to the section shown in the left panel whereas the distance between other sections illustrated in this figure is 200–300 µm. Structures in the parentheses indicate some of the areas with no prominent labelling. Abbreviations: AH, anterior hypothalamic area; BST, bed nucleus of the stria terminalis; CPu, caudate putamen (striatum); Cx, cerebral cortex; DM, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; Hip, hippocampus; IMD, intermediodorsal thalamic nucleus; IP, interpeduncular nucleus; LPB, lateral parabrachial nucleus; Me, medial amygdaloid nucleus; MHb, medial habenular nucleus; MPA, medial preoptic area; MPB, medial parabrachial nucleus; MPO, medial preoptic nucleus; Pa, paraventricular hypothalamic nucleus; PAG, periaqueductal gray; PH, posterior hypothalamic area; PV, paraventricular thalamic nucleus; Re, reuniens thalamic nucleus; Rh, rhomboid thalamic nucleus; S, subiculum; SFO, subfornical organ; SN, substantia nigra; Sol, nucleus of the solitary tract; sp5, spinal trigeminal tract; VMH, ventromedial hypothalamic nucleus. Scale bar = 2 mm.

Table 7. Adenosine A_1 and LPA_1 receptor-independent basal [^{35}S]GTPγS binding in 4-week old male Wistar rat brain. Rat brain sections (horizontal cut) were processed for [^{35}S]GTPγS autoradiography using a two-step protocol and the autoradiographs were digitized and quantified. The radioligand concentration was 32 pM in this set of incubations. Data are expressed as nCi/g equivalents with non-specific binding (determined with 10 μM GTPγS) subtracted from the total and are the mean ± S.E.M. from six individual animals (n = 6).

Structure	**[^{35}S]GTPγS binding (nCi/g)**	**Structure**	**[^{35}S]GTPγS binding (nCi/g)**
Structures with relatively low and homogeneous basal binding		*Preoptic area*	
Caudate putamen	208 ± 6	Bed nucleus of the stria terminalis	596 ± 17
Cerebral cortex	144 ± 7	Medial preoptic area	742 ± 33
Hippocampus	175 ± 7	Medial preoptic nucleus	822 ± 43
Subiculum	239 ± 9		
Substantia nigra	200 ± 10	*Amygdala*	
		Medial amygdaloid nucleus	761 ± 27
Thalamus			
Medial habenular nucleus	363 ± 18	*Midbrain*	
Intermediodorsal thalamic nucleus	342 ± 13	Dorsal raphe nucleus	545 ± 28
Paraventricular thalamic nucleus	455 ± 23	Interpeduncular nucleus	651 ± 12
Reuniens thalamic nucleus	377 ± 25	Periaqueductal gray	363 ± 17
Rhomboid thalamic nucleus	318 ± 15		
Subfornical organ	287 ± 19	*Pons*	
		Lateral parabrachial nucleus	561 ± 15
Hypothalamus		Medial parabrachial nucleus	462 ± 18
Anterior hypothalamic area	643 ± 38		
Dorsomedial hypothalamic nucleus	836 ± 28	*Medulla oblongata*	
Paraventricular hypothalamic nucleus	549 ± 39	Nucleus of the solitary tract	942 ± 44
Posterior hypothalamic area	568 ± 48		
Ventromedial hypothalamic nucleus	795 ± 31	*Spinal cord*	
		Spinal trigeminal tract	393 ± 4

The regions with prominent basal [^{35}S]GTPγS labelling seem to belong to those participating in the regulation of primitive vital functions and behaviour. For instance, the nucleus of the solitary tract plays an important role in controlling cardiovascular, respiratory, gustatory, hepatic and renal mechanisms (Lawrence & Jarrott 1996). The subfornical organ participates in the regulation of cardiovascular system and electrolyte homeostasis as well as adjusting thirst (Noda 2006, Fry & Ferguson 2007). The bed nucleus of the stria terminalis is involved in emotional behaviour induced by stressful and anxiogenic stimuli (Forray & Gysling 2004, Choi et al. 2007). Many of the studied regions, including the habenular or the parabrachial nucleus, function as an important link between the forebrain and the brainstem (Sandyk 1991, Chen et al. 2004). The parabrachial nucleus also regulates taste sensation and gastrointestinal activity as well as cardiovascular and respiratory system (Benarroch 2006). The hypothalamus has an important role in the regulation of motivated behaviour and hormone release, as well as in the maintenance of body temperature. In line with details that can be observed from the autoradiographs of

previously published papers, several hypothalamic nuclei were intensively labelled under the basal conditions, supporting our earlier argumentation that non-optimal signal-to-noise ratios are expected for receptor-dependent responses due to the high basal [^{35}S]GTPγS labelling in this region (Laitinen 2004).

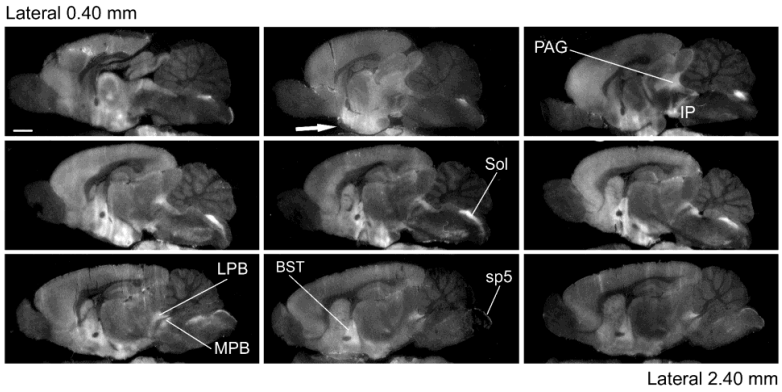


Figure 14. The adenosine A_1 and LPA_1 receptor-independent basal [^{35}S]GTPγS labelling is largely concentrated over midline structures of the brain. The numbers (Lateral) in the figure indicate the distance (mm) of the first and last section from the midline of the brain. The distance between sections illustrated in this figure is 120 µm. A prominent basal [^{35}S]GTPγS labelling is detectable in the same midline structures shown in the horizontal sections in Figure 12. The boundaries of the hypothalamic structures (arrow) are not as discrete as they are in the horizontal sections. Abbreviations: BST, bed nucleus of the stria terminalis; IP, interpeduncular nucleus; LPB, lateral parabrachial nucleus; MPB, medial parabrachial nucleus; PAG, periaqueductal gray; Sol, nucleus of the solitary tract; sp5, spinal trigeminal tract. Scale bar = 2 mm.

5.3.3 Sensitivity to NEM

Since treatment of brain sections with pertussis toxin is not feasible (our unpublished observations), the sulfhydryl-alkylating reagent NEM has been successfully used to uncouple G_i-linked receptors from their cognate G proteins in brain sections due to its ability to irreversibly alkylate the sulfhydryl groups of cysteine residues, including those in the C-terminus of the G_i protein α-subunits. Treatment with NEM therefore reveals if members of the G_i class of G proteins are involved in certain signalling processes. In [^{35}S]GTPγS autoradiography studies, both basal signal and agonist-induced responses are sensitive to NEM, as observed for several GPCRs (Waeber & Chiu 1999, Laitinen 2004, Palomäki & Laitinen 2006). In the present study, pre-treatment with NEM (1 mM) for 30 min clearly decreased the overall labelling observed under basal conditions (Figure 15), suggesting that G_i activity likely accounts for the [^{35}S]GTPγS binding to brain sections, including regions with high basal signal. Therefore, it is reasonable to speculate that the basal labelling is, at least partly, due to GPCR activity, and perhaps induced by so far unidentified receptor agonists and/or high local density of other types of proteins capable of binding [^{35}S]GTPγS. Receptor-activating ligands might be endogenously present in sections or could be generated from their precursors during the incubations. It should be emphasized, however, that the heterogeneously distributed basal signal is inherent to the [^{35}S]GTPγS autoradiography technique, and does not necessarily reflect the situation in the brain *in vivo*.

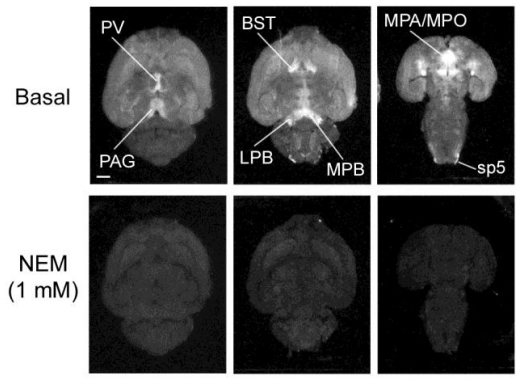


Figure 15. Treatment with the irreversible sulfhydryl-alkylating reagent *N*-ethylmaleimide (NEM) reduces overall [^{35}S]GTPγS labelling and completely abolishes the basal labelling in [^{35}S]GTPγS autoradiography. Horizontal rat brain sections were incubated for 10 min in assay buffer, followed by incubation for 30 min with 1 mM NEM in the same buffer. After a 10 min wash, [^{35}S]GTPγS autoradiography was conducted using a two-step protocol. Abbreviations: BST, bed nucleus of the stria terminalis; LPB, lateral parabrachial nucleus; MPA, medial preoptic area; MPB, medial parabrachial nucleus; MPO, medial preoptic nucleus; PAG, periaqueductal gray; PV, paraventricular thalamic nucleus; sp5, spinal trigeminal tract. Scale bar = 2 mm.

5.3.4 Constitutive receptor activity or orphan GPCRs and their ligands as a potential source of enhanced basal signal

Some GPCRs have the ability to spontaneously adopt an active conformation in the absence of receptor-activating endogenous ligand, i.e. they display constitutive activity (Bond & Ijzerman 2006). Constitutive activity can be reversed with inverse agonists that have the ability to shift the equilibrium towards the inactive receptor conformation. For instance, the histamine H_3 receptor, which can display high constitutive activity in rat brain preparations (Morisset et al. 2000, Rouleau et al. 2002), localizes to some extent in the same regions as the observed basal signalling. However, in our hands, the H_3 receptor inverse agonist thioperamide was unable to suppress the basal binding in any of these regions (data not shown). With respect to other GPCRs, our previous studies have provided no evidence for the constitutive activity of the brain-enriched adenosine A_1 or cannabinoid CB_1 receptors in [^{35}S]GTPγS binding assays (Laitinen 1999, Savinainen et al. 2003). However, these observations do not rule out the possibility that constitutive GPCR activity, mediated by still unidentified receptors, could be responsible for some of the labelling in brain regions with prominent basal signal.

Periaqueductal gray is known to be an important mediator of opioid receptor-mediated analgesia (Gray et al. 2006). In our study, the opioid receptor antagonist naloxone was not able to reduce the basal binding significantly in this, or in any other brain region examined (data not shown) indicating that tonic opioid receptor activity was not responsible for the high basal [^{35}S]GTPγS labelling in this brain region. Neither selective antagonists/ inverse agonists for cannabinoid CB_1 and CB_2 receptors nor the S1P receptor antagonist VPC 23019 were able to reduce the basal signal detectably in any of the brain regions examined. Moreover, no reduction in basal labelling was achieved with the selective neuropeptide Y Y_1 receptor antagonist BIBP 3226, the Y_2 receptor antagonist BIIE 0246 or the Y_5 receptor antagonist L-152,804, although these NPY receptor subtypes are known to be present in several brain regions, including various hypothalamic nuclei. In addition to the receptors examined in this study, Waeber and Moskowitz (1997) have tested the effect of several 5-

HT receptor antagonists on basal binding, but none of the antagonists used (NAN-190, GR-127,935, methiothepine) reduced the basal signal in the studied brain regions. Furthermore, a specific α_2-adrenoceptor antagonist RX821002 was not able to reduce basal [^{35}S]GTPγS binding (Happe et al. 2000).

The hypothalamus appears to host most of the still orphan endoGPCRs, as well as many of the known GPCRs (Vassilatis et al. 2003). On the other hand, over 30 neurotransmitters or neuromodulators have been documented to be present in the nucleus of the solitary tract (Lawrence & Jarrott 1996), which was also the most intensely labelled structure in the basal conditions used in our study. Thus it is not surprising that although selective antagonists for various brain enriched receptors were tested in our experiments, none of them was able to decrease the basal signal in any of the studied regions. In addition, some ligands, especially those with a peptide structure may dissociate slowly from their receptors, contributing to prolonged biological activity (Haskell-Luevano et al. 1996). It might be possible that binding of such slowly dissociating ligands explain some of the basal [^{35}S]GTPγS labelling.

5.3.5 Regional enrichment of heterotrimeric G proteins and/or other classes of GTP-binding proteins as a potential source of enhanced basal signal

One contributor to the heterogeneously distributed basal signal could be a high local concentration of the heterotrimeric G proteins in a particular brain region. Although [^{35}S]GTPγS autoradiography principally detects agonist-dependent activity of G_i-coupled receptors (Laitinen 2004) in brain sections and $G\alpha_o$, the most abundant member of the G_i family of G proteins shows widespread distribution throughout the rat brain neuropil (Worley et al. 1986), other classes of heterotrimeric G proteins, namely G_s, G_q and G_{12} could also contribute to the heterogeneously distributed basal signal in a region-specific manner. Although not yet systematically studied, the so far published autoradiographs representing basal [^{35}S]GTPγS binding allows one to draw the conclusion that the labelling in several regions with intense basal binding appears to be dramatically reduced in brain sections of $G\alpha_o$-deficient mice (Jiang et al. 2001). This supports the view that the G_i class of G proteins, the brain-enriched $G\alpha_o$ in particular, is the major determinant of labelling under basal conditions of [^{35}S]GTPγS autoradiography. It is not yet known to what extent other classes of GTP binding proteins, such as the monomeric G proteins might contribute to [^{35}S]GTPγS binding to tissue sections under the assay conditions of autoradiography. In addition to the above-mentioned "classical" G proteins, several classes of cellular proteins can additionally bind and utilize GTP for functional purposes (Wennerberg & Der 2004), and are therefore broadly classified as GTP-binding proteins (G proteins). Since brain cryostat sections are expected to retain also such GTP-binding proteins, the presence of such proteins may in part, explain some of the basal signal in [^{35}S]GTPγS autoradiography.

5.4 CONCLUSIONS

The present study has provided the first systematic neuroanatomical mapping of the adenosine A_1 and LPA_1 receptor-independent basal [^{35}S]GTPγS signal in the developing rat brain. Our study reveals that the enhanced basal binding is mainly localized in the phylogenetically conserved brain structures, such as several thalamic and hypothalamic nuclei that develop at an early age. The basal signal could be diminished by incubation with the irreversible sulfhydryl-alkylating reagent, NEM, which is known to abolish all known agonist-induced G protein activity in [^{35}S]GTPγS autoradiography. In contrast, selective antagonists towards various brain-enriched receptors failed to suppress the basal signal in any of the studied regions. The basal signal could reflect regional enrichment of

various members of the GTP-binding protein family and/or constitutive activity of unknown GPCRs or the presence of still unidentified ligands capable of activating their receptors.

It may prove difficult to identify individual factors contributing to the heterogeneously-distributed basal signal in brain cryosections. Nonetheless, attempts to identify these factors should be an important goal for further efforts to improve the signal-to-noise ratio of the method. Systematic approach to test several additional antagonists might be needed and based of the presently mapped anatomical structures, should now be more feasible. A more systematic manipulation of assay conditions such as buffer ion composition might also be worth testing in future experiments, as this might facilitate the detection of constitutive GPCR activity.

6 Development of LC/MS/MS Method for LPA Analysis[2]

Abstract: A highly selective and sensitive liquid chromatography-tandem mass spectrometry (LC/MS/MS) method was developed for the determination of LPAs (16:0 LPA, 18:0 LPA, 18:1 LPA, 20:4 LPA) in rat brain cryosections. After partitioning the LPAs from other lipophilic material present in the tissue with a liquid-liquid extraction, a reversed-phase column and ion pair technique was used for separating analytes with a gradient elution. An internal standard (17:0 LPA) was included in the analysis. Detection and quantification of the LPAs were carried out with a triple quadrupole mass spectrometer using negative electrospray ionization (ESI) and multiple reaction monitoring (MRM). The artificial formation of LPAs from lysophosphatidylcholines during the sample preparation procedure and instrumentation was carefully studied during the method development. The method was validated; acceptable selectivity, accuracy, precision, recovery, and stability were obtained within the calibration curve range of 36–1790 fmol of LPAs. The quantification limit of the assay was 54 fmol injected into column for each LPAs. The method was applied to comparative studies of LPA levels in rat brain cryosections after the various chemical pre-treatments of the sections.

[2] Adapted with permission of Elsevier from: **Aaltonen N, Laitinen JT, Lehtonen M. Quantification of lysophosphatidic acids in rat brain tissue by liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr B 878: 1145-1152, 2010.**

6.1 INTRODUCTION

Recent advances in LPA research have revealed its potential therapeutic and diagnostic usefulness as well as the need for development of selective and highly sensitive analytical methods, for instance in the diagnostics of the very malignant ovarian cancer (Xu et al. 1998, Meleh et al. 2007). Several methods have been developed to analyze LPA levels in biological samples. LPA has been detected from plasma, serum and other body fluids as well as from tissue homogenates. In most cases, LPA is first extracted from the biological matrix using modified liquid-liquid extraction methods as described by Folch et al. (1957) or Bligh and Dyer (1959). Extraction procedures used together with strong acids (Shan et al. 2008, Georas et al. 2007, Tokumura et al. 2009), however, raises the concern about artificial formation of LPA from LPC under highly acidic conditions (Scherer et al. 2009). There are several described methods for determining the total LPA content e.g. bioassay (Tigyi & Miledi 1992), immunoassay (Chen et al. 2000), and radioenzymatic assay (Saulnier-Blache et al. 2000) but there are some limitations in those methods. Bioassays determine the biological effects elicited by LPA, such as the changes in the calcium-dependent chloride currents in voltage-clamped *Xenopus* oocytes (Tigyi & Miledi 1992). Although sensitive, bioassays are susceptible to disturbance by interfering compounds present in biological samples. Immunoassay (Chen al. 2000) suffers from the poor selectivity of the antibody and finally the radioenzymatic assay (Saulnier-Blache et al. 2000) requires the use of radioactive reagents.

A general approach used to quantify individual LPA species from biological samples has been gas chromatographic (GC) analysis (Xu et al. 1998, Sugiura et al. 1999, Nakane et al. 2002). Nonetheless, as an indirect method, GC requires thin layer chromatography purification as well as hydrolysis and derivatization of non-volatile LPA prior to analysis and thus is extremely laborious and time-consuming. Moreover, some indirect analysis protocols have not included appropriate internal standards in the assay (Xu et al. 1998). HPLC (Holland et al. 2003) and capillary electrophoresis (Chen & Xu 2001) methods have also been used though light-scattering detection and indirect ultraviolet detection are rather insensitive and unselective.

Higher sensitivity and selectivity is obtained by mass spectrometric (MS) detection. Some authors have reported analysis of phospholipids by flow injection directly coupled to MS (Xiao et al. 2001, Yoon et al. 2003). Unfortunately, flow injection can be problematic since there is no prior chromatographic separation to avoid ion suppression effects from highly abundant phospholipid species and other matrix components (Shan et al. 2008) and also artificial conversion of other lysophospholipids to LPA at the ion source has been demonstrated (Zhao & Xu 2009). However, high-throughput shotgun lipidomics provides a powerful tool for untargeted analysis of total lipid extracts, including hundreds of molecular species of glycerophospholipids, glycerolipids, and sphingolipids (Ejsing et al. 2009, Ståhlman et al. 2009). Preferred methods for targeted quantifying of the levels of individual LPA species from biological samples are liquid chromatography-mass spectrometry (LC/MS) (Baker et al. 2001) and liquid chromatography tandem mass spectrometry (LC/MS/MS) methods (Georas et al. 2007, Shan et al. 2008, Tokumura et al. 2009, Scherer et al. 2009).

Previously, substantial amounts of LPA species were found in rat brain by GC analysis (Sugiura et al. 1999, Nakane et al. 2002). Here we report the highly selective and sensitive LC/MS/MS method for quantitative measurement of LPA species from rat brain cryosections. As far as we are aware, this is the first time that LPA has been measured from brain tissue by LC/MS/MS.

6.2 MATERIALS AND METHODS

The materials and general methods are described in Chapter 4.

6.2.1 Preparation of standards

The stock solutions of 16:0 LPA, 18:0 LPA, 18:1 LPA and 20:4 LPA and internal standard (IS) (17:0 LPA) were prepared by dissolving the compounds in methanol. The stock solutions were stored at –20 °C. Standard working solutions were prepared daily in methanol to first obtain a concentration of 2.0 μM and 0.6 μM for IS. The dilutions from calibration standard working solutions were prepared in methanol to give concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 μM. An internal standard was added into each sample to obtain a final concentration of 0.3 μM. Quality control (QC) sample working solutions (0.03, 0.1, 0.3 and 0.8 μM) were prepared in methanol. An internal standard was added into each solution to obtain a final concentration of 0.3 μM. To prepare the calibration and QC samples, 100 μl of each particular standard solution, 80 μl of 50 mM Tris-HCl (pH 7.40), and 220 μl of methanol were mixed to obtain 400 μl of solution. Each calibration and QC sample was then prepared according to Chapter 4.2.2 starting from the chloroform addition into the sample.

6.2.2 Assay validation

This method was validated in terms of selectivity, linearity, precision, accuracy, recovery, and stability (Guidance for Industry 2001, van de Merbel 2008). LPAs are endogenous compounds and a brain matrix with no analytes is not available. Therefore 4% (w/v) BSA in Tris-buffer was used as a surrogate matrix (van de Merbel 2008). Homogenized rat brain tissue was used as the authentic biological matrix. The whole brain was homogenized in 50 mM Tris-HCl (pH 7.40) buffer using a Soniprep 150 homogenizer (MSE Ultrasonic Disintegrator; MSE Scientific Instruments, Manor Royal, Crawley, Sussex, UK) and samples were prepared according to Chapter 4.2.2. The volume of the homogenate in each sample corresponded to that of the tissue cryosection samples.

The selectivity of the method was assessed by analyzing reference standards, tissue samples, buffers, and solvents for interfering peaks at the retention times of LPAs. The standard addition method was used to further study the selectivity and matrix effect by spiking two concentrations of standards (0.2 and 0.4 μM) into the brain tissue homogenate to obtain regression curves for each LPA species (van de Merbel 2008). The slopes of the curves were compared to the slopes of standard curves prepared without tissue matrix using analysis of covariance (ANCOVA). Selectivity was also studied by diluting the tissue samples 1:2, 1:5 and 1:10 with methanol before injecting into LC/MS/MS (Schuhmacher et al. 2003) and comparing concentrations of diluted samples and undiluted samples. An in-source fragmentation experiment with 18:1 LPC molecule (Little et al. 2006) was performed in order to probe ionization matrix effects by other molecular species, such as glycerophosphocholines and lysophosphatidylserines. The transition m/z 281 → 281 was utilized to monitor 18:1 fatty acid anion, which is an important fragment in all phospholipids, distinct from LPAs. Furthermore, a post-column infusion experiment was performed to evaluate the ion suppression after the injection of the tissue sample. The infusion setup consisted of a syringe pump and a post-column T-piece as reported elsewhere (Bonfiglio et al. 1999).

The linearity of the assay for each of the analytes was assessed by analyzing the calibration curves from six concentrations of calibration samples in triplicate covering the range of 36–1790 fmol of LPA injected into the column. The calibration curve included samples without analytes including IS (a blank sample) and excluding IS (a zero sample).

Unweighted linear regression analysis was used to make the calibration curve. Correlation coefficients were also calculated. The lower limit of quantification (LLOQ) was determined by calculating precision and accuracy for five LLOQ samples that were independent of the calibration curve. The intra-day precision of the assay was assessed by calculating the relative standard deviation (RSD) for the analysis of QC samples in five replicates, and inter-day precision was determined by the analysis of QC samples on three days. Accuracy was assessed by calculating the deviation of the measured value from the nominal value, which compared the calculated and known concentrations. BSA was used as a surrogate matrix to the above mentioned experiments (van de Merbel 2008) and samples were prepared according to previous sections. QC samples including LLOQ were prepared in the presence of BSA and intra-day precision and accuracy were calculated as described above. The recovery of the analytes using brain tissue homogenate where two concentrations of standards (0.2 and 0.4 μM) had been spiked was calculated with the following equation: Recovery (%) = 100×(S–U)/C. In equation S represents the measured concentration of spiked sample, U represents the measured concentration of non-spiked sample and C represents the nominal concentration of the analyte.

The stability of the analytes was studied in three replicates using brain tissue homogenate samples. The concentrations of the stability samples were compared to those of freshly prepared samples. The freeze and thaw stability was determined after three freeze–thaw cycles. The short-term temperature stability was investigated by keeping the samples for 4 h at room temperature before sample preparation. The long-term stability was evaluated by analyzing samples which had been stored up to two months at −80 °C. The stock solution stability was investigated by comparing freshly prepared standards to standards prepared from a stock which had been frozen for 30 days and kept at room temperature for 6 h after thawing. The post-preparative stability was assessed by keeping the samples in an autosampler at 10 °C for 24 h.

6.3 RESULTS AND DISCUSSION

6.3.1 Method development

During the early method development, several extraction procedures and test tube materials were tested. When modifications of chloroform-based liquid–liquid extraction methods described by Bligh and Dyer (1959) and extraction with 1-butanol (Georas et al. 2007, Scherer et al. 2009, Xiao et al. 2001, Baker et al. 2001) were compared, the best extraction efficiency and precision were obtained using a modified Bligh and Dyer method (data not shown). The majority of lipophilic material from the tissue matrix is removed by a single liquid–liquid extraction step to the chloroform phase while the LPAs stay in the water phase. During those experiments, we observed that LPA could be adsorbed easily onto the surface of test tubes, especially when plastic materials were used. The peak areas were approximately 35% lower when extractions were performed in polypropylene microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) compared to Pyrex® borosilicate glass tubes (SciLabware, Stone, Staffordshire, UK). Therefore all the following experiments were performed using glassware. In addition, a significant improvement was found in the precision and accuracy, especially in the case of 18:0 LPA, by washing glass test tubes with hydrochloric acid (6 M) between the experiments.

Several authors have used extraction procedures with strong acids to move the LPAs into the lower organic phase of the Bligh and Dyer extract (Yoon et al. 2003, Georas et al. 2007, Shan et al. 2008, Tokumura et al. 2009). This makes sample preparation laborious with a two-step extraction followed by separation and manipulation of chloroform extract. Moreover, one serious concern about acidification is the artificial formation of LPA by acid-catalyzed hydrolysis of more abundant lysophospholipids. In our hands, treatment of the sample with 0.6 M hydrochloric acid in the presence of 18:1 LPC increased the amount of

18:1 LPA during sample preparation significantly (data not shown). Overall, there were several reasons for deciding to analyze the water phase. First, sample preparation was straightforward and quick to perform. Second, without using any pH adjustment with acid, the artificial formation of LPA from LPC during the sample preparation could be avoided. Finally, the majority of LPC remained in the lower phase, being absent from analysis and MS ion source where the loss of choline from LPC has been reported (Zhao & Xu 2009). Though the majority of LPCs were washed out into the chloroform during sample preparation, about 10 % of LPC still remained in the water phase which makes us to conclude that a chromatography step has to be included in the method (data not shown). Glycerophosphocholines, such as LPC, are also reported to cause LC/MS/MS ion suppression during the analysis of biological samples (Little et al. 2006).

Different reversed phase columns and mobile phases were tested in order to obtain the best peak symmetry, selectivity, and resolution between LPAs and other lipid classes such as LPCs. Strongly retentive high resolution sub-2 μm stationary phases containing C8 and C18 were initially tested resulting in narrow and symmetric peaks. Unfortunately, the peaks started to broaden considerably after only a few injections and the column performance deteriorated, probably due to irreversible attachment of LPAs to the stationary phase. Similar results were obtained with bridged ethylsiloxane/silica hybrid technology reversed phase C8 and C18 columns (XBridge, Waters, Ireland). In reversed phase columns, sample retention depends on three characteristics of the column: type and concentration of the bonded phase and column surface area. Very hydrophobic analytes, like LPAs, are strongly retained, and in some cases their elution from a strong column (e.g. narrow bore columns with small internal volumes) may not be possible, even with mobile phases containing high percentages of organic solvent. We achieved improved chromatographic performance with a C8 column with a larger particle size (3.5 μm) and pore size of 300 Å (Zorbax 300 SB-C8, Agilent Technologies, Palo Alto, CA, USA). In this case, the less strong wide pore column allowed the convenient elution of the sample without loss of column performance. However, retention times of LPAs and LPCs were overlapping with the mobile phase gradients in use.

Initially, the mobile phase consisted of acetate buffer and acetonitrile:isopropanol (5:2, v/v). During method development, several types of gradients were tested, but resolution between LPAs and LPCs could never be achieved. We found a wide pore reversed phase column (300 Å) and methanol together with acetate buffer to sharpen chromatographic peaks and also give resolution between LPAs and LPCs. Different solvents are enriched on the surface of the bonded phase to different degrees. Among the commonly used reversed phase organic solvents, methanol is adsorbed less than acetonitrile, making the system less retentive, and more suitable for LPA analysis. To further improve the resolution between LPAs and LPCs, we tested ion pairing reagents such as triethylamine (TEA) and *N*,*N*-dimethylhexylamine. Ion-pair chromatography provides an additional opportunity for achieving selectivity. Poor chromatographic peak symmetries were found with 10 mM TEA probably due to the simultaneous retention of LPAs by both reversed-phase and ion-pair processes. Instead, a high concentration (1%) of TEA was found to improve peak symmetries due to retention being determined by the ion-pairing properties, and it also increased the intensity of the MS signal. TEA has been reported to promote the formation of molecular ion and diminish the response of adduct ion (Schug & McNair 2002). In addition, it was further hypothesized that mobile phase additives with higher proton affinity would aid in formation of negative ions by extracting hydrogen ions in negative mode ESI (Schug & McNair 2002). A narrow bore column in favour of a wide pore reversed phase column was chosen to our final ion-pair chromatography method since this further improved column efficiency and selectivity, and therefore also improved resolution between LPAs and LPCs.

It should be noted, however, that a high concentration of TEA may contaminate the instrument to some extent which could be seen especially in the subsequent measurements

in the positive ion mode (Hughes et al. 2007). Therefore careful cleaning of the instrument is needed after the measurements and the use of project-specific capillaries is highly recommended. After the measurements we cleaned the instrument carefully with mixture of water and acetonitrile supplemented with formic acid (1%). A mass spectrometer spray chamber was rinsed carefully with a mixture of isopropanol and water. In addition, after the project the HPLC instrument was cleaned with a 50:50 mixture of methanol and potassium phosphate buffer (100 mM, pH 5.0). We also cleaned the capillary with a mixture of isopropanol and water and replaced the electrospray nebulizer needle and the LC filter element (5 μm). By these cleaning procedures, the residual TEA (*m/z* 102) was almost completely removed from the instrument and there was no effect (e.g. reduced sensitivity) of following measurements in the positive ion mode.

Mass spectrometric detection was performed using a highly selective MRM technique in the negative ion mode. Using full-scan MS experiments, the deprotonated molecular ions $[M - H]^-$ for 16:0 LPA, 18:0 LPA, 18:1 LPA, 20:4 LPA and IS (17:0 LPA) were found to be *m/z* 409, *m/z* 437, *m/z* 435, *m/z* 457, and *m/z* 423, respectively. A specific and sensitive assay was developed by monitoring transitions to the most intensive product ions. The following transitions were used: *m/z* 409 → 153 for 16:0 LPA, *m/z* 437 → 153 for 18:0 LPA, *m/z* 435 → 153 for 18:1 LPA, *m/z* 457 → 153 for 20:4 LPA, and *m/z* 423 → 153 for 17:0 LPA (Figure 16), where an ion at m/z 153 is formed by loss of water from the ion at *m/z* 171.

6.3.2 Selectivity

In order to determine the selectivity of the method, the standards and tissue samples prepared with the sample preparation method were analyzed (Figure 17). The solvents did not contribute any interfering peaks or background in any of the standard chromatograms. However, in the chromatograms of tissue samples, additional peaks were observed with the same parent-to-daughter ion transitions as the LPA species (Figure 17), which has previously been reported by Shan et al. (2008). These peaks are likely due to loss of choline from the LPC molecule in the MS ion source (Zhao & Xu 2009).

When two concentrations of standards (0.2 and 0.4 μM) were added into the brain tissue homogenate and the slopes of the regression curves were compared to those prepared without tissue matrix, the slopes were found to be statistically equal for other LPA species, except for 18:0 LPA (Figure 18). This was taken as evidence of the absence of significant matrix effect or interference induced by the brain matrix. In the case of 18:0 LPA, absorption of the analyte on the surface of the test tube may explain the results to some extent. When tissue samples were diluted 1:2, 1:5 or 1:10 with methanol before injecting into LC/MS/MS, the concentrations of diluted samples were found to be constant due to simultaneous dilution of internal standard. This further ensures our observation that there is no matrix effect or interference, which could cause systematic error to the results (data not shown). Furthermore, no significant ion suppression at the retention time of analytes was found in a post-column infusion study where remarkable ion suppression occurred at a retention time of 0.5 min but this was normalized by the retention time of 3.0 min (data not shown). In-source fragmentation of 18:1 LPC gave transition of *m/z* 281 → 281 (Figure 16) which was used to optimize the resolution between LPA and other species like ion suppression-causing glycerophosphocholines (Little et al. 2006).

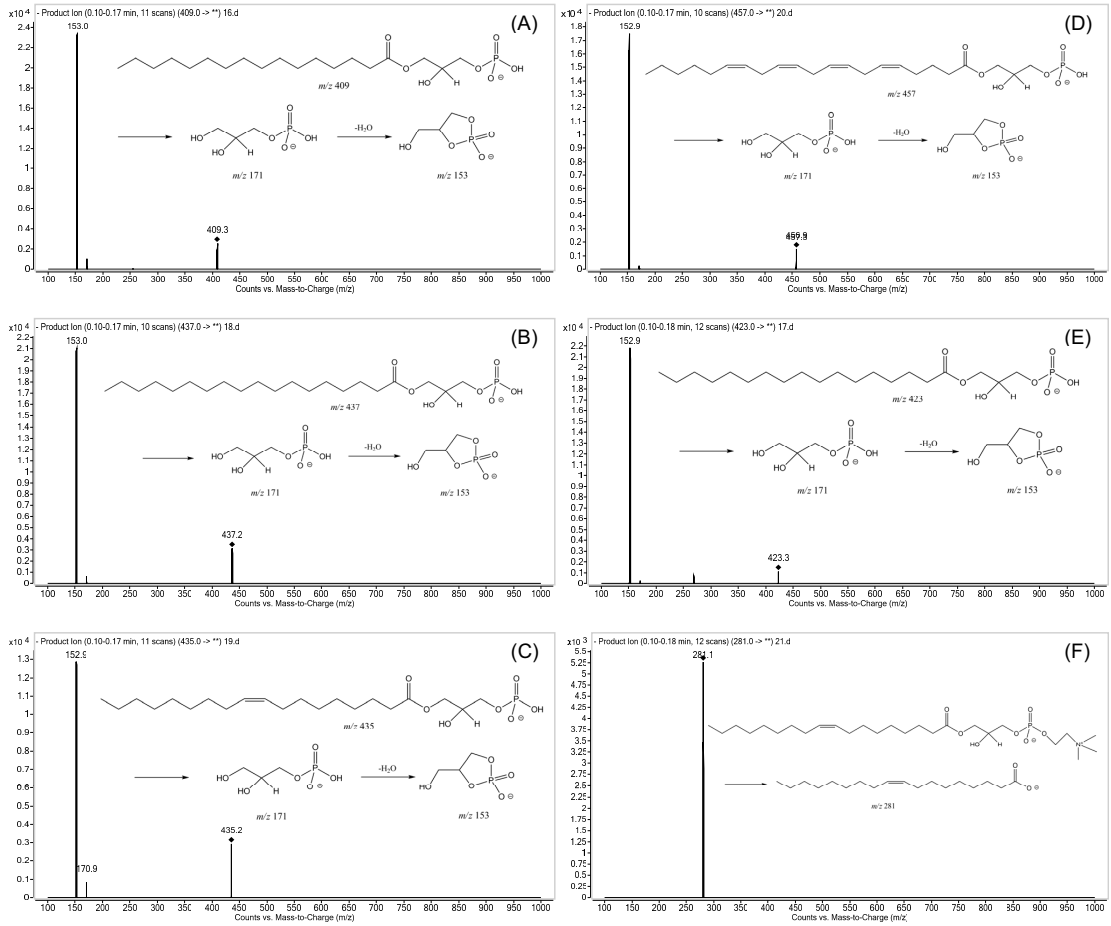


Figure 16. Product ion mass spectra and molecular structures with proposed fragmentation of (A) 16:0 LPA (*m/z* 409 →100–1000), (B) 18:0 LPA (*m/z* 437 →100–1000), (C) 18:1 LPA (*m/z* 435 →100–1000), (D) 20:4 LPA (*m/z* 457 →100–1000), (E) 17:0 LPA (*m/z* 423 →100–1000), and (F) 18:1 LPC (m/z 281 →100–1000). One microliter of standard solution in methanol (1:10 dilution from stock solutions, ~20 μM) was injected directly into the LC/MS/MS using an isocratic mobile phase consisting of 30% of 1% TEA in 50 μM ammonium acetate and 70% of 1% TEA in 90% methanol delivered at 0.3 ml/min.

6.3.3 Linearity, precision, accuracy, and recovery

The six point calibration curves were highly linear over the range of 36–1790 fmol of LPA injected into column. The calibration curve parameters with standard errors and regression coefficients are summarized in Table 8. The LLOQ with acceptable accuracy (± 10%) and precision (≤ 15% RSD) was 54 fmol of LPA injected into the column. Previously, the detection limits for LPA species achieved with LC/MS/MS analysis have been in the range of 0.01–0.03 μM (200–600 fmol/injection) (Meleh et al. 2007) whereas with flow injection, a detection limit as low as of 0.3–1.0 nM (6–20 fmol/injection) (Yoon et al. 2003) has been reported. However, only Shan et al. (2008) have reported a quantification limit for LC/MS/MS method (160–500 fmol/injection). With the radioenzymatic assay, a detection limit of 200 fmol has been reported (Saulnier-Blache et al. 2000).

The precision and accuracy of all QC samples were within the acceptable range (Table 9). The method was accurate and precise between runs and within individual runs at each QC level for all the LPAs. The method was accurate and precise at all studied levels also when

QC samples were determined in the presence of the surrogate sample matrix [4% (w/v) BSA, m/V]. No statistically significant differences (one-way ANOVA with Tukey's multiple comparison *post hoc* test with $p<0.05$ considered as statistically significant) were found between results of QC samples with or without surrogate matrix at any concentration level (data not shown). The recoveries for 16:0 LPA, 18:0 LPA, 18:1 LPA and 20:4 LPA were found to be 104%, 69%, 92%, and 86%, respectively.

Table 8. The linear range, calibration curve parameters with standard errors and regression coefficients of 16:0 LPA, 18:0 LPA, 18:1 LPA and 20:4 LPA (n=3). Unweighted linear regression analysis was used to make the calibration curve. LLOQ was determined by calculating precision and accuracy for five LLOQ samples that were independent of the calibration curve.

Compound	Linear range (fmol)	Regression parameters Slope ± S.T.D error	Intercept ± S.T.D error	R^2	LLOQ (fmol)
16:0 LPA	36-1790	1.0007 ± 0.0131	-0.0002 ± 0.0210	0.999	54
18:0 LPA	36-1790	0.6322 ± 0.0110	0.0117 ± 0.0156	0.999	54
18:1 LPA	36-1790	0.7878 ± 0.0158	-0.0177 ± 0.0253	0.999	54
20:4 LPA	36-1790	0.7355 ± 0.0171	-0.0151 ± 0.0273	0.998	54

Table 9. Within-run and between-day precision and accuracy for 16:0 LPA, 18:0 LPA, 18:1 LPA, and 20:4 LPA and their nominal values at each QC level.

Compound	Nominal conc. (μM)	Intra-day precision and accuracy Mean (n = 5) (μM)	RSD (%)	Mean accuracy (%)	Inter-day precision Mean (n = 3 days) (μM)	RSD (%)
16:0 LPA	0.03	0.03	5.0	92	0.03	22
	0.1	0.1	3.6	85	0.1	11
	0.3	0.3	2.3	90	0.3	5.4
	0.8	0.8	1.6	94	0.8	5.2
18:0 LPA	0.03	0.03	14	104	0.03	33
	0.1	0.1	2.8	103	0.1	10
	0.3	0.3	2.4	114	0.3	12
	0.8	1.0	1.2	120	0.9	9.4
18:1 LPA	0.03	0.03	6.5	108	0.03	17
	0.1	0.1	4.0	89	0.1	12
	0.3	0.3	3.0	89	0.3	9.1
	0.8	0.7	1.2	92	0.8	6.9
20:4 LPA	0.03	0.03	4.5	100	0.03	17
	0.1	0.1	2.1	83	0.1	11
	0.3	0.3	3.5	85	0.3	11
	0.8	0.7	1.3	85	0.8	11

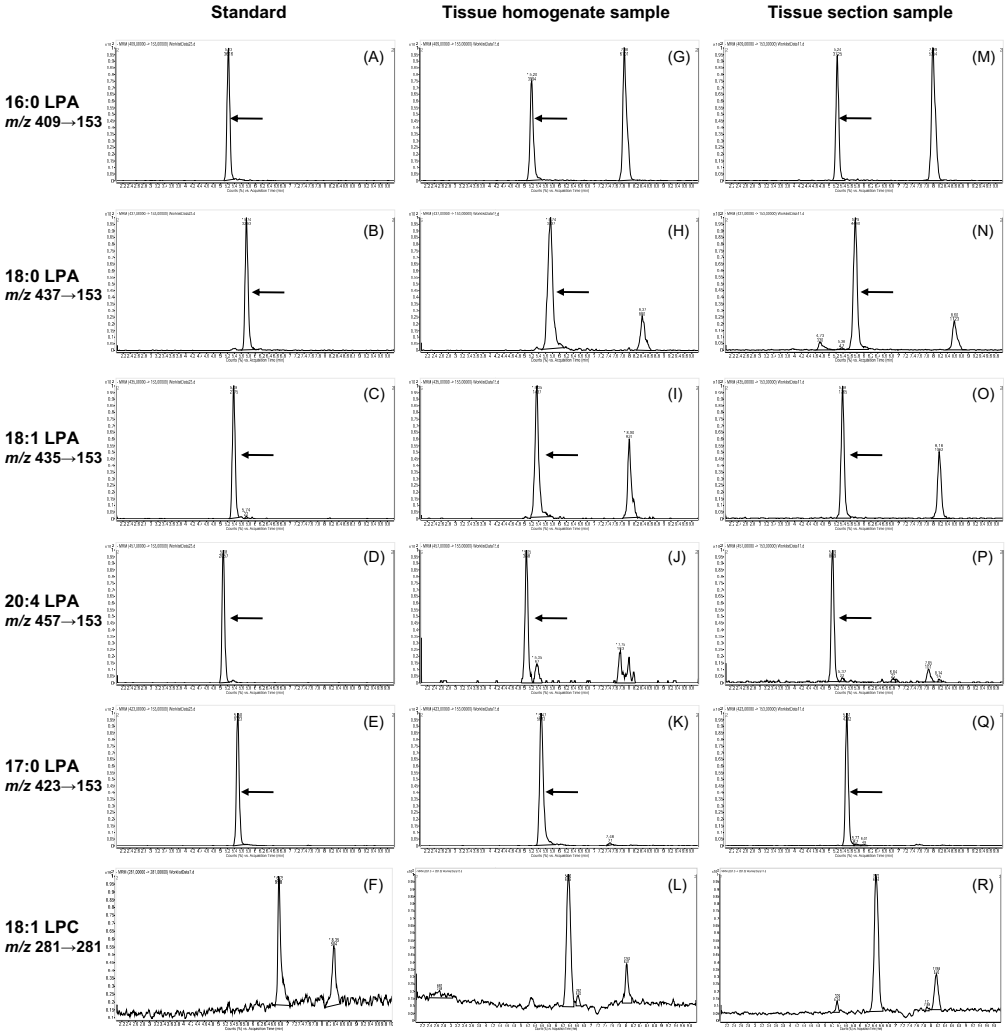


Figure 17. Representative MRM chromatograms of (A) standard sample of 16:0 LPA (0.1 μM, retention time (RT) 5.23 min), (B) 18:0 LPA (0.1 μM, RT 5.74 min), (C) 18:1 LPA (0.1 μM, RT 5.38 min), (D) 20:4 LPA (0.1 μM, RT 5.08 min), and (E) internal standard (IS) 17:0 LPA (0.3 μM RT 5.50 min). Endogenous LPA content was measured from a rat brain homogenate and brain cryosections. Representative MRM chromatograms of a rat brain homogenate of (G) 16:0 LPA (0.17 μM), (H) 18:0 LPA (0.20 μM), (I) 18:1 LPA (0.11 μM), (J) 20:4 LPA (0.04 μM), and (K) IS (0.3 μM). Representative MRM chromatograms of a rat brain cryosections of (M) 16:0 LPA (0.17 μM), (N) 18:0 LPA (0.19 μM), (O) 18:1 LPA (0.16 μM), (P) 20:4 LPA (0.08 μM), and (Q) IS (0.3 μM). Additional peaks on MRM channels of LPAs in brain homogenate and cryosections were due to artificial formation of LPAs from LPCs in MS ionization chamber (Zhao & Xu 2009). The transition *m/z* 281 → 281 monitored 18:1 fatty acid anion and it was used to optimize the resolution between LPA and ion suppression causing lipids (Little et al. 2006). Representative MRM chromatograms of (F) 18:1 LPC standard (8 μM, RT 6.75 min and 8.35 min), (L) tissue homogenate, and (R) tissue section. In the chromatograms, the individual LPAs are indicated with the arrow.

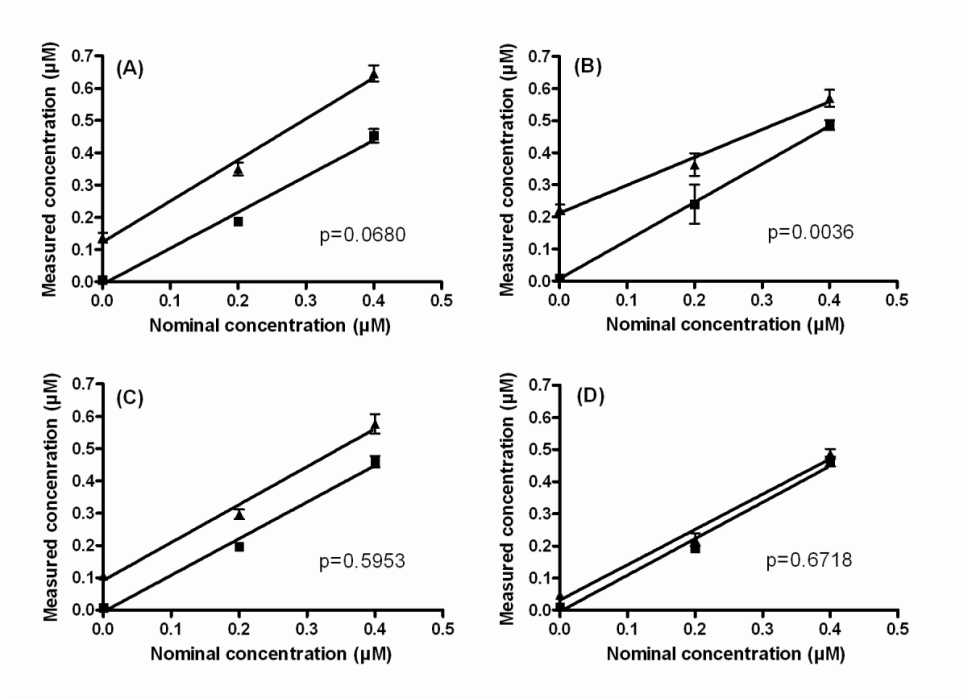


Figure 18. The matrix effect was studied with the standard addition method. Two concentrations of standards (0.2 and 0.4 µM) were spiked into the brain tissue homogenate to obtain a regression curve (triangles). Standard curve using the same concentrations was prepared without tissue matrix (squares). The standard addition method shows no matrix effect on 16:0 LPA (A), 18:1 LPA (C), and 20:4 LPA (D). Statistically significant difference was found between slopes of homogenate and reference standards of 18:0 LPA (B). This matrix effect is probably due to the adsorption of the analyte on the surface of the test tube. The statistical equality of the slopes ($p<0.05$ considered as statistically significant difference) was determined using analysis of covariance (ANCOVA) (mean ± SD, n=3).

6.3.4 Stability

There was no significant degradation of LPAs after three freeze–thaw cycles (24 h interval between the cycles) in comparison with freshly prepared samples (–10 to +17% for other LPAs and +37 % for 18:0 LPA). The short-term temperature stability showed no degradation of LPAs but, instead, increased levels of LPAs 24–101% after 4 h storage at +20 °C. The increasing amount of LPAs after storage at +20 °C has been previously described (Scherer et al. 2009). LPAs may have been formed enzymatically in the brain tissue when stored at +20 °C before the enzymatic machinery is switched off by addition of methanol and chloroform into the sample. The LPA concentrations in 30 and 60 days of long-term stability samples were within the range of –17 to +10% for other LPAs but up to –42% for 18:0 LPA. The overall divergent results of 18:0 LPA are likely to be due to adsorption of the analyte onto the surface of test tubes. The stock solutions of LPAs in methanol were stable for 30 days when stored at –20 °C and kept at room temperature for 6 h after thawing. Post-preparative stability samples were found to be stable when they were stored for 24 h at 10 °C in the autosampler.

6.3.5 Application

In preliminary experiments with rat brain cryosections, the following endogenous LPA levels were found: 3.2 ± 0.4 for 16:0 LPA, 5.8 ± 1.8 for 18:0 LPA, 2.6 ± 0.6 for 18:1 LPA, and 1.2 ± 0.4 for 20:4 LPA (nmol/g protein in tissue, mean ± SD of three replicate samples from two individual animals, n=6). According to GC analysis (Nakane et al. 2002), the four LPA species followed in our LC/MS/MS method account for 93% of LPA species in rat brain. Previously, in the GC analysis 18:1 LPA was found to be the predominant LPA species in rat brain homogenate (Nakane et al. 2002). Based on our findings, 18:0 LPA seems to predominate; this may be due to differences in the method and the age of the rats used (we used young 4-week-old rats). In preliminary experiments, the concentrations of LPA species were above the LLOQ of the method and all the measured concentrations were within the range of the method.

6.4 CONCLUSIONS

A highly selective and sensitive method using LC/MS/MS was developed for the determination of LPA species in rat brain cryosections. As far as we are aware, this is the first report where the LPA content of brain tissue has been measured using LC/MS/MS. From the analytical point of view, LPAs are a demanding group of compounds since, for example, artificial formation of LPA from LPCs has been demonstrated during sample preparation and within the instrumentation. To prevent the artificial formation of LPA, we used a single step extraction procedure without any strong acid treatment to remove lipophilic material from the water phase, which was then analyzed. This sample pre-treatment also removed most of the lipids causing the matrix effect and the method was found to be highly selective. Since residual LPC remained in the water phase after sample preparation, additional peaks were observed at the same parent-to-daughter ion transitions as the LPA species. Therefore, we developed a chromatographic method based on a narrow bore reversed phase column and ion-pair technique to optimize the efficiency of the column and to achieve resolution between LPAs and LPCs. Mass spectrometric detection was performed using a highly selective MRM technique in the negative ion mode. The method was validated and acceptable accuracy, precision, recovery, and stability were obtained for concentrations within the range of the calibration curve for all of the studied LPAs. According to the validation results and data from the preliminary study, we conclude that the LC/MS/MS method described in this paper is applicable for the targeted quantitative analysis of LPA species in rat brain sections.

7 Identification of Enzymatic Pathways Degrading LPA in Brain Sections[3]

Abstract: The lifetime of the signalling pool of LPA is controlled by the equilibrium between synthesizing and degrading enzymatic activity. In the current study, we have characterized these enzymatic pathways in rat brain by pharmacologically manipulating the enzymatic machinery required for LPA synthesis and degradation. In rat brain cryosections, the lifetime of bioactive LPA was found to be controlled by Mg^{2+}-independent, NEM-insensitive phosphatase activity, attributed to LPPs. Pharmacological inhibition of this LPP activity amplified LPA_1 receptor signalling, as revealed using functional autoradiography. Although two LPP inhibitors, sodium orthovanadate and propranolol, locally amplified receptor responses, they did not affect global brain LPA phosphatase activity (also attributed to Mg^{2+}-independent, NEM-insensitive phosphatases), as confirmed by P_i determination and by LC/MS/MS. Interestingly, the phosphate analog, aluminium fluoride (AlF_x^-) not only irreversibly inhibited LPP activity thereby potentiating LPA_1 receptor responses, but also totally prevented LPA degradation, however this latter effect was not essential in order to observe AlF_x^--dependent potentiation of receptor signalling. We conclude that vanadate- and propranolol-sensitive LPP activity locally guards the signalling pool of LPA whereas the majority of brain LPA phosphatase activity is attributed to LPP-like enzymatic activity which, like LPP activity, is sensitive to AlF_x^- but resistant to the LPP inhibitors, vanadate and propranolol.

[3] Adapted from **Aaltonen N, Lehtonen M, Varonen K, Arrufat Goterris G, Laitinen JT. Lipid phosphate phosphatase inhibitors locally amplify lysophosphatidic acid LPA_1 receptor signalling in rat brain cryosections without affecting global LPA degradation. BMC Pharmacology 12:7, 2012**.

7.1 INTRODUCTION

Previous studies have demonstrated that brain sections retain the capacity to generate endogenous GPCR agonists, such as adenosine and LPA, during autoradiography incubations, resulting in tonic adenosine A_1 and LPA receptor activity in anatomically defined brain regions (Chapter 5, Laitinen 1999, Palomaki & Laitinen 2006). The LPA-evoked [^{35}S]GTPγS binding response in rat brain sections reflects LPA_1 receptor activity, as it is sensitive to the $LPA_{1/3}$-selective antagonist Ki16425 and is restricted to the developing white matter tracts (Chapter 5, Waeber & Chiu 1999, Laitinen 2004). This labelling pattern faithfully mirrors the known expression pattern of LPA_1 receptors in the developing rat brain (Allard et al. 1998, Weiner et al. 1998, Handford et al. 2001, Cervera et al. 2002).

It was recently demonstrated that a comprehensive elimination of the enzymatic hydrolysis of the endocannabinoid 2-AG in brain sections leads to 2-AG accumulation and subsequent cannabinoid CB_1 receptor activation, as successfully revealed using functional autoradiography (Palomaki et al. 2007). Using this approach, we have characterized the enzymatic pathways and their role in tonic LPA_1 receptor activity by pharmacologically manipulating the enzymatic machinery required for LPA degradation. The two degradative pathways generating inorganic phosphate (P_i) as a result of LPA degradation are depicted in Figure 19.

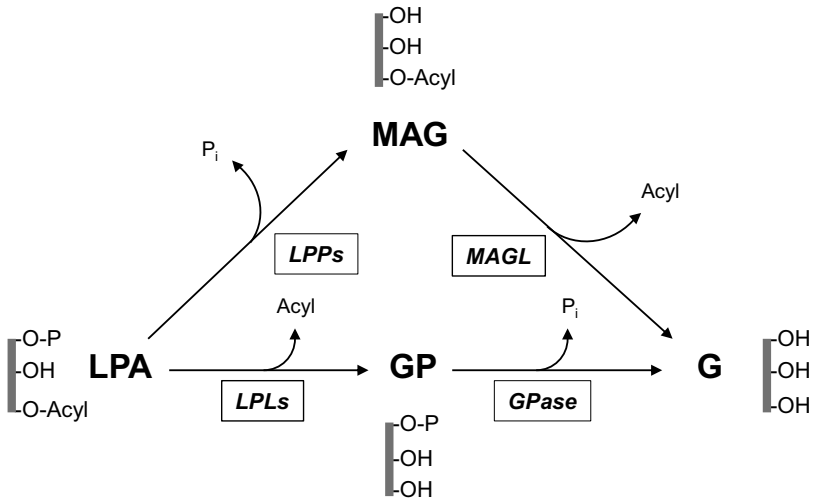


Figure 19. The two enzymatic pathways generating inorganic phosphate (P_i) as a result of LPA degradation. Abbreviations: G, glycerol; GP, glycerol phosphate; LPA, lysophosphatidic acid; LPL, lysophospholipase; LPP, lipid phosphate phosphatase; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase.

7.2 MATERIALS AND METHODS

The materials and general methods are described in Chapter 4.

7.2.1 Determination of P_i and glycerol

To estimate LPA-degrading phosphatase activity under the assay conditions mimicking functional autoradiography, triplicate slides with two or three horizontal brain sections underwent an autoradiography-mimicking protocol (except that DPCPX, GDP and the radioligand were omitted). The experiments with Na_3VO_4, propranolol, and nadolol (as a negative control for propranolol) were performed with and without NEM pretreatment. The protocol consisted of a 10 min pre-incubation in the assay buffer, a 30 min incubation in the assay buffer with or without 5 mM NEM, then a washing step with assay buffer, and finally a 90 min incubation in the presence of chemicals of interest as well as 0.1% BSA and 1 mM DTT. In NEM-treated sections, Mg^{2+} was omitted from the assay buffer. In experiments with NaF and AlF_x^-, the protocol consisted of two sequential 40 min incubations (steps 1 and 2) and finally a 90 min incubation (step 3) in the presence of the chemicals of interest as well as 0.1% BSA and 1 mM DTT. The LPA species used was 18:1 LPA. In all experiments, after the final 90 min incubation step, the postincubation buffer was collected quantitatively and the P_i content was determined in duplicate using the Fiske-Subbarow method, as described in Esmann (1988) after TCA-precipitation of BSA which interferes with method. Absorbances (λ = 700 nm) were read with a Tecan Spectrafluor microplate reader.

To clarify the enzymatic routes responsible for LPA degradation in our experimental setting, P_i formation was determined using enzyme-coupling fluorescent method (Vazquez et al. 2003). The glycerol content was determined using a coupled enzyme reaction (Free Glycerol Reagent, Sigma, Cat.# F6428) with the exception that H_2O_2 production was coupled to peroxidase-dependent formation of the fluorescent dye resorufin (Navia-Paldanius et al. 2012). Briefly, rat cerebellar membranes (1 μg/well on a 96-well plate), prepared as described in Saario et al. (2004), were pretreated with the serine hydrolase inhibitors MAFP (1 μM) or JZL184 (100 μM) for 30 min and then incubated with or without 18:1 LPA, GP, or (2S)-OMPT (10 μM final concentration). The fluorescence (λ_{ex} = 530 nm, λ_{em} = 590 nm) was monitored kinetically for 90 min at 10 min intervals using Tecan Infinite M200 fluorometer.

7.3 RESULTS AND DISCUSSION

7.3.1 Tonic LPA_1 receptor activity under basal conditions of [^{35}S]GTPγS autoradiography is not due to PLD or autotaxin activity

Our previous study suggested that tonic LPA_1 receptor activity in rat brain sections might be due to LPA generated via PLD activity, based on the sensitivity of this response to 1-butanol (Palomäki & Laitinen 2006). However, 1-butanol is known to have several cellular off-targets and it is an inefficient PLD inhibitor, and therefore butanol-sensitivity alone is invalid as a proof for PLD involvement in a given cellular process or *vice versa*. Therefore, as the initial step of the present studies, we explored in more detail the possible enzymatic pathways that could generate LPA under the assay conditions employed.

We first used various approaches to address the role of PLD, but in addition to the 1-butanol-sensitivity of this response, found no additional evidence for the involvement of the PC-PLD → PA-$PLA_{1/2}$→ LPA pathway as a source of tonic LPA_1 signalling. A summary of these studies is presented in Figure 20. Briefly, we found that exogenous addition of the PLD substrate PC or the LPA precursor PA failed to boost LPA_1 receptor signalling. Similarly, the PLD activators oleate and PIP_2 did not enhance LPA_1 receptor activity, and the PIP_2-depletor, neomycin, failed to reduce activity. Although 1-butanol has been previously demonstrated not to affect LPA_1 receptor signalling in response to exogenous LPA (Palomäki & Laitinen 2006), we found here that 1-butanol readily blunted the responses to sub-threshold concentrations of LPA. Finally, the selective PLD inhibitor,

compound 4k (Monovich et al. 2007, Su et al. 2009), did not reduce tonic LPA_1 receptor activity.

We next examined whether tonic LPA_1 activity in brain sections is due to LPA formed as a result of ATX activity. However, we found that exogenously added ATX substrate LPC did not boost tonic LPA_1 receptor activity (Figure 20). In addition, LPA could theoretically be generated either from glycerophosphate or from MAG in reactions catalyzed by glycerophosphate acyl transferase or monoacylglycerol kinase, respectively. In brain sections, however, catabolic rather than *de novo* synthesizing pathways are likely to be dominating. In previous experiments, neither exogenous 2-AG, (partially converted to 1(3)-AG during the incubations) nor inhibitors of 2-AG degradation were able to induce [^{35}S]GTPγS binding in the LPA_1 receptor-enriched white matter areas of rat brain sections (Palomaki et al. 2007), suggesting that monoacylglycerol kinase was not involved. Neither exogenous glycerol-3-phosphate was able to induce LPA_1 receptor mediated [^{35}S]GTPγS binding, providing no evidence of involvement of glycerophosphate acyl transferase (data not shown). Collectively, these studies indicate that with compounds used, we could not affect LPA-generating pathways in brain sections. We next explored if the tonic LPA_1 receptor activity could be boosted by inhibitors of LPA degradation.

7.3.2 The LPP inhibitors Na_3VO_4 and propranolol locally amplify LPA_1 receptor signalling without affecting global LPA degradation

The LPP-mediated degradation of LPA is susceptible to the phosphatase inhibitor sodium orthovanadate (Na_3VO_4) (Simon et al. 2002) and to propranolol (Roberts et al. 1998, Simon et al. 2002), better known as a classical β-adrenoceptor blocking agent. Another β-blocker, nadolol, has no demonstrable LPP inhibiting capacity (Simon et al. 2002) and therefore can serve as a useful control compound. We tested the effects of these compounds on basal LPA_1 receptor signalling. Treatment of brain sections with propranolol (1 mM) or Na_3VO_4 (100 μM) resulted in stimulated [^{35}S]GTPγS binding responses that were restricted to the white matter areas of 4 week-old rat brain (Figure 21a). The observed labelling pattern was fully reproduced by the addition of exogenous LPA (50 μM) and all the evoked responses, including the tonic LPA_1 receptor activity observed under basal conditions, were abolished by treatment with the $LPA_{1/3}$ receptor selective antagonist Ki16425 (5 μM) (Figures 21a and b). As expected, treatment with nadolol (1 mM) had no effect on basal LPA_1 receptor signalling (Figure 21c). Both Na_3VO_4 and propranolol, but not nadolol, amplified the LPA-evoked (0.5 μM to 50 μM) binding responses (Figure 21c). Since propranolol and vanadate amplified LPA_1 receptor signalling only when present in the [^{35}S]GTPγS labelling step, these drugs presumably inhibit LPPs in a reversible manner.

To rule out the direct agonism of propranolol and vanadate at the LPA_1 receptor, we performed classical filtration-based [^{35}S]GTPγS binding assay and found that neither compound was able to stimulate [^{35}S]GTPγS binding to the rat cerebellar membranes, whereas exogenous LPA evoked a dose-dependent response (data not shown). We additionally observed that the LPA_3 receptor-preferring agonist (2S)-OMPT (0.5 μM to 50 μM) induced only a weak response in the brain of a 4-week old rat when compared to signal achieved with exogenous LPA (10 μM) (data not shown). This lends further support to the argument that observed LPA-evoked signalling is reflecting the activity of the myelin-enriched LPA_1 receptors instead of LPA_3 receptors that are expressed to a lesser degree in the brain (Choi et al. 2010).

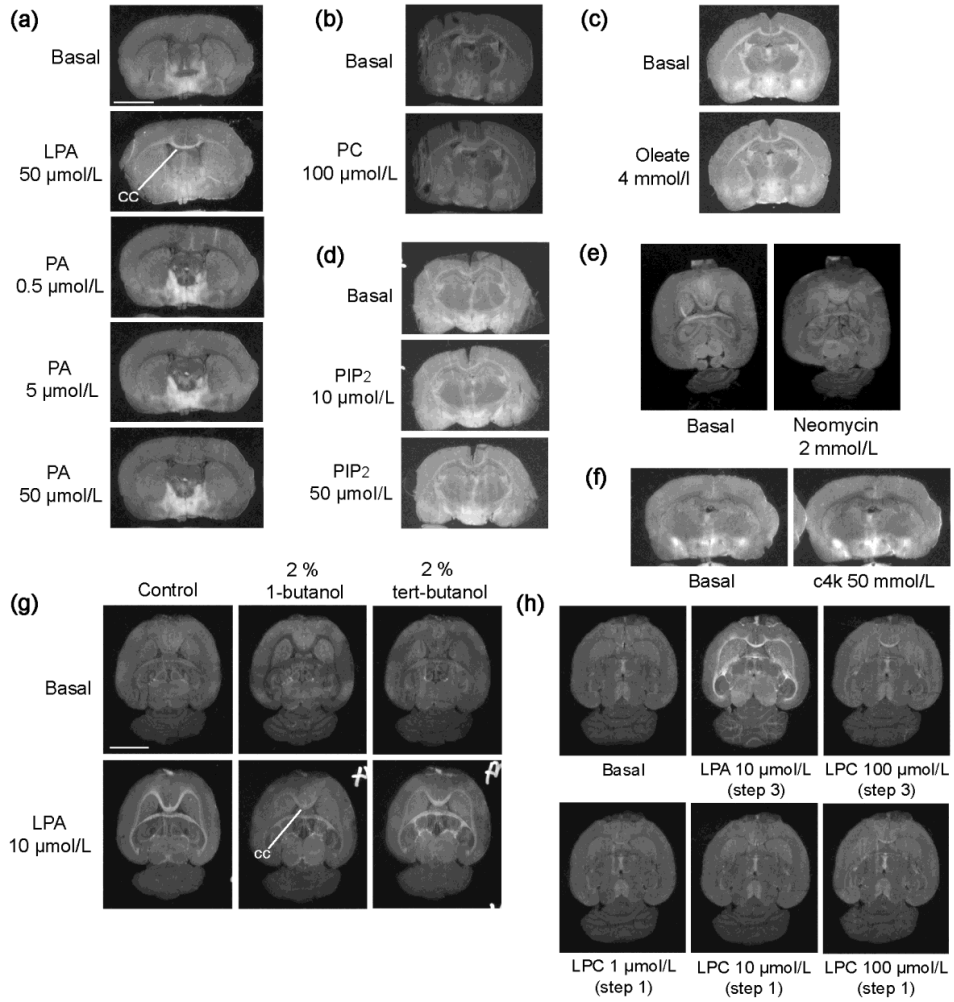


Figure 20. Evidence indicating that tonic LPA_1 receptor activity under basal conditions of [^{35}S]GTPγS autoradiography is not due to PLD or autotaxin activity. Inclusion of the LPA precursors phosphatidic acid (PA) (present in step 3) (a), or phosphatidylcholine (PC) in step 3 (b) does not boost tonic LPA_1 receptor activity. The PLD activators oleate (step 3) (c), and PIP_2 (step 3) (d) do not enhance tonic LPA_1 receptor activity, and the PIP_2-depletor neomycin (present in steps 2 and 3) (e) does not reduce it. The selective PLD inhibitor, compound 4k (c4k) (steps 1–3), does not reduce tonic LPA_1 receptor activity (f). The non-specific PLD inhibitor 1-butanol (step 3) blunts the response evoked by exogenous LPA while the control compound tert-butanol does not have any effect (step 3) (g). Incubation of brain sections with the autotaxin substrate LPC (step 1 or 3) does not affect tonic LPA_1 receptor activity (h) (cc, corpus callosum). Scale bars = 5 mm.

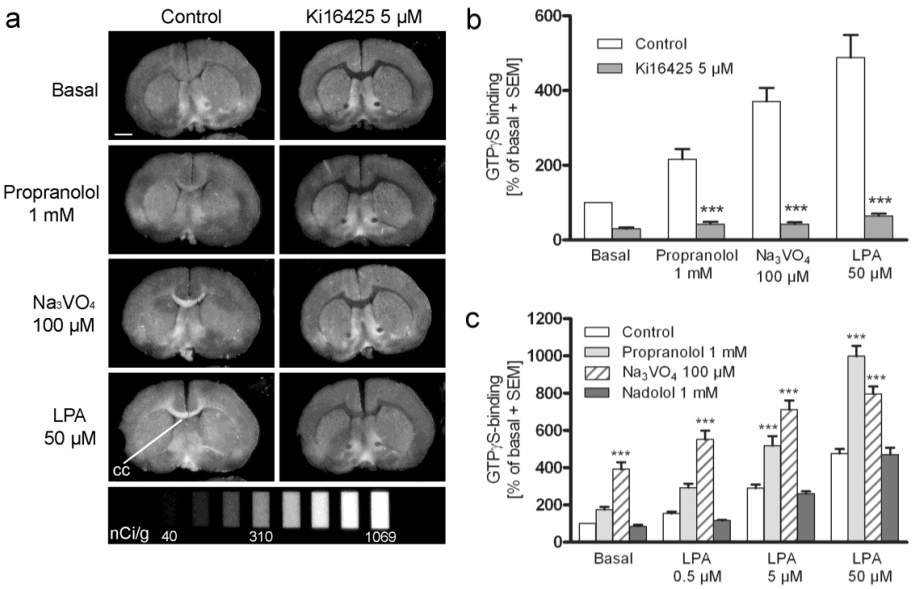


Figure 21. Vanadate and propranolol evoke LPA-mimicking [^{35}S]GTPγS binding response that is sensitive to $LPA_{1/3}$ receptor antagonist. (a) Test chemicals were included during the [^{35}S]GTPγS labelling step (step 3). Treatment with propranolol or Na_3VO_4 results in G protein activity in the LPA_1 receptor enriched white matter tracts, a response that is mimicked by exogenous LPA. The $LPA_{1/3}$ receptor antagonist Ki16425 abolishes all the evoked responses in the white matter regions, including the tonic LPA_1 signal observed under basal conditions (cc, corpus callosum). Scale bar = 2 mm. The eight-point [^{14}C] standard used in the quantification is shown at the bottom of the image. (b) and (c) Quantitative data of the binding responses including the dose response for exogenous LPA with or without the simultaneous treatment with the inhibitors. Autoradiography films were digitized and [^{35}S]GTPγS binding was quantified from the corpus callosum of coronal rat brain sections. Significance level: (b) ***$p < 0.001$ compared to the treatment without Ki16425 (c) ***$p < 0.001$ compared to control in each LPA concentration (n=6).

The LPPs catalyze the hydrolysis of the phosphate group of their lipid substrates resulting in the generation of P_i. The measurement of the released P_i offers a straightforward way to monitor LPP activity (McDermott et al. 2006). Studies with LPA (50 µM) indicated that LPP-like phosphatase activity accounted for the majority of LPA degradation, as ~ 93% of the LPA-derived P_i was formed as a result of Mg^{2+}-independent, NEM-resistant phosphatase activity (Figure 22). In the routine assay buffer containing Mg^{2+}, 46 ± 1 % (mean ± SEM, n=3) of exogenous LPA (50 µM, corresponding to 47.5 nmol potentially available P_i per slide) was degraded during the 90 min incubation whereas in the Mg^{2+}-free assay buffer supplemented with NEM (5 mM), the respective figure was 44 ± 3 %. However, neither the LPP inhibitors Na_3VO_4 (100 µM), propranolol (1 mM), nor nadolol (1 mM) affected total LPA phosphatase activity in a statistically significant manner, assessed at the bulk level of brain sections (Figure 22). In line with this, when the LPA content of brain sections treated with Na_3VO_4 or propranolol was analyzed using LC/MS/MS, there was no significant accumulation of LPA when compared to control sections that were incubated in the absence of these inhibitors (data not shown). In brain sections, LPP activity appears to locally control the lifetime of the signalling pool of LPA,

and LPPs must therefore reside in close proximity to the LPA_1 receptors, as propranolol and vanadate had no effect on LPA degradation when assessed at the bulk brain level.

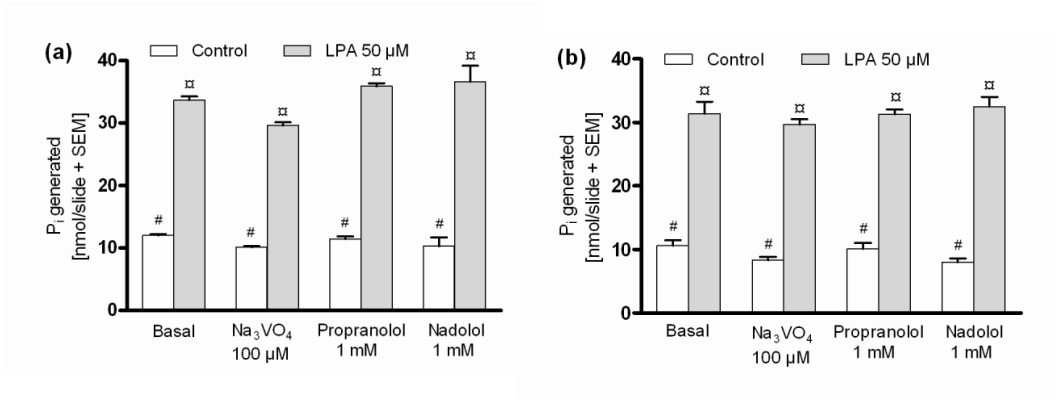


Figure 22. Brain tissue dephosphorylates LPA through LPP-like phosphatases that are resistant to propranolol and Na_3VO_4. Slides with three horizontal rat brain sections underwent the autoradiography-mimicking incubation protocol, (a), or were pretreated with NEM (5 mM) followed by the autoradiography-mimicking protocol with the exception that Mg^{2+} was omitted from the incubation buffer (b). Following a 90 min incubation in the presence of 0.1% BSA together with the indicated combinations and concentrations of the compounds, the assay buffer was quantitatively collected and the P_i content was determined. Note that the bulk of LPA degradation is due to NEM-resistant and Mg^{2+}-independent phosphatase activity (compare a and b) and that Na_3VO_4, propranolol or nadolol do not affect basal or LPA-derived P_i generation in a statistically significant manner. Significance level: Each bar indicated with ¤ shows a significant difference ($p < 0.001$) to the corresponding bar indicated with # while there is no significant difference between the bars marked with the same symbol (n=3).

7.3.3 Aluminium fluoride amplifies LPA_1 receptor signalling and totally prevents LPA degradation

We undertook a search for additional inhibitors in an attempt to identify compounds that could comprehensively target the global pool of LPA phosphatases in brain sections. Sodium fluoride (NaF) is commonly used as a phosphatase inhibitor and aluminium fluoride (AlF_x^-) acts as a transition stage phosphate analog also capable of inhibiting several phosphatases (Li 2003). AlF_x^- forms spontaneously in aqueous solutions in the presence of Al^{3+} and Fl^- ions. We pretreated brain sections with NaF or AlF_x^- in order to test whether these compounds could affect LPA receptor activity and/or LPA degradation. Interestingly, when sections were pretreated with AlF_x^- ($AlCl_3$ 50 µM + NaF 10 mM), a stimulated [^{35}S]GTPγS binding response throughout the LPA_1 receptor-enriched white matter regions was evident (Figures 23a and b). This labelling pattern was fully mimicked by addition of exogenous LPA (0.5 µM to 50 µM) (Figures 23a and b), and was blocked by Ki16425 (5 µM) (Figures 23a and c). Similarly, pretreatment of brain sections with NaF (10 mM) resulted in stimulated [^{35}S]GTPγS binding responses throughout the white matter regions (Figure 23a). Pretreatment with these compounds was sufficient to evoke LPA_1 receptor signalling, suggesting that, in contrast to the reversibly acting inhibitors Na_3VO_4 and propranolol (that needed to be present during the [^{35}S]GTPγS labelling step), AlF_x^- and NaF inhibited LPP activity in an irreversible manner. AlF_x^- is known to mimic the chemical structure of P_i and therefore affects the activity of several phosphoryl transfer enzymes (Li 2003). As a phosphate analog, AlF_x^- might bind to the P_i-recognizing binding pocket of the LPPs and by this mechanism lead to irreversible inhibition.

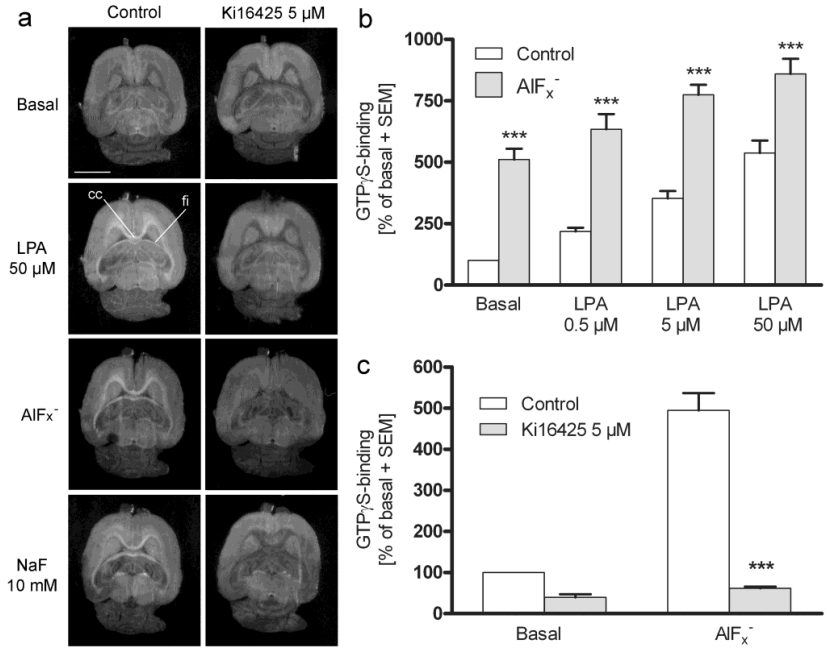


Figure 23. AlF_x^- and NaF evoke LPA-mimicking [^{35}S]GTPγS binding response that is sensitive to $LPA_{1/3}$ receptor antagonist. (a) AlF_x^- (NaF 10 mM + $AlCl_3$ 50 µM) was included during the 40 min preincubation step (step 1), NaF was included in step 2, and LPA in step 3, whereas Ki16425 was present throughout all steps. Treatment with AlF_x^- and NaF results in region-specific G protein activity that is restricted to the LPA_1 receptor-enriched white matter tracts, a response that is mimicked by exogenous LPA and that is sensitive to Ki16425 (cc, corpus callosum; fi, fimbria of the hippocampus). Scale bar = 5 mm. (b) and (c) Quantitative data of the binding responses. Autoradiography films were digitized and [^{35}S]GTPγS binding was quantified from corpus callosum of the coronal rat brain sections. Significance level: (b) ***p < 0.001 compared to control in each LPA concentration (c) ***p < 0.001 compared to the treatment without Ki16425 (n=6).

We wished to explore in more detail the mode of inhibition of these compounds, as well as the behaviour of NaF in our experimental setting. This was justified by the fact that aluminium is a common constituent of glassware and F^- can etch it from the glass. Deforoxamine mesylate (DFOM) is an aluminium and iron(III) chelator that can be used in experimental settings to reveal if aluminium is present in the system. When brain sections were treated with DFOM (50 µM), the responses to AlF_x^- and NaF (10 mM) in functional autoradiography were totally abolished (Figure 23a). It is noteworthy that DFOM needed to be added together with AlF_x^- or NaF in order to achieve this reversal; if added after pretreatment with AlF_x^- or NaF, DFOM was ineffective (data not shown). These studies indicate that AlF_x^- acts as an irreversible inhibitor of brain LPP activity thereby amplifying tonic LPA_1 receptor activity. The general phosphatase inhibitor, NaF per se, did not inhibit LPPs, but mimicked the action of AlF_x^- based on the ability of F^- to etch Al^{3+} from the glass slides.

We were curious to examine whether AlF_x^- could also inhibit LPA degradation at the bulk brain level. When sections were pretreated with AlF_x^- but then omitted from all subsequent steps, AlF_x^- readily facilitated LPA_1 receptor signalling (Figure 23, Figure 24a), but such a pretreatment did not inhibit LPA degradation in a statistically significant manner. Degradation of exogenous LPA (50 µM) alone yielded 22.6 ± 1.2 nmol P_i per slide

whereas pretreatment with AlF_x^- followed by incubation with exogenous LPA yielded 21.4 ± 0.4 nmol P_i per slide (mean ± SEM, n=3). However, when added together with LPA, AlF_x^- totally (and NaF partially) blocked the formation of LPA-derived P_i, thus providing evidence of the ability of these compounds to inhibit the vanadate- and propranolol-insensitive pool of LPA phosphatases in a reversible manner (Figure 24b). Treatment with DFOM (50 μM) totally prevented the ability of AlF_x^- and NaF to inhibit the degradation of LPA (Figure 24b), indicating that AlF_x^-, rather than NaF, was the active compound.

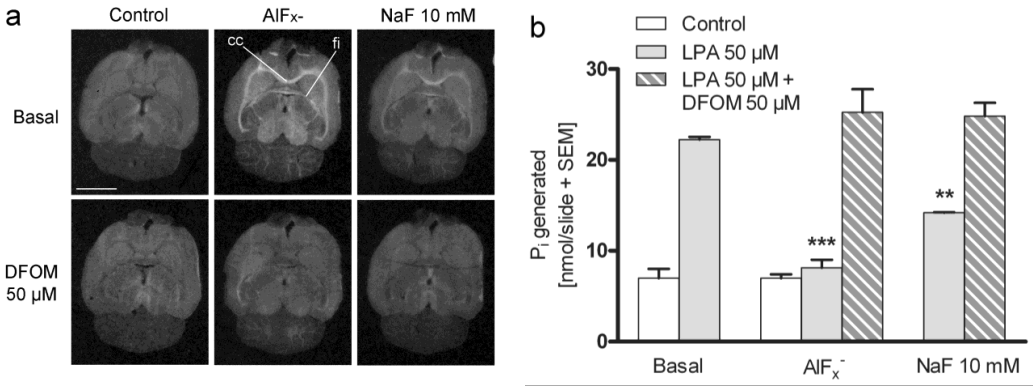


Figure 24. AlF_x^- (not NaF) generates the LPA_1 receptor-mediated response; AlF_x^- also blocks the LPA phosphatase activity. (a) AlF_x^- (NaF 10 mM + $AlCl_3$ 50 μM) was included during the 40 min pre-incubation step (step 1), NaF was present in step 2 whereas deferoxamine mesylate (DFOM) was present throughout steps 1–3 and in addition during a 10 min pre-incubation prior to AlF_x^- treatment. Both NaF and AlF_x^- induce [^{35}S]GTPγS binding in the LPA_1 receptor-enriched white matter areas. The responses to AlF_x^- and NaF are totally abolished with the aluminium and iron(III) chelator DFOM (cc, corpus callosum; fi, fimbria of the hippocampus). Scale bar = 5 mm. (b) Slides with two horizontal brain sections underwent the three-step autoradiography-mimicking protocol. AlF_x^- (NaF 10 mM + $AlCl_3$ 50 μM) and NaF were present during the 90 min incubation step (step 3). DFOM was present in steps 2 and 3. After the final incubation step, the assay buffer was quantitatively collected and the P_i content was determined. Note that NaF partially and AlF_x^- totally inhibit LPA-derived P_i formation and that DFOM reverses these actions. Significance level: ***p < 0.001 and **p < 0.01 compared to the treatment with LPA alone (n=3).

To further explore the consequences of total inhibition of LPA phosphatase activity, AlF_x^--treated brain sections were incubated for 40 min in autoradiography buffer, the buffer was removed and tissue LPA content extracted using chloroform-methanol, followed by LC/MS/MS analysis. Four LPA species with different acyl substitutions (16:0 LPA, 18:1 LPA, 18:0 LPA and 20:4 LPA) were examined in the present study. The relative abundances of the four LPA species in brain sections incubated under control conditions were 20:4 LPA ≈ 16:0 LPA < 18:1 LPA < 18:0 LPA. The amounts of three of these species (16:0 LPA, 18:1 LPA and 20:4 LPA) were significantly increased after AlF_x^- treatment when compared to control sections (Figure 25). These experiments indicate that total blockade of LPA phosphatase activity with AlF_x^- treatment resulted in accumulation of several endogenous LPA species at the bulk brain level. However, no such bulk LPA accumulation was required to observe the AlF_x^--evoked potentiation of LPA_1 receptor signalling.

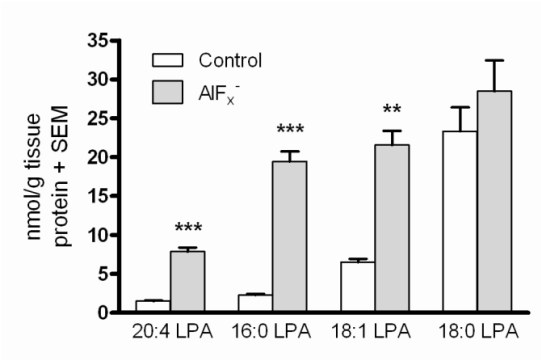


Figure 25. Treatment with AlF_x^- results in accumulation of several endogenous LPA species in rat brain sections. The slides with two horizontal brain sections were treated with AlF_x^- (NaF 10 mM + $AlCl_3$ 50 μM) for 40 min followed by extraction of LPA species. The samples were analyzed with LC/MS/MS for four endogenous LPA species. The amounts of three of these LPA species are increased in a statistically significant manner in AlF_x^--treated sections as compared to control sections. Significance level: ***$p < 0.001$ and **$p < 0.01$ compared to control (t test, n=3).

7.3.4 The LPA → MAG → glycerol pathway efficiently degrades exogenous LPA

In addition to the LPA → MAG → glycerol pathway (Figure 19), another P_i-generating pathway for LPA degradation involves its deacylation to form glycerophosphate which is further dephosphorylated by glycerophosphatases to glycerol (the LPA → GP → glycerol pathway) (Figure 19). Since there are two degradative pathways for LPA that potentially release P_i, we wished to clarify which pathway would account for LPA degradation in our model. We performed kinetic monitoring of P_i production from exogenous LPA and glycerol 3-phosphate (GP) after incubation with rat cerebellar membranes in a 96-well-format. In this setting, cerebellar membranes generated P_i from LPA (10 μM) but there was no P_i generation from GP (10 μM) (Figure 25a), indicating that the P_i formed from LPA in our experimental setting is principally due to LPP/LPP-like activity. As a further proof, we used the dephosphorylation-resistant thio-analog of LPA, (2S)-OMPT, that is also an LPA_3 receptor-preferring agonist. As expected, there was no P_i generation from (2S)-OMPT (10 μM) (Figure 26a).

Since both of the two P_i-releasing pathways for LPA degradation finally produce glycerol, we assessed cerebellar membrane-dependent glycerol generation from exogenous LPA in brain tissue. MAGL is believed to be mainly responsible for the MAG → glycerol conversion. In addition, ABHD6 and ABHD12 have been identified to hydrolyze brain endocannabinoid 2-AG (Blankman et al. 2007), and together the three serine hydrolases account for ~99% of the brain 2-AG hydrolase activity (Savinainen et al. 2012). It is therefore likely that in addition to MAGL, ABHD6 and ABHD12 are involved in the degradation of both 1- and 2-monoacylglycerols. To delineate the relative contributions of the three hydrolases, we pretreated cerebellar membranes with two serine hydrolase inhibitors, methylarachidonoylfluorophosphonate (MAFP) and JZL184. The former is a potent, non-selective inhibitor of MAGL (Saario et al. 2004, Savinainen et al. 2010) that also inhibits ABHD6/ABHD12, whereas the latter is a MAGL-selective inhibitor (Long et al. 2009). As expected, in rat cerebellar membranes incubated together with LPA (10 μM), glycerol production closely matches P_i generation (Figures 26a and 26b), indicating that MAG → glycerol conversion takes place under the assay conditions employed. With MAFP pretreatment (1 μM), LPA-derived glycerol production was decreased by 91% (Figure 26b). With JZL184 pretreatment (100 μM), the corresponding reduction was 71% (Figure 26b).

The selectivity of the inhibitors towards MAGL likely explains the difference in the inhibition of glycerol production from LPA between the two inhibitors. Since there was no P_i generation from exogenous glycerol 3-phosphate, it seems that LPA is predominantly degraded by the LPA → MAG → glycerol pathway in our experimental setting whereas the LPA → GP → glycerol pathway plays a minor role. According to our findings, both phosphohydrolases (LPPs/LPP-like) as well as MAGL and related hydrolases (ABHD6/ABHD12) seem to be active.

Previously, NEM-insensitive LPA phosphohydrolase activity was studied in the nuclear fraction isolated from rabbit cerebral cortex (Baker et al. 2000). This activity was found to be present also in the microsomal fraction. In the nuclear fraction, phosphohydrolase activity was found to be sensitive to NaF (50 mM) but virtually insensitive to propranolol (0.5 mM). Dephosphorylation by phosphohydrolases was found to be more active route for LPA degradation when compared to deacylation by lysophospholipases. It was also indicated that followed by dephosphorylation of LPA, monoacyl product is rapidly converted to glycerol by monoglyceride lipase. These findings support our present findings concerning the active pathways involved in LPA degradation in brain as well as the existence of LPP-like, propranolol- and vanadate-insensitive phosphohydrolase activity.

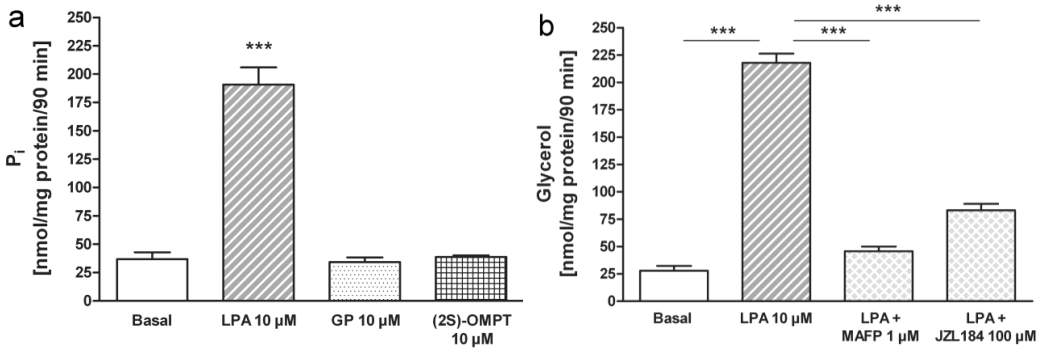


Figure 26. Rat cerebellar membranes principally metabolize LPA via the LPA → MAG → glycerol pathway. (a) Rat cerebellar membranes (1 µg/well) were incubated in the absence (basal) or presence of LPA, glycerol 3-phosphate (GP), or the phosphatase-resistant LPA analog (2S)-OMPT. P_i generation was kinetically monitored for 90 min using a fluorescent assay. Rat cerebellar membranes generate P_i from exogenous LPA but not from exogenous GP or (2S)-OMPT. Significance level: ***$p < 0.001$ compared to basal (n=4 for basal and LPA, n=3 for GP and (2S)-OMPT). (b) Rat cerebellar membranes were pretreated for 30 min with DMSO or the broad-spectrum serine hydrolase inhibitor MAFP or the MGL-specific inhibitor JZL184. This was followed by 90 min incubation in the absence (basal) or presence of LPA. Glycerol generation was kinetically monitored for 90 min using a fluorescent assay. Rat cerebellar membranes readily generate glycerol from exogenous LPA; this response is largely blocked by MAFP and partially so by JZL184. Significance level: ***$p < 0.001$ compared to either basal (a) or as indicated (b) (n=4).

7.4 CONCLUSIONS

The two most important pathways to generate LPA are thought to be LPA generation either from lysophospholipids by autotaxin or from phosphatidic acid by the PLD → $PLA_{1/2}$ pathway. Our findings indicated, however, that tonic LPA_1 activity in brain sections was not due to LPA generated as a result of autotaxin or PLD activity, since exogenous precursors of LPA as well as various compounds reported to affect autotaxin and/or PLD activity failed to affect the basal tone of LPA_1 receptor activity. We observed that in brain sections, the lifetime of bioactive LPA is controlled by Mg^{2+}-independent, NEM-insensitive phosphatase activity attributable to LPPs. Pharmacological inhibition of this LPP activity by AlF_x^-, propranolol or sodium orthovanadate amplified basal and LPA-stimulated LPA_1 receptor signalling, as revealed using functional autoradiography. We conclude that LPP acts locally to control the lifetime of the signalling pool of LPA in the vicinity of LPA_1 receptors whereas the majority of brain LPA phosphatase activity is attributable to additional LPP-like enzymatic activity. LPP-like enzymatic activity, like LPP activity, is sensitive to AlF_x^- but appears to be resistant to the two other LPP inhibitors, vanadate and propranolol. Finally, we demonstrated that degradation of exogenous LPA is almost entirely channelled via the LPA → MAG → glycerol pathway and that MAGL accounts for the majority of oleylglycerol-hydrolyzing activity in brain tissue. The P_i- and glycerol-generating enzymatic routes involved in LPA degradation are summarized in Figure 27.

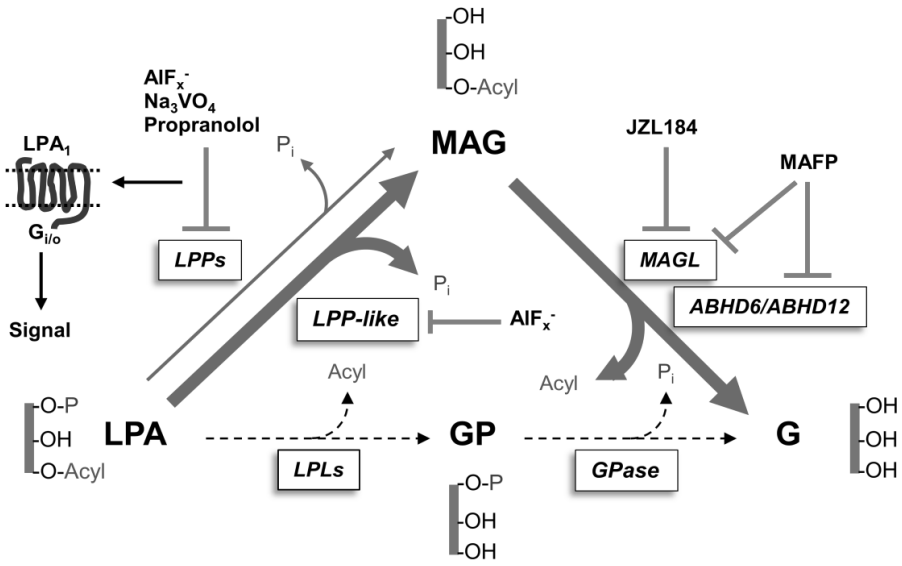


Figure 27. Summary of the inorganic phosphate (P_i)- and glycerol-generating enzymatic pathways potentially involved in brain tissue-dependent LPA degradation. The width of the arrows indicates the relative activity of the pathway in the present experimental setting. According to the present study, the LPA → MAG → glycerol pathway is predominantly responsible for LPA degradation in rat brain whereas the LPA → GP → glycerol pathway plays a minor role. The majority of brain LPA phosphatase activity is attributed to LPP-like enzymatic activity. The blunt arrows indicate the enzyme inhibition. Abbreviations: ABHD, α/β-hydrolase domain-containing protein; G, glycerol; GP, glycerol phosphate; LPA, lysophosphatidic acid; LPL, lysophospholipase; LPP, lipid phosphate phosphatase; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase, MAFP; methylarachidonoylflurophosphonate.

8 Identification of Enzymatic Pathways Generating 2-AG in Brain Sections[4]

Abstract: The principal brain endocannabinoid, 2-AG, is enzymatically produced by postsynaptic neurons and then activates presynaptic CB_1 receptors in a retrograde manner. The primary pathway for 2-AG generation is believed to be conversion from the DAGs by two *sn*-1-specific lipases, DAGLα and DAGLβ. Previous studies with DAGL-deficient mice indicate that DAGLα is the major enzyme needed for retrograde synaptic 2-AG signalling. The current study investigated whether the CB_1 receptor-mediated G_i protein activity is altered in brain cryosections of DAGL-deficient mice when compared to wild-type mice and whether the *sn*-1-specific DAGLs are able to generate 2-AG in brain cryosections. Functional autoradiography indicated that brain regional CB_1 receptor-G_i-activity largely remained unaltered in DAGLα-knockout and DAGLβ-knockout mice when compared to wild-type littermates. Following comprehensive pharmacological blockade of 2-AG hydrolysis, brain sections generated sufficient amounts of 2-AG to activate CB_1 receptors throughout the regions endowed with these receptors. As demonstrated by LC/MS/MS, this pool of 2-AG was generated via tetrahydrolipstatin-sensitive enzymatic pathways distinct from DAGLα or DAGLβ. We conclude that in addition to the *sn*-1-specific DAGLs, additional 2-AG-generating enzymatic pathways are active in brain sections.

[4] Adapted with permission of Elsevier from: **Aaltonen N, Riera Ribas C, Lehtonen M, Savinainen JR, Laitinen JT. Brain regional cannabinoid CB_1 receptor signalling and alternative enzymatic pathways for 2-arachidonoylglycerol generation in brain sections of diacylglycerol lipase deficient mice. Eur J Pharm Sci 51:87-95, 2014.**

8.1 INTRODUCTION

Convincing evidence has been provided to show that DAGLα is the major enzyme involved in the biosynthesis of 2-AG needed for retrograde synaptic signalling. It was observed that the retrograde synaptic suppression was absent in several brain regions of DAGLα-KO mice whereas it remained intact in the brain of DAGLβ-KO mice (Gao et al. 2010, Tanimura et al. 2010, Yoshino et al. 2011). Furthermore, there were up to 80 % reductions in 2-AG levels in DAGLα-KO mice brain whereas in the brains of DAGLβ-KO mice, the 2-AG levels remained unaltered (Tanimura et al. 2010, Yoshino et al. 2011), or were reduced up to 50 % (Gao et al. 2010). However, despite the chronic reduction in 2-AG levels, there was no evidence of CB_1 receptor up-regulation in the studied brain regions, as evidenced by unaltered levels of mRNA (Gao et al. 2010) and Western blotting of receptor protein (Tanimura et al. 2010). However, it has not been studied whether the brain regional CB_1 receptor–G_i signalling axis remains intact in DAGL-deficient mice.

We previously demonstrated that pharmacological elimination of 2-AG hydrolytic activity in rat brain sections leads to an accumulation of endogenous 2-AG and subsequent CB_1 receptor activation, as revealed using [^{35}S]GTPγS autoradiography (Palomäki et al. 2007). It was postulated that the DAGL activity generated 2-AG in sufficient amounts to activate CB_1 receptors. The 2-AG accumulation was susceptible to two recognized inhibitors of the DAGLs, tetrahydrolipstatin (THL) and compound RHC80267, and CB_1 receptor activity was modestly amplified by two DAGL activators, calcium and glutathione (Palomäki et al. 2007). Since only nonselective pharmacological tools were previously available to explore the possible role of DAGLs, we were curious to extend these findings by examining whether 2-AG accumulation takes place in cryosections of DAGLα-KO or DAGLβ-KO mice brain when compared to WT brain and whether there would be alterations in the functionality in CB_1 receptor-G_i axis between the genotypes.

8.2 MATERIALS AND METHODS

The materials and general methods are described in Chapter 4.

8.2.1 DAGL-deficient mice

Frozen brains of DAGLα-KO, DAGLβ-KO and WT mice were a gift from Dr. Masanobu Kano, University of Tokyo, Tokyo, Japan and from Dr. Kenji Sakimura, Niigata University, Niigata, Japan. The generation of the DAGL-KO mice has been described by Tanimura et al. (2010). The brains were removed from 3 to 6 months old mice representing combination of both males and females of all genotypes. The mice were sacrificed by decapitation, and within the next 3 min, the whole brain was dissected out, dipped briefly either in isopentane (chilled on dry ice) or in liquid nitrogen, and stored at –80 °C. Both isopentane and liquid nitrogen were used to freeze the brains in order to compare the two freezing methods. Since our preliminary experiments demonstrated that there was no difference in the outcome between the brains processed in these two different ways (data not shown), a combination of both brain types were used in following experiments. Horizontal brain sections (20 μm thick) were cut at –20 °C using a Leica cryostat, thaw-mounted onto Superfrost®Plus slides (Menzel-Gläser, Germany), dried for 1–2 h at room temperature under a constant stream of air and stored thereafter at –80 °C.

8.2.2 LC/MS/MS for eCB determination

Sample preparation

Triplicate slides each with five brain sections underwent the autoradiography-mimicking protocol (except that DPCPX, GDP, DTT and radioligand were omitted). The protocol consisted of 20 min pre-incubation in the assay buffer, 60 min incubation in the assay buffer with or without the lipase inhibitors and 0.5% (w/v) BSA, and finally a 90 min incubation in the assay buffer supplemented with 0.1% (w/v) BSA. Between the steps, the slides were rinsed either twice (after the first step) or once (after the second step) with the assay buffer. After the final 90 min incubation step, the postincubation buffer was quantitatively collected. The slides were rinsed twice (2 min each time) in ice-cold washing buffer, dipped for 30 s in ice-cold deionized water and air-dried.

To determine the 2-AG and AEA content, the brain sections were moistened with methanol and the tissue was scraped manually from the slides with a spatula into a plastic test tube. To determine 2-AG and AEA content in the incubation buffer, 500 µl of incubation buffer was transferred to a plastic test tube. Extraction of the analytes from the sample matrix has been previously described (Lehtonen et al. 2011). Briefly, 500 µl of ice-cold methanol was added to samples, which were then homogenized with a Soniprep 150 homogenizer (MSE Ultrasonic Disintegrator; MSE Scientific Instruments, Manor Royal, Crawley, Sussex, UK). Samples were transferred into screw-capped glass test tubes and the lipids were extracted by adding chloroform and water to yield a methanol/chloroform/water ratio of 1:2:1 (v/v/v). Samples were centrifuged at 1500 x *g* for 10 min at 10 °C to obtain a sharp phase separation. The upper aqueous layer was discarded and the lower organic layer was transferred to a screw-capped glass test tube. This liquid extraction was repeated once, and the organic layers were combined. The sample was then evaporated to dryness under nitrogen at room temperature and the residue was reconstituted in 50 µl of ice-cold acetonitrile. The sample was dissolved for 5 min and then 20 µl of water was added. After centrifugation at 10 000 x *g* for 10 min at 10 °C, the supernatant was transferred to an HPLC sample vial for LC/MS/MS analysis, as described below.

Liquid chromatography/tandem mass spectrometry (LC/MS/MS)

The LC/MS/MS instrumentation used in the eCB analysis has been previously described (Lehtonen et al. 2011). The HPLC system consisted of an Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Waldbronn, Germany) with a solvent micro vacuum degasser, a binary pump, a thermostatted column compartment, and an autosampler. The mass analysis was made with an Agilent 6410 Triple Quadrupole MS equipped with an electrospray ionization source (Agilent Technologies, Palo Alto, CA, USA). Ten microliters of the sample solution were injected onto a reversed-phase HPLC column (Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 mm x 50 mm, 1.8 µm) (Agilent Technologies, Palo Alto, CA, USA) using an isocratic mobile phase consisting of H_2O/acetonitrile/formic acid (33:67:0.1, v/v/v), delivered at 150 µl/min. Column temperature was maintained at 40 °C and the autosampler tray temperature was set at 10 °C. The following ionization conditions were used: ESI positive ion mode, drying gas (nitrogen) temperature 300 °C, drying gas flow rate 10 l/min, nebulizer pressure 50 psi, and capillary voltage 4000 V. Detection was performed using multiple reaction monitoring with the following transitions: m/z 348 → 62 for AEA, m/z 356 → 63 for AEA-d8, m/z 379 → 287 for 2-AG, and 387 → 294 for 2-AG-d8. Fragmentor voltage and collision energy for AEA, AEA-d8, 2-AG, and 2-AG-d8 were 120 and 10 V, 120 and 12 V, 130 and 8 V, and 125 and 10 V, respectively. Dwell time was 100 ms for each transition, and mass resolutions for MS1 and MS2 quadrupoles were 2.4 and 1.2 FWHM, respectively, for AEA, and 0.7 and 0.7 FWHM,

respectively, for AEA-d8, 2-AG, and 2-AG-d8. Deuterated internal standards, AEA-d8 and 2-AG-d8, were used for quantification, and peak area ratios of the analyte to the internal standard were calculated as a function of the concentration ratios of the analyte to the internal standard.

8.3 RESULTS AND DISCUSSION

8.3.1 The functionality of CB_1 receptor–G_i-axis remains unaltered in DAGLα-KO and DAGLβ-KO mice when compared to WT animals.

We assessed basal and CB_1 receptor-dependent G protein activity in cryosections of DAGLα-KO, DAGLβ-KO and WT mice by analyzing selected CB_1 receptor-enriched brain regions (Herkenham et al. 1991), i.e. the cingulate cortex, caudate putamen, globus pallidus, somatosensory cortex, substantia nigra, cerebellar molecular layer, hippocampus-overall, hippocampus-CA1, hippocampus-CA3, and hippocampus-dentate gyrus, in a quantitative manner. There were no statistically significant differences in basal G protein activity in examined brain regions in the DAGLα-KO, DAGLβ-KO and WT mice (Figures 28a and b). Pretreatment with the broadly-acting serine hydrolase inhibitor MAFP (10 μM) induced a robust [^{35}S]GTPγS binding response throughout the CB_1 receptor-enriched brain regions (Figure 28a). Evidently, after comprehensive blockade of 2-AG hydrolysis, mice brain cryosections were able to generate sufficient amounts of 2-AG to activate CB_1 receptors. Previously, by classical filtration based [^{35}S]GTPγS binding assay it was demonstrated that MAFP has no direct effect on CB_1 receptor activation (Savinainen et al. 2003, Palomäki et al. 2007). No statistically significant differences were observed in the MAFP-evoked CB_1 receptor response between the genotypes (Figure 28b).

The synthetic CB_1 receptor agonist CP55,940 induced concentration-dependent (0.5 μM, 5 μM, 50 μM) [^{35}S]GTPγS binding responses in the studied brain regions (Figure 28a). The dose-response for CP55,940 was chosen to extend up to 50 μM due to the high degradative capacity of brain sections, where millimolar concentrations of GDP are needed to observe agonist-driven G_i protein activity (Laitinen 2004). No statistically significant difference was observed in CP55,940-evoked regional CB_1 receptor activity between DAGLβ-KO and WT mice whereas in certain hippocampal regions of DAGLα-KO mice (hippocampus-overall, hippocampus-CA3 and hippocampus-dentate gyrus), sub-maximal concentrations of CP55,940 evoked a statistically significant increase in CB_1 receptor activity (Figure 28b). In agreement with the previous findings with rat brain cryosections (Palomäki et al. 2007, Aaltonen et al. 2013), the observed labelling patterns evoked by both MAFP and CP55,940 were abolished with the CB_1 receptor-selective antagonist AM251 (1 μM), confirming that responses were mediated via the CB_1 receptors (Figure 29).

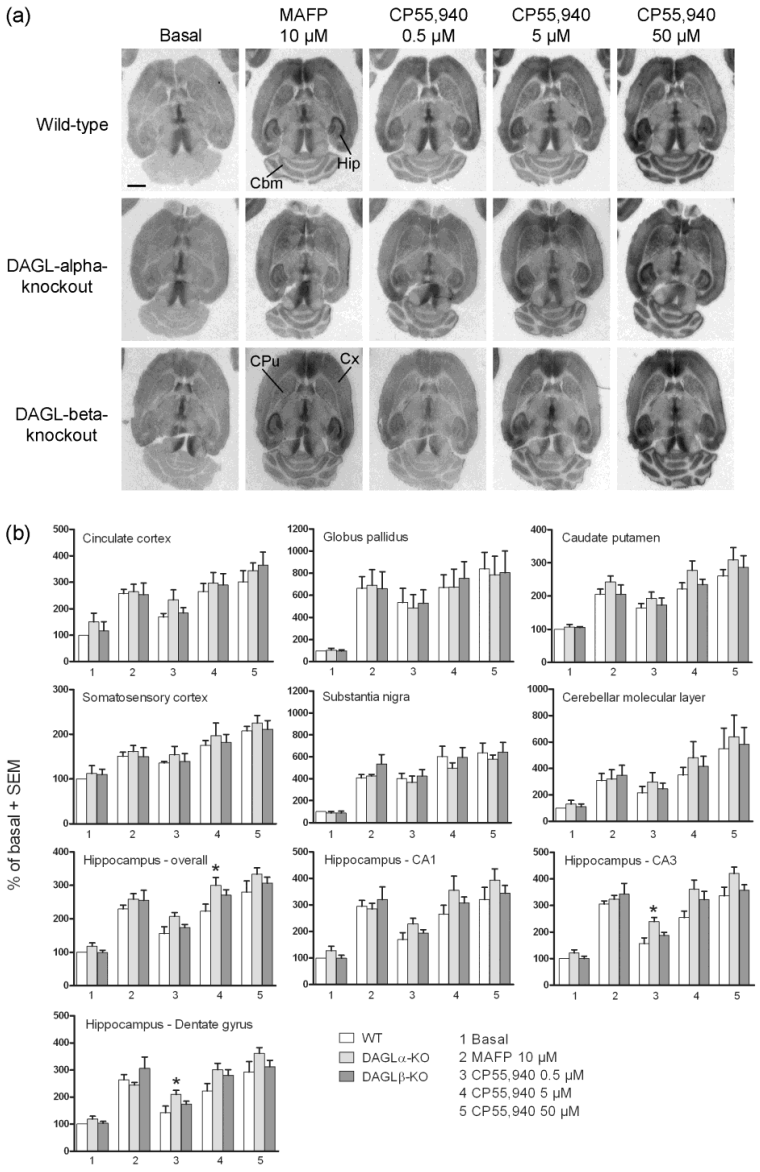


Figure 28. Blockade of endocannabinoid hydrolysis by the irreversibly acting inhibitor MAFP evokes CP55,940-mimicking [^{35}S]GTPγS binding responses throughout the CB_1 receptor-enriched brain regions of DAGLα-KO, DAGLβ-KO and WT mice brain sections. MAFP was included during step 2 whereas CP55,940 was included during the [^{35}S]GTPγS labelling step. (a) In basal conditions, there is no difference in [^{35}S]GTPγS labelling between the genotypes. Treatment with MAFP induces G protein activity in the CB_1 receptor-enriched brain regions; this response is similar in the three genotypes. The MAFP-evoked response is dose-dependently mimicked by CP55,940 (Cx, cerebral cortex; CPu, caudate putamen; Hip, hippocampus; Cbm, molecular layer of the cerebellum). Scale bar = 2 mm. (b) Quantitative data of the [^{35}S]GTPγS binding responses. No statistically significant difference is observed in basal or MAFP-evoked G protein activity between WT and DAGLα-KO or DAGLβ-KO mice. In certain hippocampal structures, treatment with CP55,940 induces increased binding response in DAGLα-KO mice when compared to WT mice (*$p < 0.05$) (n=5).

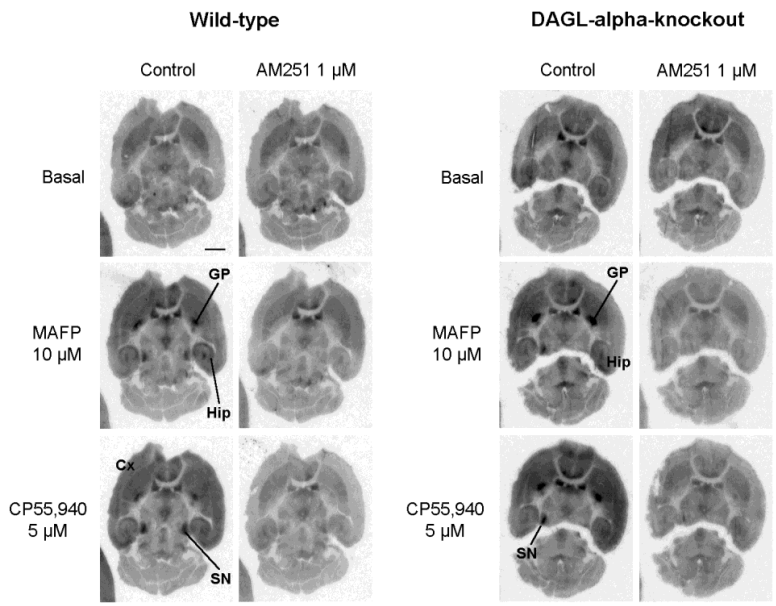


Figure 29. [^{35}S]GTPγS binding responses evoked by MAFP and CP55,940 are sensitive to CB1 receptor-selective antagonist AM251. MAFP was included during the step 2 whereas CP55,940 and AM251 were included during the [^{35}S]GTPγS labelling step (Cx, cerebral cortex; GP, globus pallidus; Hip, hippocampus; SN, substantia nigra). Scale bar = 2 mm.

Previous studies with DAGL-deficient mice revealed that both global and regional 2-AG levels in brain were significantly reduced and that 2-AG mediated retrograde synaptic suppression was absent in DAGLα-KO mice brain (Gao et al. 2010, Tanimura et al. 2010, Yoshino et al. 2011). One would expect that chronic and extensive 2-AG reduction would lead to up-regulation of CB_1 receptors as a compensatory response. However, mRNA expression pattern and protein levels of CB_1 receptors were found to be normal in the studied brain regions (Gao et al. 2010, Tanimura et al. 2010). In contrast, global genetic knockout of MAGL resulted in a dramatic increase in 2-AG levels in the nervous system that lead to a reduction of CB_1 receptor density, as well as pharmacological tolerance to the cannabinoid agonists, indicating desensitization of CB_1 receptors (Chanda et al. 2010, Taschler et al. 2011). Down-regulation of CB_1 signalling was also observed in some, but not all regions following chronic treatment of mice with the selective MAGL-inhibitor JZL184 (Schlosburg et al. 2010). Likewise, chronic treatment of rats with Δ^9-THC significantly reduced cannabinoid agonist-stimulated responses in several brain regions enriched with the CB_1 receptors whereas acute Δ^9-THC treatment had no such effect (Sim et al. 1996). Our present findings indicate that agonist-induced CB_1 receptor signalling is unaltered in all tested brain regions of DAGLβ-KO mice and in the majority of tested brain regions of DAGLα-KO mice as compared to their WT littermates. In certain hippocampal regions of DAGLα-KO mice brain, however, increased CB_1 receptor activity was observed. Evidently, in the majority of brain regions, the loss of DAGL does not affect the functionality of the CB_1-receptor-$G_{i/o}$ axis or the receptor density but it is conceivable that in hippocampus, chronic reduction of 2-AG leads to activation of compensatory mechanisms. Previously, hippocampus has been demonstrated to be one of the brain structures that is very sensitive to desensitization and down-regulation of CB_1 receptors both in mice (Sim-Selley et al. 2006, McKinney et al. 2008, Schlosburg et al. 2010) and humans (Hirvonen et al. 2013) and similarly this structure might also be susceptible to receptor up-regulation. Nonetheless, the chronic reduction of 2-AG levels in the CNS does not seem to induce any major

compensatory effects to such an extent as seen after the chronic elevation of 2-AG levels described in several animal models.

8.3.2 Mice brain sections generate 2-AG via THL-sensitive enzymatic pathways distinct from DAGLα or DAGLβ.

To investigate the origin of 2-AG capable of activating the CB_1 receptors in MAFP-treated sections, brain sections of DAGLα-KO, DAGLβ-KO and WT mice were treated with MAFP or with the combination of MAFP and the established DAGL inhibitor THL using autoradiography-mimicking conditions. After the incubation, the tissue and buffer lipids were extracted using chloroform-methanol and 2-AG and AEA levels were determined with LC/MS/MS. Pretreatment with MAFP (10 μM) resulted in an approximately 9-fold increase in the tissue 2-AG content (Figure 30a) and an approximately 100-fold increase in 2-AG levels in the incubation buffer (Figure 30b). Inclusion of THL (10 μM) blocked the 2-AG generation in MAFP-treated sections; this was evident in both tissue and incubation buffer samples (Figures 30a and b). There were no statistically significant differences in 2-AG levels between WT and DAGLα-KO or DAGLβ-KO mice in any of the tested conditions (Figures 30a and b). These results indicate that in the present experimental setting, brain sections generate 2-AG via THL-sensitive enzymatic pathways distinct from DAGLα or DAGLβ. In addition to MAGL, MAFP potently inhibits other serine hydrolases including the AEA-degrading enzyme FAAH (McKinney and Cravatt 2005). When relative levels of AEA were determined after pre-incubation with MAFP (10 μM), there was an approximately 7-fold increase in the AEA content when compared to basal situation in tissue samples (Figure 31a) and an approximately 3-fold increase in incubation buffer samples (Figure 31b). Treatment with THL (10 μM) had no effect on the MAFP-induced increases in AEA levels (Figures 31a and b). There were no statistically significant differences in AEA contents between WT and DAGLα-KO or DAGLβ-KO mice in any of the tested conditions (Figures 31a and b).

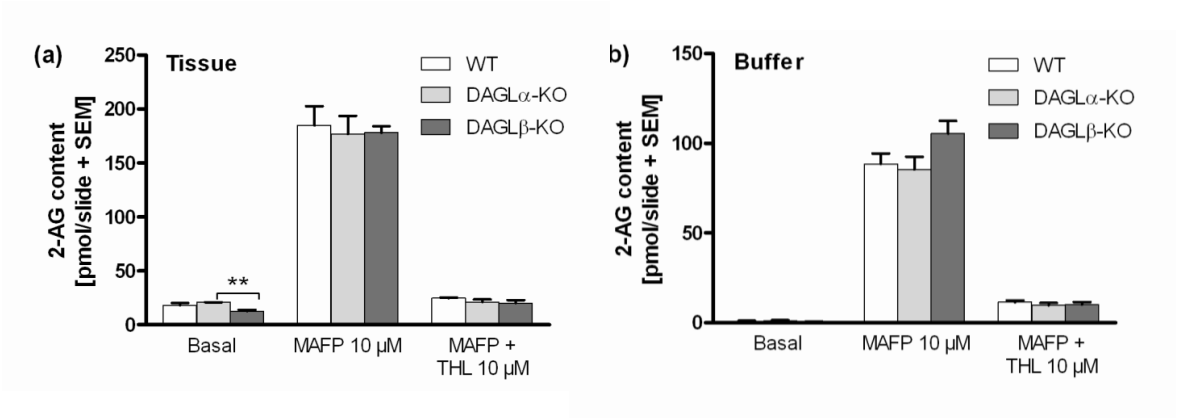


Figure 30. Mouse brain sections generate 2-AG via THL-sensitive enzyme pathway(s) distinct from DAGLα or DAGLβ. (a) and (b) Slides with five horizontal sections of DAGLα-KO, DAGLβ-KO and WT mice brain underwent the three-step autoradiography-mimicking incubation. MAFP and THL were present during the 60 min incubation step (step 2). (a) The tissue was scraped from the slides and lipids extracted using chloroform-methanol followed by 2-AG determination with LC/MS/MS. (b) After the final incubation step, the assay buffer was quantitatively collected and lipids extracted using chloroform–methanol followed by 2-AG determination with LC/MS/MS. Significance level: $^{**}p < 0.01$ (n=4).

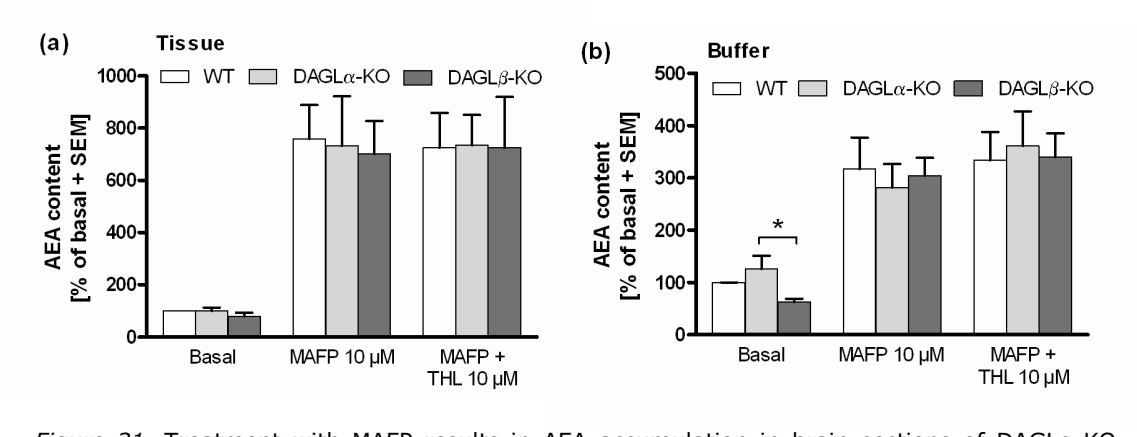


Figure 31. Treatment with MAFP results in AEA accumulation in brain sections of DAGLα-KO, DAGLβ-KO and WT mice in THL-insensitive manner. (a) and (b) Slides with five horizontal sections of DAGLα-KO, DAGLβ-KO and WT mice brain underwent the three-step autoradiography-mimicking incubation. MAFP and THL were present during the 60 min incubation step (step 2). (a) The tissue was scraped from the slides and lipids extracted using chloroform–methanol followed by determination of relative content of AEA with LC/MS/MS. (b) After the final incubation step, the assay buffer was collected and lipids extracted using chloroform-methanol followed by determination of relative content of AEA with LC/MS/MS. Significance level: $*p < 0.05$ (n=4).

In the comparison of 2-AG and AEA levels between unprocessed cryosections of DAGLα-KO, DAGLβ-KO and WT mice brains, the sections were thawed, the tissue lipids were extracted using chloroform–methanol and the endocannabinoid content was determined with LC/MS/MS. Unexpectedly, we found approximately 2-fold elevated levels of 2-AG in sections of DAGLα-KO brain when compared to WT and DAGLβ-KO brain (Figure 32a). There was no statistically significant difference in the 2-AG content between the sections of WT and DAGLβ-KO brain (Figure 32a). When AEA levels were determined, there were no statistically significant differences in the AEA content between the three genotypes (Figure 32b).

Although MAFP elevates tissue levels of both 2-AG and AEA under the conditions employed, our previous studies indicated that treatment of brain sections with URB597, a potent and specific inhibitor of FAAH, failed to mimic MAFP in evoking the CB_1 receptor activity response (Palomäki et al. 2007). This is true also for another FAAH inhibitor, PF-750 (our unpublished observation). Furthermore, exogenous AEA, even in the presence of URB597, generated only relatively weak responses as opposed to the robust responses evident in MAFP-treated sections (Palomäki et al. 2007). These data strongly argue against the possibility that the observed responses were mediated by AEA, the other major CB_1 receptor-activating eCB recognized to date. Moreover, we recently demonstrated that the potency of the MAGL-selective inhibitor JZL184 is not sufficient to afford visualization of the eCB-driven CB_1 receptor activity (Aaltonen et al. 2013). However, this is possible using a truly potent MAGL-selective inhibitor (JJKK-048) (Aaltonen et al. 2013) indicating that 2-AG is the endocannabinoid responsible for activating CB_1 receptors in brain sections. However, not even comprehensive elimination of MAGL activity is sufficient to generate the full MAFP-mimicking response, as MAGL accounts for ~85 % of brain 2-AG hydrolysis and additional hydrolases (ABHD6 and ABHD12) remain active after selective MAGL-blockade (Blankman et al. 2007, Savinainen et al. 2012). Only MAFP seems to be capable of comprehensively prevent brain 2-AG hydrolysis.

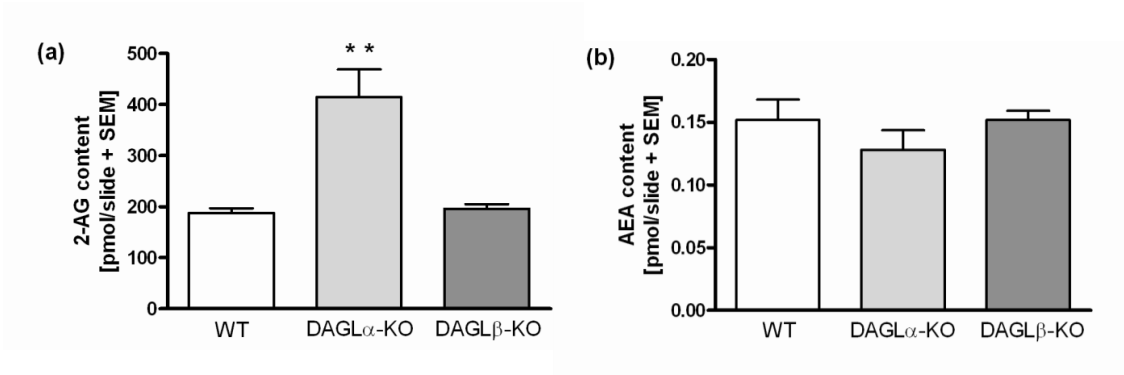


Figure 32. Tissue 2-AG content is higher in unprocessed brain sections of DAGLα-KO mice when compared to WT and DAGLβ-KO mice, there is no difference in tissue AEA content between the genotypes. (a) and (b) Slides with five horizontal cryosections of DAGLα-KO, DAGLβ-KO and WT mice brain were thawed, tissue scraped from the slides, and lipids extracted using chloroform-methanol followed by endocannabinoid determination with LC/MS/MS. Note the difference to figures 30 and 31; no incubations have been conducted with this set of slides. Significance level: $^{**}p < 0.01$ compared to wild-type (n=4).

Accumulation of 2-AG, but not that of AEA, was sensitive to THL, indicating that mouse brain has THL-sensitive enzymatic machinery that is able to generate 2-AG in sufficient amounts to activate CB_1 receptors. Since there was no statistically significant difference in the 2-AG contents between the genotypes in MAFP-treated brain sections, we conclude that this pool of 2-AG is not being generated via DAGLα or DAGLβ. These results therefore indicate that alternative THL-sensitive enzymatic pathways capable of generating 2-AG are active in brain cryosections. Since previous reports indicated that both global and regional 2-AG levels in brain were significantly reduced in DAGLα-KO mice brain (Gao et al. 2010, Tanimura et al. 2010, Yoshino et al. 2011), currently observed ability of brain sections of DAGLα-deficient mice to generate 2-AG, as well as increase in 2-AG levels in unprocessed brain sections of DAGLα-KO mice, are unexpected. Since we measured 2-AG and AEA contents in brain sections that were incubated at room temperature for a prolonged period of time, and it has been demonstrated that post-mortem rat brain can generate 2-AG rapidly after decapitation (Sugiura et al. 2001), the eCB contents likely reflect the biochemical reaction taking place in dead neural tissues. It is possible that lipids leak out from damaged or dead cells in brain sections and 2-AG is produced by enzymes other than DAGLs. In DAGL-KO mice, the activities of these enzymes might have been elevated in order to compensate for the lack of DAGLs. It has been demonstrated that there were no changes in mRNA or protein levels of DAGLα in DAGLβ-KO and *vice versa* (Gao et al. 2010), suggesting that the lack of one isotype is not inducing up-regulation of the other isotype. Alternative pathways for 2-AG generation could include conversion from 2-arachidonoyl lysophosphatidic acid by a phosphatase or generation from 2-arachidonoyl-phospholipids by sequential actions of PLA_1 and lyso-PLC (Piomelli 2003, Bisogno 2008). However, these pathways are apparently not involved in the 2-AG generation required for retrograde synaptic suppression (Tanimura et al. 2010), though there is evidence indicating that *sn-1*-DAGLα is not involved in hippocampal inhibitory synaptic transmission that is believed to be mediated by 2-AG (Min et al. 2010b). We assume that the pool of 2-AG generated in mice brain cryosections is different from the DAGLα-dependent pool that is used for retrograde 2-AG signalling in the living brain. We therefore wish to emphasize that brain cryosections do not represent a physiological model for eCB generation or retrograde signalling in the living brain. However, this study clearly demonstrates that as

well as the *sn-1* specific DAGLs, additional 2-AG generating enzymatic pathways are active in brain sections.

It should be noted that in addition to their signalling functions, eCBs have been indicated to possess supplemental roles (Piomelli 2003, Alger & Kim 2011). A detectable amount of 2-AG is present in non-stimulated brain without permanent activation of CB_1 receptors (DiMarzo and Petrosino 2007, Gao et al. 2010, Tanimura et al. 2010) reflecting that not all of the 2-AG is used for retrograde signalling. Since 2-AG acts at the crossroads of multiple biochemical pathways of lipid metabolism, it is likely that a proportion of 2-AG generated in brain serves as a lipid intermediate in these pathways. Moreover, although the signalling pool of 2-AG is apparently operating between post- and presynaptic neurons, another pool might be present in other cell types and not be involved in retrograde signalling. It has been demonstrated that mouse microglia (Walter et al. 2003, Carrier et al. 2004, Witting et al. 2004) as well as astrocytes (Walter et al. 2004) have the capacity to produce 2-AG. Thus, it might be that non-neuronal cell types account for the production of 2-AG in brain cryosections. Further studies are therefore warranted in order to identify the cell types generating this alternative pool of 2-AG in brain tissue. Finally, it is noteworthy that in some brain regions highly enriched with the CB_1 receptors (such as the substantia nigra) (Herkenham et al. 1991), DAGLα is only sparsely expressed, as evidenced by weak labelling with the DAGLα antibodies in immunohistochemical studies (Uchigashima et al. 2007, Tanimura et al. 2010). This further supports the existence of alternative biochemical routes that could generate CB_1 receptor-activating eCBs in brain regions with sparse DAGLα expression.

8.4 CONCLUSIONS

The present findings indicate that the functionality of the CB_1 receptor–G_i-axis remains unaltered in various brain regions of DAGLα-KO and DAGLβ-KO mice when compared to WT animals. Furthermore, we demonstrate that mice brain cryosections are able to generate sufficient amounts of 2-AG to activate CB_1 receptors throughout the brain regions endowed with these receptors. As demonstrated by LC/MS/MS, this pool of 2-AG is generated via THL-sensitive enzymatic pathway(s) but evidently not via DAGLα or DAGLβ. This pool of 2-AG must therefore be different from the DAGLα-dependent pool that is used for retrograde 2-AG signalling in living brain. We conclude that in addition to DAGLs, brain tissue has additional THL-sensitive enzymatic sources capable of generating 2-AG.

9 General Discussion

9.1. General comparison of LPA and eCB signalling

The present study provides novel information about regulation of LPA and eCB signalling systems in brain tissue. Though both LPA and eCBs are bioactive lipids that signal through specific GPCRs, they are usually discussed separately in the literature. There is apparent similarity between the two lipid systems, but certain differences can also be found.

From the historical point of view, LPA and eCB research has been evoked from different origins. The natural source of cannabinoids, the *Cannabis sativa* plant, has been used for millennia for its intoxicating and medicinal properties. Identification of the active component of *Cannabis sativa* (Gaoni & Mechoulam 1964) and further efforts to explain cannabinoid actions in the body led to the discovery of cannabinoid receptors and their endogenous ligands, the eCBs. The LPA research was initiated upon the early observations that members of the endogenous phospholipid class of compounds can act as extracellular effectors in modulating blood pressure (Sen et al. 1968, Tokumura et al. 1978). From the first observations, it took three decades until the first LPA receptor was cloned. The first cannabinoid receptor was cloned in 1988 (Devane et al. 1988) whereas the first LPA receptor was cloned approximately ten years later, in 1996 (Hecht et al. 1996). The discovery of the specific receptors instigated an intense research activity on both fields.

Currently, there are more GPCRs identified for LPA (six receptors) (Table 2) than for eCBs (two receptors). In addition to the currently recognized receptors, there might be other receptors for both LPA and eCBs among the large group of orphan GPCRs; so far more candidates have appeared for LPA. According to phylogenetic models of GPCRs, LPA receptors appear to have evolved from two distinct lineages whereas CB_1 and CB_2 receptors evolved from a single lineage (Figure 3) (Shimizu 2009). The LPA signalling is mediated via all four main G protein subfamilies whereas eCB receptors principally signal via G_i type G proteins. Due to a larger number of receptors and signalling partners, LPA signalling is somewhat more complicated than eCB signalling. In the brain, the CB_1 receptor is one of the most abundant GPCRs showing a robust expression pattern in the gray matter areas (Herkenham 1991). The LPA_1 receptor is considered to be the primary LPA receptor in the brain but it shows restricted expression pattern mainly in white matter tracts of the developing brain (Choi et al. 2010). The role of eCB signalling as a modulator of neurotransmission is rather well understood whereas the role of LPA in neurotransmission largely remains to be elucidated. Both LPA and eCBs play major roles in brain development.

Studies on receptor and enzyme knockout animals have revealed certain differences between LPA and eCB functions. The CB receptor knockouts as well as knockouts of eCB-synthesizing and -degrading enzymes show a normal phenotype (Zimmer et al. 1999, Buckley et al. 2000, Buckley et al. 2008, Tanimura et al. 2010, Gao et al. 2010, Chanda et al. 2010, Yoshino et al. 2011, Tascler et al. 2011). The LPA_1 receptor knockouts, instead, show 50% embryonic lethality (Contos et al. 2000) whereas ATX knockouts as well as LPP3 knockouts are embryonically lethal (Tanaka et al. 2006, Escalante-Alcade et al. 2003). The embryonic lethality occurs also when another lysophospholipid receptor, $S1P_1$, is knocked out (Liu et al. 2000). Based on these observations one could conclude that lysophospholipid signalling is more essential for development than eCB signalling. In post-natal life, it seems that eCB-mediated signalling become especially important during disease.

Following the discovery of receptors mediating the actions of eCBs and LPA, the characterization of their biosynthetic and degradative enzymes has provided essential information about the functionality of these lipid systems and has remarkably increased the

knowledge on how exogenous agents can affect these systems. Enzymatic networks reveal the closeness of the systems to each other; theoretically, when 2-arachidonoyl-LPA is dephosphorylated by phosphatase activity, the product will be 2-AG (Figure 33). The enzymatic conversion of 2-arachidonoyl-LPA to 2-AG has been demonstrated to take place in brain homogenates (Nakane et al. 2002) but currently, there is no direct evidence to link the LPPs to cannabinoid signalling (Brindley & Pilquil 2009). In aggressive cancer cells, the 2-AG-hydrolyzing enzyme MAGL generates lipid precursors for the production of LPA (Nomura et al. 2010). MAG acts as a substrate for COX and by COX activity, MAGL products can also be used for eicosanoid production (Nomura et al. 2011) (Figure 33), linking both LPA and eCB systems to the eicosanoid system.

Figure 33. The interconnection between three lipid-GPCR signalling systems involving LPA, endocannabinoid and eicosanoid signalling. $C_{20:4}$ LPA can be converted to 2-AG by a phosphatase activity. The hydrolysis of 2-AG produces AA, which can be used as a precursor for the eicosanoid production (modified from Brindley & Pilquil 2009).

The dysregulation of LPA and eCB signalling is involved in the pathology of several common diseases (Tables 3 and 4). In the majority of these diseases, however, the main functions of LPA and eCBs are opposite. In cancer, LPA generally induces cancer progression whereas eCBs suppress cancer. LPA evokes pain whereas eCBs relieve pain. Both LPA and CB-receptors are widely expressed in the body, which makes it challenging to achieve an organ-specific effect by using exogenous receptor agonists or antagonists. Lipid GPCRs likely bind their ligands laterally via the lipid bilayer (Hanson et al. 2012, Hurst et al. 2010, Hurst et al. 2013), suggesting that exogenous ligands should be first targeted to the cell membrane. Furthermore, the ligands are susceptible to the rapid enzymatic degradation. These issues challenge the development of lipid ligands and for drug discovery purposes, receptor agonists or antagonists either for LPA or eCB receptors are not currently the principal targets. Instead, allosteric modulators as well as enzyme inhibitors for lipid biosynthetic and degradative enzymes are under intense investigation. There are several pharmacological inhibitors developed especially targeting the eCB-degrading enzymes (Table 5) but currently only few compounds exist which inhibit LPA synthesis or degradation. There are certain requirements for a pharmacological inhibitor until it can be used as a drug; for instance, it should be potent, selective over other enzymes, soluble, stable *in vivo*, and preferentially orally active. Only a few inhibitors developed so far meet these requirements, for many their *in vivo* profile has not been made publicly available. Much of work still needs to be done to fully utilize the therapeutic potential of these lipid systems.

9.2. Studying the life cycle of LPA and 2-AG

Brain sections presumably preserve the functional enzymatic machinery required for synthesis and degradation of lipid GPCR ligands, in a suitable microenvironment along with their native signalling partners. In the present study, a novel pharmacological

application of functional autoradiography was utilized in studies of the enzymatic pathways responsible for the lipid messenger synthesis and degradation in brain sections. For additional information, functional autoradiography was combined with supporting methods. The accumulation of endogenous compounds in tissue sections was verified by combining the results of functional autoradiography with a sophisticated LC/MS/MS analysis. Additionally, the degradation of LPA and 2-AG was studied by assaying their degradation products. The measurement of P_i has been previously used when determining degradation of adenine and guanine nucleotides during autoradiography incubations (Laitinen et al. 2001, Laitinen 2004). Here, this approach was utilized for the first time for studying LPA degradation. The determination of glycerol is a straightforward way to monitor the degradation of glycerophospholipids and was utilized when studying the enzymatic pathways involved in the degradation of LPA.

Characterization of the [^{35}S]GTPγS autoradiography method

[^{35}S]GTPγS autoradiography measures the first step in the receptor-G_i protein signalling cascade, the guanine nucleotide exchange. Inherent to the method is [^{35}S]GTPγS binding under basal conditions that may possibly mask some receptor responses. Adenosine is continuously generated in brain sections during the incubations and it can cause an A_1 receptor-dependent [^{35}S]GTPγS binding response predominantly in grey matter areas (Laitinen 1999, Moore et al. 2000). The primary source for adenosine in brain sections is degradation of adenine nucleotides such as ATP (Laitinen 1999). It has been demonstrated that adenosine A_1 receptors exhibit no constitutive activity in the absence of agonist (Savinainen et al. 2003). Thus either the adenosine A_1 receptor-selective antagonist DPCPX or the adenosine-hydrolyzing enzyme adenosine deaminase need to be routinely included in the protocol to eliminate this adenosine-derived signal. In addition to the adenosine A_1 receptors, LPA_1 receptors have been observed to be tonically active in brain sections contributing to the white matter-enriched [^{35}S]GTPγS binding in basal conditions. This labelling can be eliminated by using an $LPA_{1/3}$ receptor-selective antagonist (Palomäki & Laitinen 2006).

In the present study, the majority of the adenosine A_1 and LPA_1 receptor-independent basal labelling was found to be located in the brain regions participating in the regulation of primitive vital functions and behaviour, such as in thalamic and hypothalamic nuclei. Due to the sensitivity to NEM, the basal signal appears to be G_i protein-mediated. The sensitivity to NEM cannot be construed as an absolute proof of involvement of G_i proteins, since in addition to cysteine in the C-terminus of G_i, NEM has the potential to alkylate cysteines in other proteins (Laitinen 2004). In principle, basal labelling could reflect regional enrichment of various members of the G protein family, that could bind [^{35}S]GTPγS also in a manner independent of any interaction with GPCR. Furthermore, constitutive activity of unknown GPCRs or the presence of still unidentified ligands capable of activating their receptors could affect basal labelling. Interestingly, the majority of currently orphan receptors are considered to be located in the hypothalamus, which was the region that was most intensively labelled under basal conditions. None of the antagonists used in this study could abolish the basal labelling, indicating that it is not induced by the activity of histamine H_3, cannabinoid CB_1/CB_2, $S1P_1$, or neuropeptide $Y_1/Y_2/Y_5$ receptors. Since LPA_1 receptors are expressed and the [^{35}S]GTPγS binding response is clearly visible in the juvenile brain (Laitinen 2004, Choi et al. 2010), we performed the present studies using brain sections of young, 4 week-old rats. A preliminary comparison indicated that identical brain structures were also labelled in the adult rat brain. However, a more detailed study would be needed to make it possible to conduct a quantitative comparison of the regional signal intensity between young and adult animals. It should be emphasized that the male Wistar rats were sacrificed at one particular circadian time (8–9 hrs after lights on a 12:12 hour light–dark cycle). Although not yet studied, factors such as diet, sex and the circadian

time could in principle affect the distribution and/or intensity of the basal [^{35}S]GTPγS labelling. It should be emphasized, that the current anatomical mapping does not necessarily reflect the situation in the brain *in vivo*.

In addition to white matter-enriched LPA_1 receptors, [^{35}S]GTPγS autoradiography is suitable for studying other G_i-coupled lipid receptors, including $S1P_1$ and CB_1 receptors. The activation of both $S1P_1$ and CB_1 receptors induces robust responses, predominantly in gray matter areas (Waeber & Chiu 1999, Laitinen 2004, Sim-Selley et al. 2009). In contrast to the LPA_1 receptor, receptors for S1P or endocannabinoids display no tonic activity visible in functional autoradiography. Our unpublished results indicate that brain sections are not capable of generating S1P during the incubations, whereas they do seem to be able to generate LPA and 2-AG. The generation of S1P requires phosphorylation of sphingosine by sphingosine kinase (Pyne et al. 2009); it is likely that catabolic rather than *de novo* synthesizing pathways are active in brain sections. If one wishes to clarify the pathways that require kinase activity, methods based on living cells presumably would be needed. Furthermore, since functional autoradiography detects receptor-mediated G protein activity, detection of G protein-independent, e.g. β-arrestin-mediated, GPCR activity would require some additional technique.

[^{35}S]GTPγS autoradiography as a pharmacological approach to study enzymatic activity regulating lipid GPCR signalling

Traditionally, functional autoradiography has been performed using exogenous receptor agonists or antagonists. In addition to exogenous ligands, endogenous lipid mediators pre-existing in the sections or generated during the incubations, are able to activate their cognate receptors and induce the [^{35}S]GTPγS binding response. The use of pharmacological inhibitors of enzymes that degrade these ligands or, alternatively, stimulators of their biosynthetic enzymes, provides a novel way to extend the usefulness of the method.

2-AG is known to be efficiently generated in the post-mortem rat brain tissue after decapitation (Sugiura et al. 2001). Previously, brain sections were found to efficiently degrade 2-AG (Savinainen et al. 2001). Further studies indicated that the elimination of 2-AG degradative pathways by MAFP evoked accumulation of endogenous 2-AG and the subsequent CB_1 receptor activation as revealed using [^{35}S]GTPγS autoradiography (Palomäki et al. 2007). Prolonging the pre-incubation time prior to MAFP treatment had no effect on the [^{35}S]GTPγS binding responses evoked by the subsequent MAFP treatment (Palomäki et al. 2007), and it was postulated that 2-AG pre-existing in the sections had become depleted during the prolonged pre-incubation and the endocannabinoid, activating the CB_1 receptors, had been synthesized during the incubations. Since it is a broadly-acting serine hydrolase inhibitor, MAFP blocks virtually all 2-AG-degradating enzymes, and during the following 90 minute [^{35}S]GTPγS labelling step, 2-AG-synthesizing enzymes have sufficient time in which to operate. The current study attempted to clarify wether the MAFP-evoked response was due to DAGL activity by using brain sections of DAGLα-KO and DAGLβ-KO mice. Unexpectedly, the obtained results indicate that some THL-sensitive enzymes other than DAGLs are responsible for 2-AG generation in brain sections. Since DAGLα-mediated 2-AG generation is believed to be responsible for the retrograde synaptic signalling, it is likely that cell types other than neurons are responsible for synthesizing the 2-AG in brain sections.

An additional goal was to identify compounds that could boost the tonic LPA_1 signal in brain sections and in this way increase the understanding of the regulation of the brain LPA-GPCR signalling. Since known precursors of LPA as well as various compounds reported to affect the autotaxin and/or PLD activity failed to affect the basal tone of LPA_1 receptor activity, the tonic LPA_1 activity in brain sections is apparently not due to LPA generated by autotaxin or PLD activity. Instead, it was possible to manipulate the tonic LPA activity by pharmacologically inhibiting LPA degradation. The results indicated that

LPPs guard the signalling pool of LPA but the majority of LPA degradation in brain sections is due to other, LPP-like, enzymes. The LPP activity served to limit the actions of endogenously generated LPA but not exogenously applied LPA. AlF_x^-, propranolol and vanadate were found to inhibit LPPs; AlF_x^- in an irreversible manner and propranolol and vanadate in a reversible manner. Additionally, the results suggested that AlF_x^- is able to inhibit the LPP-like enzymes in a reversible manner. The three inhibitors used are rather unspecific towards LPPs, due to the the lack of commercially available specific inhibitors of LPPs. However, inhibitor-induced [^{35}S]GTPγS binding responses were restricted to the LPA_1 receptor-enriched white matter areas, no responses were observed in other brain regions. Furthermore, the observed [^{35}S]GTPγS labelling was sensitive to a $LPA_{1/3}$ receptor-specific antagonist, evidence for the absence of unspecific responses.

The presently described approach is well suited for studying the lipid class of compounds, which are synthetized on demand and rapidly enzymatically degraded. The approach is not likely to be applicable for classical hydrophilic vesicle pre-packed neurotransmitters, as previously demonstrated with muscarinic cholinergic receptors (Palomäki et al. 2007). It should be noted that different enzymatic routes may be active *in vivo* than *in vitro*. Brain sections do not tend to represent a physiological model but provide a platform for studies of metabolic pathways regulating the lifetime of bioactive lipids, especially when combined with analytical methods.

LC/MS/MS as a method to quantify LPA levels in the brain tissue

It was wished to provide direct evidence that the endogenous LPA could accumulate in brain sections followed by a treatment with pharmacological inhibitors of LPA degradation. In previous reports, LPA content in the brain tissue had been determined by gas chromatography (GC) (Xu et al. 1998, Sugiura et al. 1999, Nakane et al. 2002), but this was an indirect method requiring first thin layer chromatographic purification, and then GC, all rather time-consuming. There have been a few existing LC/MS/MS methods published in the literature for the determination of LPA from biological fluids (Georas et al. 2007, Shan et al. 2008, Tokumura et al. 2009, Scherer et al. 2009) but not from the brain tissue. There have also been some limitations in the previously published methods, and so an LC/MS/MS method was developed that was optimized to the current experimental demands.

From an analytical point of view, LPAs are a demanding group of compounds, and several important issues were encountered during the method development. LPA is sticky, binding to many surfaces, e.g. test tubes and chromatographic columns. It was noticed that LPA may easily bind to several test tube materials (e.g. polypropylene microcentrifuge tubes and certain glass test tubes) and residual LPA might lead to variations in the results. When Pyrex® borosilicate test tubes were used and washed with hydrochloric acid between the experiments, sufficient repeatability was obtained. With several chromatographic columns tested, LPA evidently bound to the columns and their performance deteriorated after a few runs. With a C8 column with a large particle size (3.5 μm) and a pore size of 300 Å, it was possible to obtain good chromatographic performance.

In the development of analytical methods, it is important to find means to eliminate interfering compounds prior to the detection. LPC, a compound that is present in high concentrations in tissues, can be converted to LPA during sample preparation and within the instrumentation, especially if acidic extraction conditions are used. LPC is also known to induce an ion suppression effect. There was some concern that in some previously published methods, LPC and the artificially formed LPA may have affected the results (Scherer et al. 2009, Zhao & Xu 2009). We first extracted LPC into a separate phase from LPA and finally removed the residual LPC by chromatographic separation. When the C8 column was combined with an ion pair reagent in the mobile phase, LPCs were separated from LPAs. It was demonstrated that the chromatographic separation prior to detection was necessary and thus the direct flow injection could not be utilized.

The present method was validated and found to be suitable for LPA analysis from the brain tissue. In addition to brain tissue, the method could be applied to studies of other biological samples after optimization of the extraction procedure to each particular tissue or fluid. It is essential to have quantitative methods to determine endogenous lipids in experimental settings. Importantly, lipids could be used as biomarkers to predict certain diseases; e.g. there are published reports describing the use of LPA as a biomarker in the diagnosis of ovarian cancer (Xu et al. 1998, Meleh et al. 2007).

Future perspectives

The present anatomical mapping of adenosine A_1 and LPA_1 receptor-independent [^{35}S]GTPγS labelling in basal conditions represented the first step in the improvement of the signal-to-noise ratio of the method. In future experiments, a systematic approach with various receptor antagonists would be needed to reveal which factors contribute to the basal labelling. The described anatomical mapping should facilitate these efforts. The constitutive receptor activity can be affected by sodium concentration, and testing different ion composition of the incubation buffer would provide information whether constitutive activity could affect the basal labelling. It is noteworthy that there might be orphan receptors activated by currently unknown ligands in the mapped brain regions. The present mapping provides a potential anatomical location of these orphan receptors and may facilitate the search for endogenous ligands capable of activating these receptors.

The present results indicate that there are LPP-like enzymatic routes existing in the brain that are capable of degradating LPA. Further identification of these routes would be needed in future experiments. Likewise, a more detailed characterization of the 2-AG-generating enzymes in the brain would be beneficial. The use of brain sections from animals having both DAGLα and DAGLβ knocked-out would strengthen the conclusion about the existence of DAGL-independent routes that are able to generate 2-AG in the brain.

There is a clear need for the development of specific and potent inhibitors targeting enzymes that regulate the lifetime of bioactive lipids. Currently, there are more specific/unspecific inhibitors reported to target the eCB system than to target the LPA biosynthetic/degradative enzymes. It seems that enzyme-selective inhibitors represent potential drug molecules. In experimental systems, specific inhibitors would provide direct evidence for the involvement of a particular enzyme without concerns about activation of compensatory mechanisms that may take place in transgenic animals. Since both LPA and 2-AG are involved in the regulation of many organ systems as well as in the pathology of several diseases, defining the mechanisms of their synthesis/degradation is especially important for understanding these processes.

10 Conclusions

The first aim of the present study was to develop and optimize the methodology used for studying lipid-GPCR signalling and the second aim was to characterize the enzymatic pathways responsible for the lipid messenger synthesis and degradation. The main technique utilized throughout the study was functional autoradiography; this was exploited in a novel way to examine the enzymatic pathways that regulate the brief lifetime of bioactive lipids.

The main conclusions of the present study are as follows:

1. The majority of the adenosine A_1 and LPA_1 receptor-independent basal [^{35}S]GTPγS labelling in the juvenile rat brain sections were located deep in the brainstem as well as in several thalamic and hypothalamic nuclei. The basal signal appeared to be G protein-mediated and could reflect regional enrichment of various members of the GTP-binding protein family, constitutive activity of unidentified GPCRs and/or the presence/synthesis of receptor ligands.

2. A LC/MS/MS method was developed for quantitative determination of LPA species from brain tissue samples. Special attention was paid to the separation of interfering compounds prior to detection.

3. LPPs degrade the signalling pool of LPA in rat brain sections. In addition to LPPs, there seemed to be alternative LPP-like enzymes existing in the brain that degrade LPA at the bulk brain level.

4. The CB_1 receptor-dependent G_i-activity remained unaltered in several brain regions of DAGL-deficient mice when compared to WT mice. Alternative enzymes other than DAGLs appeared to be responsible for synthesizing 2-AG in brain sections.

5. The enzymatic machinery synthesizing and metabolizing membrane-derived lipid mediators was well preserved in brain cryosections. Especially when combined with analytical methods, functional autoradiography could be used as a pharmacological tool to reveal the enzymatic pathways that regulate lipid-GPCR signalling.

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Niina Aaltonen

Functional Autoradiography as a Pharmacological Approach for Studying G Protein-Coupled Lipid Receptor Signalling

Bioactive lipids act as important signalling molecules both in the central nervous system and the periphery. Bioactive lipids are produced by multistep enzymatic pathways and after they exert their effect by activating their specific receptors, they are rapidly enzymatically degraded. Altered lipid signalling is linked to the pathology of several serious diseases. In the present study, functional autoradiography was applied in a novel way to examine the enzymatic pathways that synthesize and degrade signalling lipids in brain sections.

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