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Vliet, D. van der

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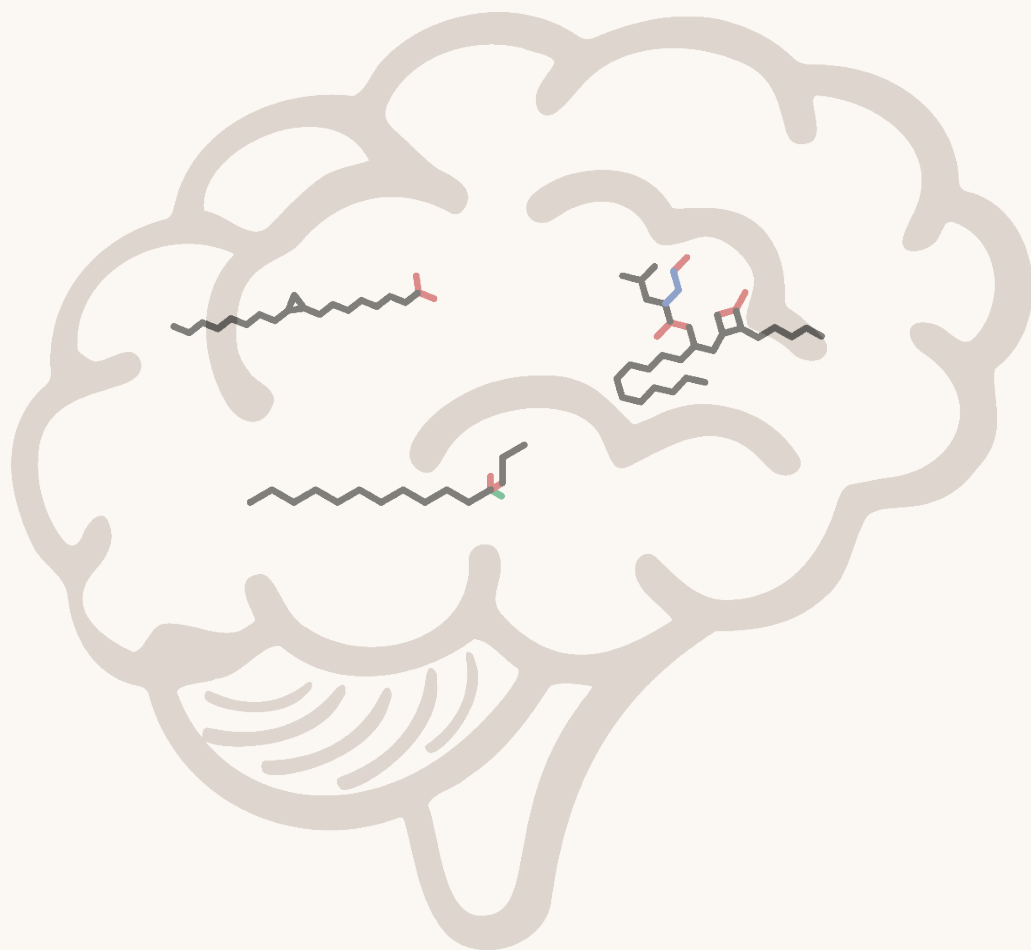
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Chemical probes to control and visualize lipid metabolism in the brain

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Introduction

Historically, lipids were viewed as metabolic and structural membrane components to support neuronal function in the brain. In recent years, a diverse set of signaling lipids has been discovered to interact with specific receptors to regulate many neurophysiological processes. These lipid messengers, such as endocannabinoids, prostaglandins, sphingosine-1-phosphate and lysophosphatidic acid, have emerged as key regulators of neurodevelopment, synaptic plasticity and inflammation. These lipid messengers fundamentally differ from classic neurotransmitters, most notably in the way they are synthesized and released. Classical neurotransmission is governed by the release of hydrophilic neurotransmitters, such as glutamate and GABA, from presynaptic vesicles into the synaptic cleft and reuptake by dedicated transporter proteins. In contrast, it is proposed that lipid messengers are not stored in vesicles, but are synthesized *on demand* and that their lifespan is regulated by dedicated metabolic enzymes. This implies that the biosynthetic- and metabolic rates of the involved enzymes are crucial in determining the flux of lipid messengers, thereby controlling the magnitude and duration of their signaling and physiological response. Consequently, lipid biosynthetic and metabolic pathways are often tightly regulated through post-translational modifications (PTMs), ion-cofactors, protein-protein interactions and the subcellular localization of their substrates and proteins. Experimental methods used to study classical neurotransmitters, like immunohistochemistry or RNA-expression analyses, are less suitable for lipid messengers, because they fail to capture the spatiotemporal dynamics of enzyme activities regulating signaling lipids. Instead, chemical biological approaches have emerged as a driving force to study lipid messengers in the brain. The development of small-molecule inhibitors, activity-based probes and bio-orthogonal lipids, have allowed researchers to investigate lipid messengers and their enzymes with a spatiotemporal resolution which was not previously possible. In this review, an overview is presented of how chemical probes can be employed for the study of lipid signaling systems in the brain. Focus is placed on the development and use of chemical probes to study endocannabinoid signaling by *i*) inhibiting the biosynthetic and metabolic enzymes; *ii*) visualizing the activity of these enzymes; and *iii*) controlling the release and transport of the endocannabinoids.

Visualizing and targeting lipid metabolizing enzymes

The enzymatic machinery controlling endocannabinoid signaling

There are two main endocannabinoids: 2-arachidonoylglycerol (2-AG) and *N*-arachidonylethanolamine (AEA, or anandamide)¹⁻³. Both endocannabinoids bind to the cannabinoid CB1 and CB2 receptors to regulate neurotransmission and various forms of synaptic plasticity, such as depolarization-induced suppression of inhibition (DSI) and long-term depression⁴. Each endocannabinoid has its own independent biosynthetic and metabolic pathway (Figure 1a). 2-AG is synthesized from phospholipids in a Ca^{2+} -dependent manner that involves the subsequent actions of phospholipase C and diacylglycerol lipase α or β (DAGL α and DAGL β)⁵. The majority of the 2-AG pool is inactivated through hydrolysis of the ester-bond by monoacylglycerol lipase (MAGL), and to a lesser extent by ABHD6 and ABHD12⁶.

The biosynthesis of AEA is more complex, since it can be synthesized via multiple pathways⁷. Phospholipase A1/2 Acyl Transferase (PLAAT) 1-5 and PLA2G4E perform the first step in which arachidonic acid from phosphatidylcholine is transferred to the free amine of phosphatidylethanolamine, thereby generating the central precursor *N*-arachidonoylphosphatidylethanolamine (NAPE)⁸.

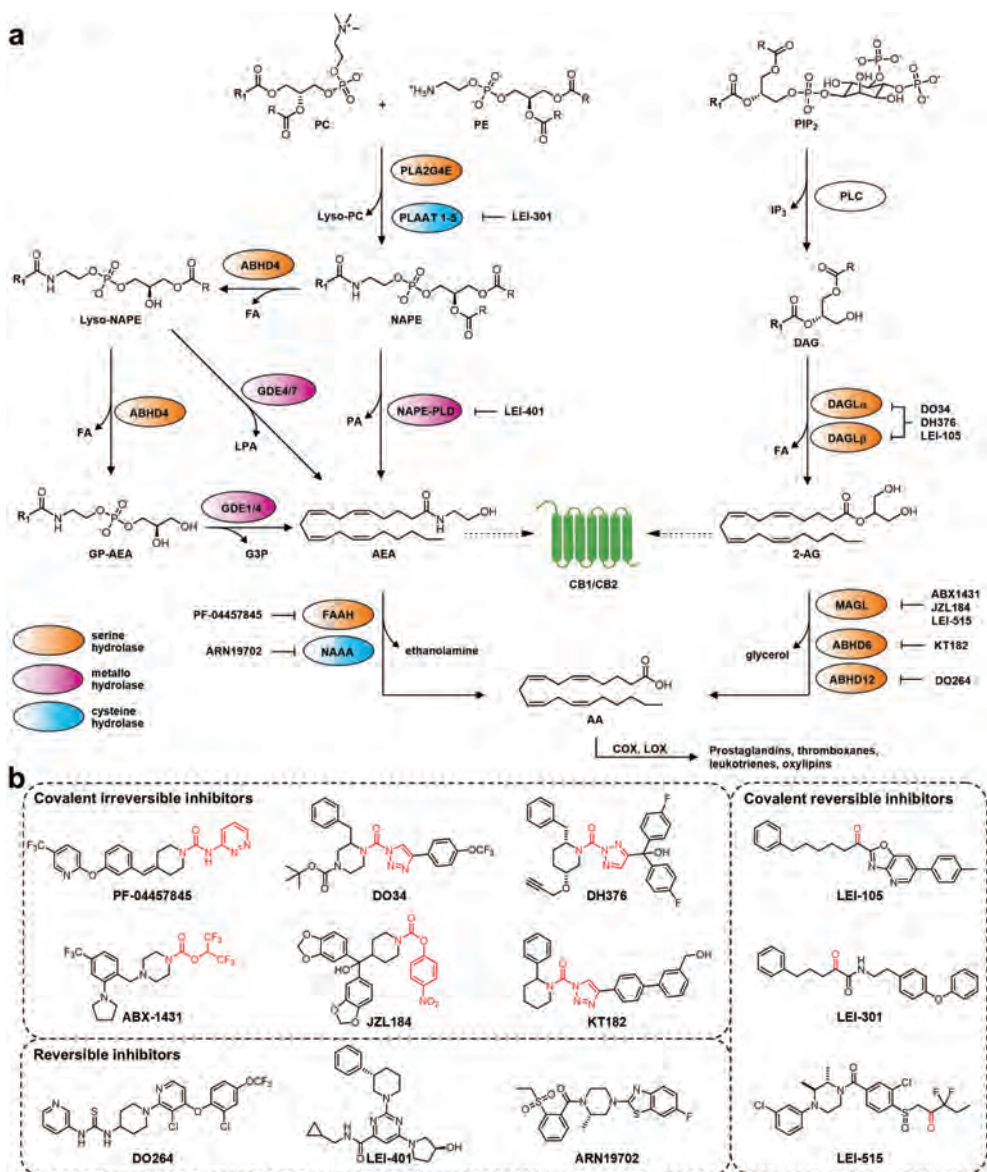


Figure 1. Overview of the metabolic pathways of the endocannabinoid system. **a**, $R_1 = C_{19}H_{31}$. Abbreviations: 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ABHD, α,β -hydrolase domain containing protein; AEA, *N*-arachidonoyl ethanolamine; CB1/CB2, cannabinoid CB1 or CB2 receptor; DAG, diacylglycerol; DAGL, diacylglycerol lipase; FA, fatty acid; FAAH, fatty acid amide hydrolase; G3P, glycerol-3-phosphate; GDE, glycerophosphodiesterase; GP-AEA, glycerophospho-AEA; IP₃, inositol 1,4,5-triphosphate; MAGL, monoacylglycerol lipase; NAAA, *N*-acylethanolamine acid amidase; NAPE-PLD, *N*-acylphosphatidylethanolamine phospholipase D; NAPE, *N*-arachidonoyl phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLA, phospholipase A; PLAAT, phospholipase A1/2 acyl transferase; PLC, phospholipase C. **b**, chemical structures of inhibitors depicted in **a**. The functional group for covalent interaction is indicated in red.

PLA2G4E is the rate-limiting enzyme in neurons that produces NApEs *on demand* in a Ca^{2+} -dependent manner⁸, whereas the PLAAT enzymes are Ca^{2+} -independent and responsible for basal NApE levels⁹. In the second step, NApE is hydrolyzed by NApE-phospholipase D (NAPE-PLD) or, alternatively, by ABHD4 and subsequently GDE1, GDE4 or GDE7, to release AEA⁷. Fatty acid amide hydrolase (FAAH) is the primary enzyme responsible for the hydrolysis of the amide bond in AEA, thereby inactivating this lipid messenger¹⁰. *N*-acylethanolamine acid amidase (NAAA) is also able to hydrolyze AEA, but to a lesser extent¹¹.

A complete overview of the enzymatic pathways of the endocannabinoids and their function in the brain has been described in detail in other reviews^{7,12–15}, but there remain many open questions. For instance, is there any cell type- or regional specificity of their contributions? Is there a bias for one of the two endocannabinoids to be mobilized: when, where and why? How does aberrant endocannabinoid signaling lead to disease and can the inhibitors of endocannabinoid biosynthesis and metabolism be used for therapeutic purposes? The development of chemical probes has contributed to answer some of these questions as outlined in this review.

Development of activity-based probes to discover and map endocannabinoid hydrolase activity

The enzymatic machinery of both endocannabinoids predominantly consists of serine hydrolases and cysteine hydrolases (Figure 1a, b), which use a catalytic serine or cysteine residue, respectively, to hydrolyze esters or amides¹⁶. These hydrolases can be targeted by broad-spectrum activity-based probes (ABPs), which were first developed by Cravatt and colleagues in 1999¹⁷. An ABP consists of a warhead that covalently reacts with the catalytically active residue of an enzyme in a mechanism-based manner, a linker and a reporter moiety (Figure 2a). When coupled to fluorescent reporter groups, ABPs enable visualization of enzyme activities in complex proteomes by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Instead, a biotin reporter group enables affinity enrichment and identification of enzyme activities by mass spectrometry (MS)–based proteomics (chemical proteomics). An ABP informs on the abundance of active enzymes in complex proteomes. The prototypic ABPs for serine hydrolases utilize a fluorophosphonate (FP) as a warhead, which is a broad-spectrum inhibitor of this enzyme family (Figure 2b, c). Recently, ABPs with a β -lactone or β -lactam group as alternative warheads have been reported to react with hydrolases not targeted by FP-probes, such as DAGL α and cysteine hydrolases (e.g. NAAA and PLAATs)^{18–20}. These ABPs can be used in activity-based protein profiling (ABPP) and chemical proteomic experiments in a *comparative* or *competitive* setting to assess the functional state of entire enzyme classes in native biological systems.

In *comparative* ABPP different biological samples (e.g. healthy vs diseased or different brain regions) are compared to each other to facilitate target identification (Figure 2d). For example, PLA2G4E, ABHD4, ABHD6 and ABHD12 have been discovered using comparative ABPP as enzymes involved in the biosynthesis and metabolism of endocannabinoids^{6,8}. Furthermore, comparative ABPP has been applied to map endocannabinoid hydrolase activity across different brain regions²¹. This revealed that FAAH activity was highest in the hippocampus, and MAGL activity was most pronounced in the frontal cortex, whereas DAGL α was most active in the cerebellum²¹. Cell type-specific activities of endocannabinoid hydrolases were studied by Viader *et al*²². They found a functional segregation of the enzymes across different CNS cell types. For example, high levels of active DAGL α and MAGL were found in neurons, whereas DAGL β and ABHD12 were mainly found in microglia, suggesting the presence of distinct pools of 2-AG, possibly exerting distinct physiological effects. Comparison of the activity profiles with global expression data revealed a poor correlation, which could indicate post-

translational regulation of the endocannabinoid hydrolases. Indeed, FP-based probes are able to capture the Ca^{2+} -dependency of PLA2G4E⁸, showcasing that ABPP can detect dynamic enzymatic activity in relation to the physiological environment.

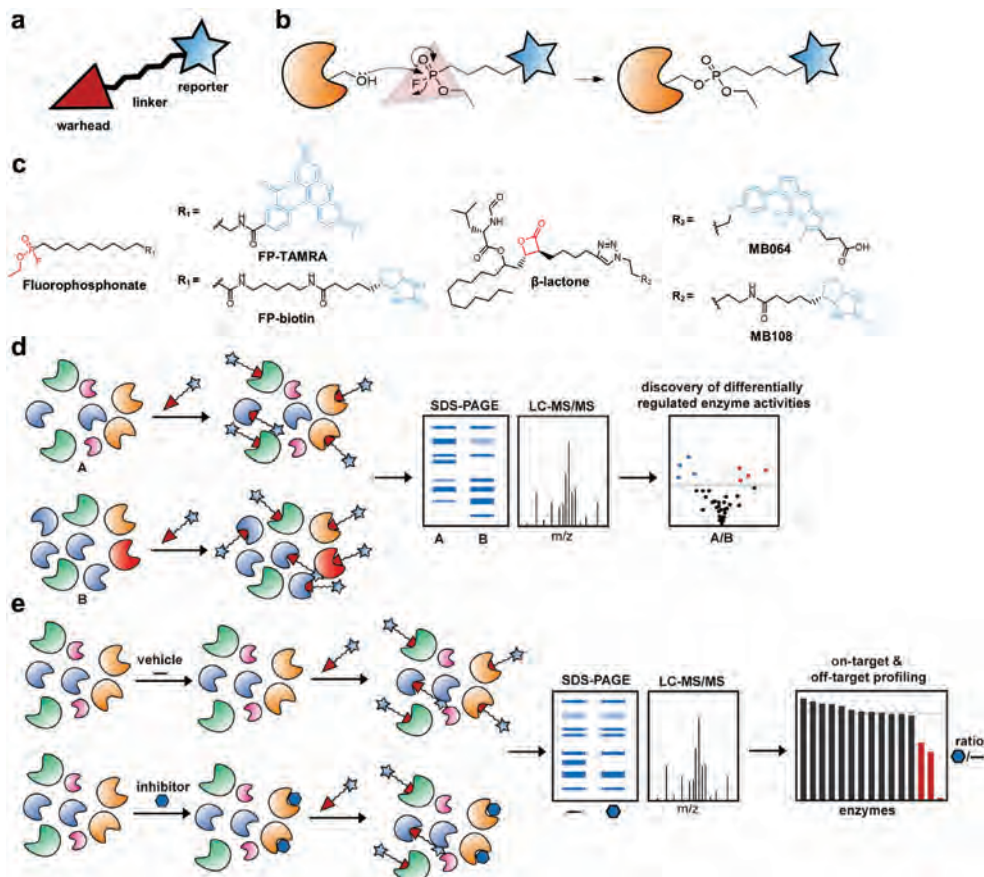


Figure 2. Competitive and comparative Activity-based protein profiling. **a**, General schematic overview of a typical activity-based probe. **b**, Mechanism of serine hydrolase targeting by fluorophosphonate probes. **c**, Structures of fluorophosphonate- and β -lactone probes, equipped with either a fluorophore or a biotin group (in blue). **d**, General schematic workflow for comparative ABPP. A and B represent any two biologically different samples. **e**, General schematic workflow for competitive ABPP.

ABPP aids the discovery of inhibitors to study endocannabinoids in the brain

The endocannabinoids are involved in many (patho)physiological functions, such as sleep, appetite, memory formation, anxiety and pain sensation^{12,13,23}. These biological roles can in principle be studied by genetically modified mice lacking specific endocannabinoid metabolizing enzymes. However, long-term and constitutive inactivation of these enzymes renders the mouse models poorly suited to study rapid and dynamic changes in endocannabinoid signaling. For example, chronic disruption of MAGL results in down-regulation of the CB1 receptor, thereby altering neurotransmission and development of physical dependence^{24,25}. Furthermore, compensatory mechanisms in NAPE-PLD knock-out animals have been detected²⁶. Small molecule inhibitors provide, on the other hand, a powerful way to

assess the temporal consequences of acute enzyme blockade on the physiological response. Since many lipid metabolizing enzymes belong to the class of serine hydrolases, it is essential to have highly selective inhibitors to attribute specific functional roles of lipid metabolizing enzymes.

Competitive ABPP is a powerful technique to characterize inhibitors of lipid metabolizing enzymes (Figure 2e). The inhibitor abrogates labeling of enzymes by the ABP either after *in vitro* incubation or *in vivo* treatment. Competitive ABPP enables the possibility to map the on- and off-target activity of small molecules, leads or drug candidates (and their metabolites) in their native physiological context. To guide inhibitor characterization, both gel-based and chemical proteomics-based protocols for the evaluation of serine hydrolase inhibitors using ABPP have been reported^{27,28}.

Competitive ABPP has been instrumental in the discovery of highly selective inhibitors of the biosynthetic and metabolic enzymes of 2-AG and AEA (Figure 1b). For instance, the β -lactone probe MB064 guided the discovery of the first selective DAGL inhibitor LEI-105, which enabled demonstrating that DSI was dependent on the *on demand* production of 2-AG in hippocampal slices²⁹. Furthermore, MB064 and FP-probes were employed to guide the discovery of DH376 and DO34, the first centrally active DAGL inhibitors³⁰.

These compounds are now widely used to study the role of DAGL in various (patho)physiological processes, such as synaptic plasticity, food intake, neuroinflammation, alcohol and cocaine addiction, epilepsy, stress and anxiety²³. Interestingly, selective DAGL α or DAGL β inhibitors have not been discovered yet, but would be valuable to understand the contribution of each protein subtype to endocannabinoid signaling in the brain³¹.

Centrally active MAGL inhibitors, such as JZL184 and ABX-1431 (Lu AG06466), were identified and optimized by a competitive ABPP-screen from a library of activated serine hydrolase-directed carbamates^{32,33}. These inhibitors showed efficacy in animal models of inflammatory and neuropathic pain and in various models of neuroinflammatory diseases, including Alzheimer's disease, Parkinson's disease and Multiple Sclerosis¹³. PF-04457845, a highly selective FAAH inhibitor, was also discovered by ABPP^{34,35}. This compound showed cannabinoid receptor dependent anti-nociceptive effects in animal models of inflammatory pain. Although inhibition of FAAH and MAGL in the mouse brain raises the levels of AEA or 2-AG, respectively, neither induces the full spectrum of behavioral changes typically observed by CB1 receptor agonists, such as Δ^9 -tetrahydrocannabinol (THC), the psychoactive constituent of *cannabis sativa*. Interestingly, simultaneous inhibition of both FAAH and MAGL does mimic the behavioral effects of THC. This indicates that 2-AG and AEA have both distinct and overlapping roles in controlling CB1 receptor signaling³⁶. Thus, selective FAAH or MAGL inhibitors may have therapeutic benefit without inducing the full spectrum of psychoactive effects observed with THC. Currently, ABX-1431 and PF-04457845 are investigated in phase 2 clinical trials for multiple indications, thereby demonstrating the translational value of using ABPP in the drug discovery process¹².

Another example that showcased the general utility of ABPP in drug discovery was a study in which the selectivity profile of the FAAH inhibitor BIA10-2474 was reported³⁷. In a phase 1 clinical trial of the experimental drug one of the healthy volunteers died, and four others suffered brain damage³⁸. Competitive ABPP experiments with BIA10-2474 mapped its serine hydrolase interaction landscape in human cortical neurons and human brain tissue from subjects not associated with the trial. In these experiments, BIA10-2474 was shown to not be selective for FAAH, but had several different lipases as off-targets and the compound disrupted cellular lipid networks in cultured neurons³⁷. This emphasizes

the need for preclinical selectivity testing in human cells and tissues in an early drug discovery stage and highlights ABPP as a valuable technology to guide therapeutic development.

Finally, competitive ABPP using photo-reactive probes enables the profiling of compounds that do not target serine or cysteine hydrolases. These probes lack an electrophilic warhead, but are instead equipped with a photo-activatable crosslinker, which covalently binds the target upon UV irradiation. Such probes have been employed to study selective CB2 receptor agonists (*vide infra*) and cellular target engagement of NAPE-PLD inhibitors^{39,40}. The latter assisted the characterization of LEI-401, the first *in vivo* active NAPE-PLD inhibitor. Using LEI-401, it was demonstrated that AEA biosynthesis in mouse brain was dependent on NAPE-PLD. LEI-401 activated the hypothalamus–pituitary–adrenal axis and impaired fear extinction, thereby emulating the effect of a CB1 receptor antagonist, which could be reversed by a FAAH inhibitor. These findings highlight the distinctive role of NAPE-PLD in AEA biosynthesis in the brain and suggest the presence of an endogenous AEA tone controlling emotional behavior⁴⁰.

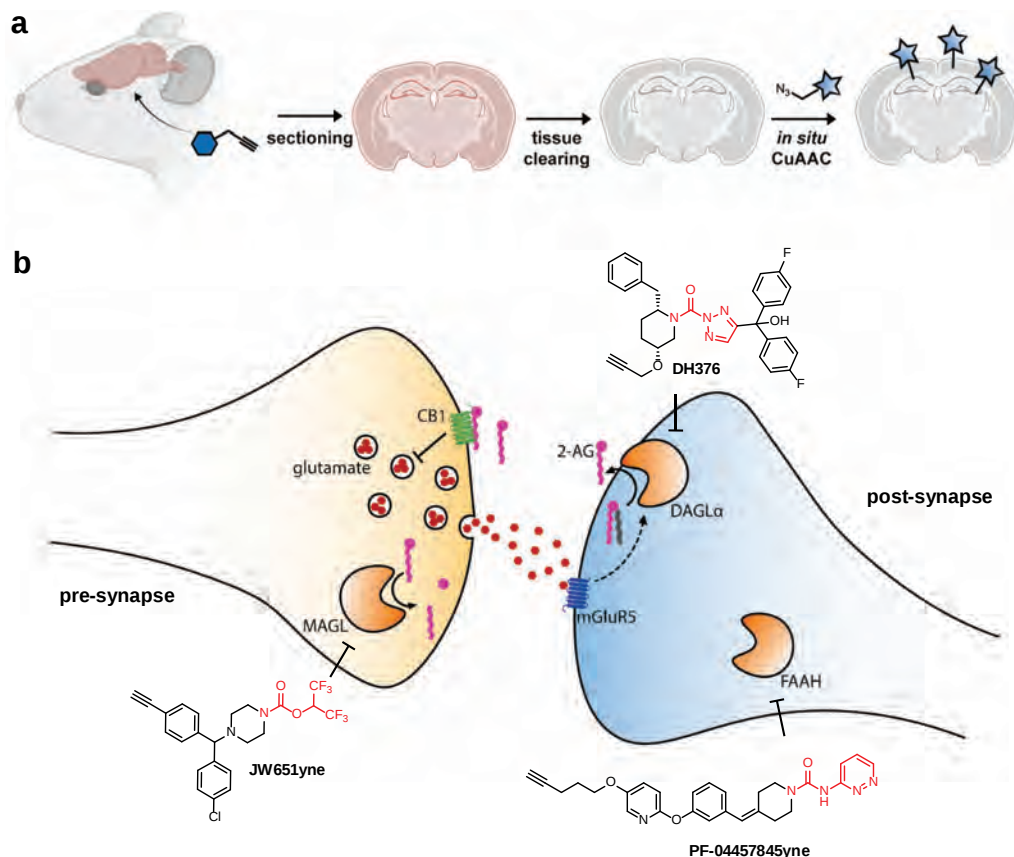


Figure 3. Visualizing nanoscale enzyme activity using activity-based probes. a, The CATCH workflow for *in situ* CuAAC. **b,** Schematic overview of a synapse with an active endocannabinoid system. Suitable ABPs for targeting pre- and post-synaptic enzymes are shown. **a** was created using biorender.com

Tailor-made ABPs allow to study lipid metabolizing enzymes with high spatial resolution

Broad-spectrum ABPs have provided an unprecedented view on enzymatic activity of entire protein families in cells and tissues. However, workflows based on broad-spectrum probes require sample homogenization for analysis, thereby losing spatial information on the (sub)cellular localization of the lipid metabolizing enzymes. Understanding the role of lipid messengers in brain function requires, however, detailed knowledge of their spatiotemporal activities in a region and cell type-specific manner. Therefore, there is a need for tailor-made, highly selective ABPs to visualize lipid metabolizing enzyme activity with enhanced spatial resolution.

To this end, two types of probes can be used: one- or two-step probes. One-step ABPs contain a fluorophore already attached to the probe which can be used to directly visualize the target. Alternatively, two-step ABPs harbor a bio-orthogonal ligation handle, to which the fluorophore is attached only after the ABP has covalently bound to its target. The archetypical ligation tag for ABPs is the alkyne, which can be coupled to reporter groups via copper-catalyzed azide-alkyne cycloaddition (CuAAC, also known as a “click” reaction)⁴¹. This avoids issues of cell permeability and reduced target affinity caused by a fluorophore. Two-step probes usually have improved pharmacokinetic properties and may penetrate the blood-brain-barrier. On the other hand, direct visualization of enzyme activity by one-step fluorescent probes avoids the secondary ligation step, simplifies the protocol and is suitable for live-imaging. Both strategies have been employed to study (sub)cellular localization and distribution of active enzymes with confocal microscopy or flow cytometry^{39,42–45}.

Multiple one-step probes for visualizing 2-AG metabolizing enzymes have been reported: DH379 and HT-01 for DAGL α and β ^{30,46}, and LEI-463-Cy5 and JW912 for MAGL^{47,48}. DH379 is based on the previously mentioned DAGL inhibitor DH376, but has not been applied to visualize DAGL activity in living cells yet. DH379 was, however, instrumental to discover the short *in vivo* half live of DAGL α protein (2–4 h), which was rapidly degraded and replaced by newly synthesized enzyme in mouse brain³⁰. This ongoing production of DAGL α generates a strong, tonic flux of 2-AG in the brain. LEI-463-Cy5, based on the selective MAGL inhibitor JW651⁴⁷, was recently used to visualize the activity of single MAGL molecules in cells by using a super resolution imaging method termed PharmacoSTORM⁴⁸. Although one-step probes can be used *in vivo* in the brain by intraventricular injection⁴⁵, such methods are technically challenging, and therefore two-step probes may be more suitable for *in vivo* labeling of active enzymes.

Recently, Pang *et al.* reported an elegant method to visualize enzyme activity in brain sections using two-step probes. They administered analogs of FAAH inhibitors PF-04457845 and BIA10-2474 equipped with an alkyne to mice, and subsequently coupled the fluorophore *ex vivo* through CuAAC⁴⁴. They developed a tissue clearing technique termed CATCH that delipidates the brain using sequential hydrogel based fixation and detergent-based washing to accelerate the CuAAC reaction (Figure 3a, b). They showed that FAAH activity was mainly found in the somas of Ctip2+ excitatory neurons in the somatosensory cortex, but not in astrocytes or inhibitory neurons. Furthermore, they visualized the opposing synaptic localization of FAAH and MAGL in post- and pre-synaptic terminals, respectively, in line with earlier work using immunohistochemistry⁴⁹. Interestingly, BIA10-2474yne, but not PF-04457845yne, was found to bind to the reticulotegmental nucleus of the pons in FAAH knock-out mice, which suggests that off-targets of BIA10-2474 are located in this brain region. Development of novel selective ABPs in combination with the CATCH method, offers the exciting opportunity to map active enzymes in the brain with cell type- and synapse-specific resolution, contributing to a spatiotemporal understanding of lipid metabolism at the nanoscale.

Visualizing and controlling lipid transfer and action

Lipid reporter functionalities

ABPs report on the enzymes that biosynthesize or metabolize lipid messengers, but they do not provide information on the lipid messengers themselves. To this end, it would be desirable to develop tools to directly visualize lipid messengers. Lipids cannot, however, be equipped with genetically encoded fluorescent markers like proteins. In addition, due to their small size and hydrophobicity, it has been challenging to directly visualize and quantify the lipid messengers in real time. Instead, lipids, including 2-AG and AEA, have traditionally been studied through isotopic analogues containing either radioactive (^{14}C , ^3H) or stable (^{13}C , ^2H) isotopes. Yet, these radiolabeled lipid messengers cannot be visualized in a cellular context since they inherently require sample homogenization and are thus mainly restricted to end point measurements. Furthermore, isotopic messengers provide insufficient spatiotemporal resolution to adequately investigate the rapid and local action of lipid signaling.

To circumvent these problems, lipid probes that carry a fluorescent tag have been developed that are suitable for real time visualization. For example, AEA-TAMRA contains a dye in the ethanolamine head group of AEA to visualize cellular uptake⁵⁰. Alternatively, upon treatment with biotin-AEA and subsequent fixation, streptavidin dyes report on AEA location and its protein interaction partners were identified through pull down experiments⁵¹. However, a major drawback of these tagged probes are the substantial alterations of steric bulk and polarity with respect to the endogenous lipid, and therefore deviating biological effects or artifacts may occur. Thus, these directly tagged lipid messengers should be used with caution to explore poorly characterized aspects of endocannabinoid signaling.

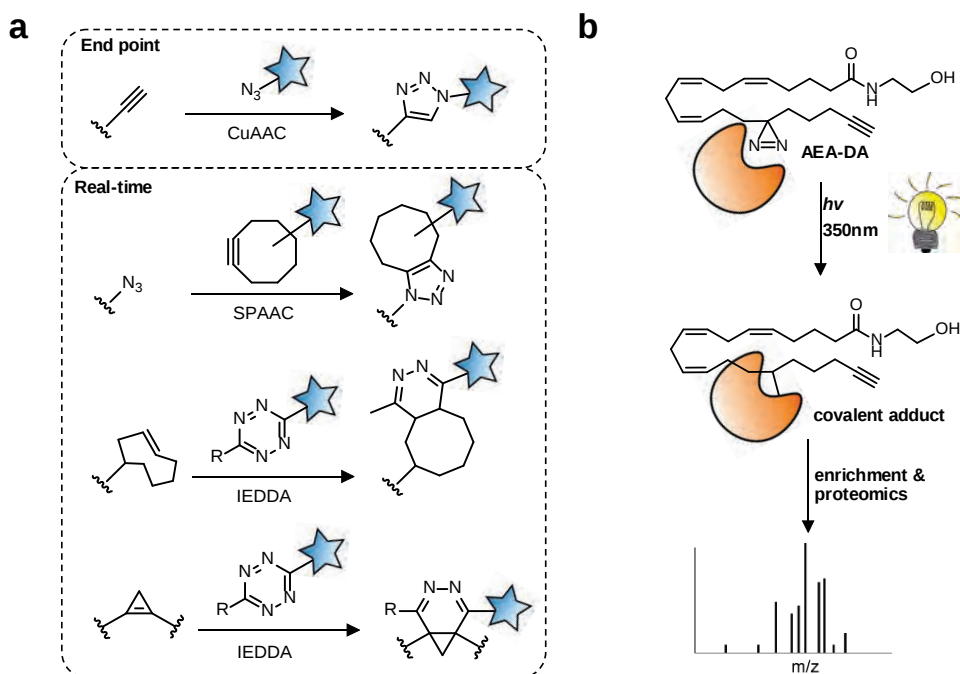


Figure 4. An outline of lipid functionalization strategies. a, Bio-orthogonal reactions used to functionalize lipids. **b,** An AEA photo-affinity probe (AEA-diazirine) to identify interaction partners of AEA.

To minimize the negative effect of a tagged analogue, recent research has focused on the introduction of small ligation handles that can be functionalized in downstream analysis through bio-orthogonal chemistry (Figure 4a). The previously mentioned alkyne moiety is a popular tag for omega-terminal fatty acid chain functionalization, due to its similarity in size and hydrophobicity with respect to the substituted alkyl group. A variety of alkyne lipids has been employed as probes to study the metabolism of their endogenous counterparts, which are distinguished from the alkyne probe by MS-based methods⁵². Alternatively, through ligation to a biotin, alkyne lipids that are incorporated into proteins can be enriched to elucidate the lipidation PTM pattern using proteomics.

Alkyne moieties are also incorporated in photo-affinity probes, to identify the biomolecular interaction landscape of signaling lipids (Figure 4b). In such probes, following covalent attachment to proximate biomolecules, the alkyne serves as a functionalization handle for enrichment and subsequent proteomics. The Cravatt lab developed two distinct AEA photo-affinity probes to map the AEA interaction protein landscape⁵³. Through this approach, a wide range of novel putative AEA protein interaction partners were identified, including NUCB1 which was found to function in NAE metabolism. In addition, the platform lend itself to *in situ* pharmacological characterization of the discovered AEA targets. Recently, this design was exploited to investigate the AEA reuptake inhibitor WOBE437⁵⁴. By using a photo-affinity-WOBE437 probe, previously unknown off-targets of WOBE437 were identified. Although these proteins preferentially bound AEA, they were not responsible for the cellular uptake of AEA. These results illustrate the importance and utility of photo-affinity lipids to profile inhibitor selectivity.

Importantly, even an omega-terminal alkyne can profoundly influence the biological behavior of the probe in comparison to the endogenous lipid. For instance, an arachidonic acid alkyne probe was processed differently in eicosanoid metabolism and had weaker immuno-stimulatory effects⁵⁵. These results emphasize that the altered behavior of a tagged substrate and its metabolites within a biological context is not limited to perturbed enzyme recognition, but may include modulatory effects on endogenous receptors. In addition, the visualization of alkyne lipids has largely been restricted to end-point analysis, since their application to living systems is limited due to the requirement of cytotoxic Cu(I) for the CuAAC⁵⁶.

Conversely, strain-promoted alkyne azide cycloaddition (SPAAC) does not require additional reagents as it relies on the energetically favored ligation of a cyclooctyne to an azide (Figure 4a). Similarly, the inverse electron demand Diels Alder (IEDDA) occurs between an electron poor diene, usually a tetrazine, and an electron-rich alkene. The IEDDA has superior ligation kinetics and thus improved temporal resolution over SPAAC. Although both SPAAC and IEDDA may rely on relatively bulky tags, these can be supplied as exogenously labeled precursors which are then metabolically incorporated into the lipid of interest. Taumara and colleagues utilized azido-choline and spatially restricted cyclooctyne fluorophores to detect organelle specific phosphatidylcholine lipid transport in live cells⁵⁷. Likewise, the group of Baskin has exploited the promiscuity of PLD enzymes to transphosphatidylate bio-orthogonal alcohols for the real time visualization of PLD activity and their phospholipid substrates⁵⁸.

Recently, the IEDDA substrate scope was expanded using sterculic acid, a natural oleic acid analog containing a 1,2-cyclopropene as a bio-orthogonal click handle, for live cell imaging employing various quenched tetrazine-fluorophores⁵⁹. This enabled the visualization of its distribution in live cells and the capture of sterculic acid-modified proteins.

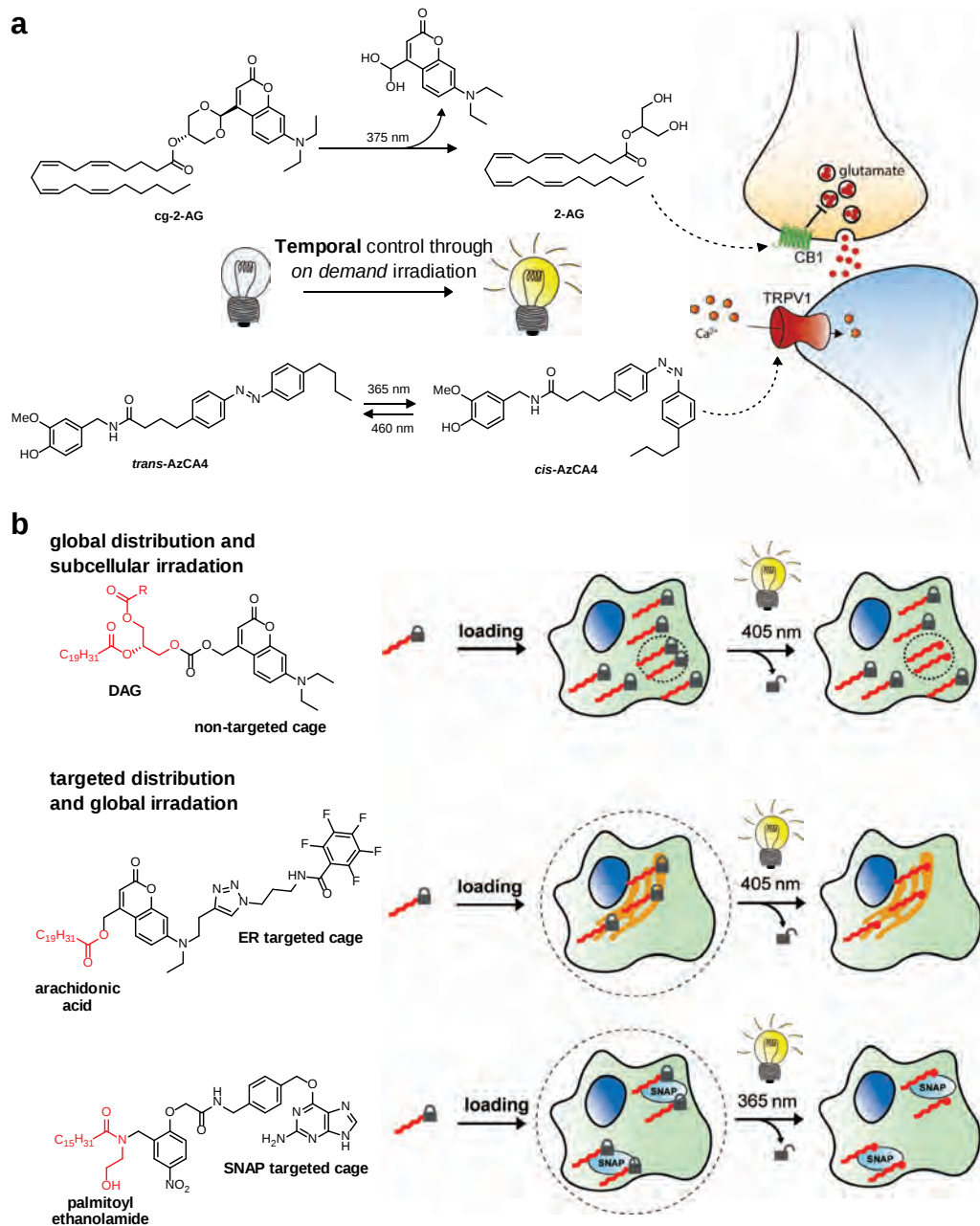


Figure 5. Controlling the spatiotemporal aspects of lipid probes. **a**, Lipids can either be functionalized with a photo-cleavable cage (cg-2-AG) or a photo-switchable azobenzene (*cis*-AzCA4). Upon irradiation, the probe is converted to its active form to exert its biological function. **b**, Spatial control over lipid release can be achieved through caged-lipid strategies with either targeted irradiation or through a subcellularly targeted cage.

Control over spatiotemporal release of lipid derived tools

In addition to visualizing lipid species, there is a need to control the spatiotemporal release of lipids. This is especially relevant for the endocannabinoids, since these lipids will immediately elicit a response when introduced to a biological context, making it challenging to record their initial effect.

A prominent design to release lipids *on demand* is to attach a photo-labile protecting group that renders the initial molecule biologically inactive (Figure 5a). Upon irradiation at the appropriate wavelength, this photo-cage is removed and the initial response to the bioactive molecule can be recorded. As early as 2005, Heinbockel and colleagues synthesized an AEA analogue with a nitrobenzoyl protecting group⁶⁰. Using this tool, they were among the first to recognize the millisecond time scale of endocannabinoid signaling.

Recently, the Schultz lab has expanded this principle to a coumarin-protected 2-AG⁶¹, nitrobenzoyl protected DAGs⁶² and a coumarin-caged DAG photo-affinity probe⁶³. Notably, the latter trifunctional probe identified many DAG interaction partners that were not previously captured through standard chemical proteomics strategies. This suggests that photo-caged probes allow to study more short-lived and low-affinity interactions.

Alternatively, photo-switchable probes may also be used to study rapid cellular responses of lipid messengers (Figure 5a). Typically, an azobenzene moiety will be incorporated in the hydrophobic tail of the lipid, which undergoes *trans* to *cis* isomerization upon light irradiation. The *cis*-azobenzene mimics the bend conformation of poly-unsaturated fatty acids, while *trans*-azobenzene more closely resembles saturated fatty acids. This has motivated the development of probes to control the activation of the ion channel TRPV1⁶⁴ and protein kinase C⁶⁵. Recently, Frank and coworkers have created photo-switchable probes for the *on demand* activation of the CB1⁶⁶ and CB2 receptors⁶⁷.

Photo-caged and -switchable lipid probes also provide spatial control through irradiation of particular cellular areas in the biological specimen (Figure 5b). Wagner *et al.* have extended this concept by designing coumarin-caged arachidonic acid probes containing an additional moiety that targets the probe to specific organelles⁶⁸. Precise targeting was also achieved with an optically-cleavable targeted palmitoylethanolamide (OCT-PEA) analogue by Tobias *et al.*⁶⁹ OCT-PEA carries a guanine motif that binds to a genetically encoded spatially restricted SNAP-tag and thus in theory could be targeted to any membrane or protein. OCT-PEA was, however, only located at the plasma membrane, due to its membrane impermeability, where it was released to function as a GPR55 agonist.

In conclusion, the development of photo-caged and -switchable probes has proven useful to control the release and function of the signaling lipid *on demand*, thereby allowing to study the acute cellular responses of the probe.

Spatiotemporal readout of lipid action

Following the precisely controlled release of lipids, it is paramount to have tools with equivalent spatiotemporal capability to record their action on biological systems. Although there are well-established tools available for electrical recordings and the characterization of second messengers, it has been more challenging to directly visualize CB1 and CB2 receptor activation or location. For this purpose a CB2 receptor selective probe, LEI-121, was developed which harbors a diazirine for covalent cross-linking and an alkyne for downstream functionalization (Figure 6a, LEI-121)³⁹. As the first GPCR photo-affinity probe, LEI-121 lend itself useful for pharmacological profiling of CB2 ligands via SDS-PAGE and proteomics.

combination of genetically encoded sensors such as eCB2.0 and control over lipid activation, metabolism and localization will allow the deciphering of endocannabinoid signaling with nanoscale spatial – and millisecond temporal resolution.

Summary and outlook

Lipid signaling has been inherently difficult to study because of the hydrophobicity of lipid messengers, their rapid metabolism, and promiscuity of metabolic networks controlling these lipids. In this review, chemical biology approaches to control and visualize lipid messengers, specifically the endocannabinoids, were described.

The extensive use of ABPP in this field has resulted in the development of excellent chemical probes to perturb and visualize enzyme activity *in vitro* and *in vivo*. Highly selective inhibitors are required to decipher the functional roles of the various enzymes in lipid networks. Additionally, these will form the basis to develop the next generation *in vivo* active ABPs. Combined with recent technical advances in visualization, such as CATCH, PharmacoS^{TORM} and correlative light-electron microscopy, this will allow researchers to visualize enzymatic activity with unprecedented resolution and specificity.

Lipid probes with photo-caged or photo-switchable moieties can be released *on demand* in a spatially controlled manner, thereby allowing to study the acute cellular responses of signaling lipids. Next-generation lipid probes should be developed for live cell compatibility, real-time visualization and minimal structural deviation from the endogenous lipid. Future exploration of the bio-orthogonal reaction space should be focused on the physiological environment in which lipids reside, such as the plasma membrane or the endoplasmic reticulum.

The release of endogenous endocannabinoids can now be monitored in high spatiotemporal resolution with the GRAB sensor eCB2.0. These genetically encoded tools report on real time endocannabinoid signaling and will complement the ABPs and lipid probes to complete the molecular tool set for imaging lipid signaling. GRAB sensors that are specific to 2-AG or AEA activation are currently under development and would be highly valuable to dissect the individual roles of 2-AG and AEA in the brain.

To conclude, the lack of appropriate tools to study lipid signaling has long hampered their study compared to other biomolecules. Recent advances in the chemical biology of lipids now provide researchers with the tools to more accurately track lipid metabolism, location and action. Together with innovations in the fields of bio-imaging, these will form the future basis to uncover the underlying mechanisms of lipid signaling. Finally, it is also envisioned that the chemical biology approaches described in this review will facilitate the translation of the fundamental discoveries into clinical solutions for brain diseases with aberrant lipid signaling.

References

- Devane, W. A. *et al.* Isolation and Structure of a Brain Constituent That Binds to the Cannabinoid Receptor. *Science* **258**, 1946–1949 (1992).
- Mechoulam, R. *et al.* Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemical Pharmacology* **50**, 83–90 (1995).
- Sugiura, T. *et al.* 2-Arachidonoylglycerol: A Possible Endogenous Cannabinoid Receptor Ligand in Brain. *Biochemical and Biophysical Research Communications* **215**, 89–97 (1995).
- Katona, I. & Freund, T. F. Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. *Nat Med* **14**, 923–930 (2008).
- Bisogno, T. *et al.* Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *Journal of Cell Biology* **163**, 463–468 (2003).
- Blankman, J. L., Simon, G. M. & Cravatt, B. F. A Comprehensive Profile of Brain Enzymes that Hydrolyze the Endocannabinoid 2-Arachidonoylglycerol. *Chemistry & Biology* **14**, 1347–1356 (2007).
- Hussain, Z., Uyama, T., Tsuboi, K. & Ueda, N. Mammalian enzymes responsible for the biosynthesis of N-acylethanolamines. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1862**, 1546–1561 (2017).
- Ogura, Y., Parsons, W. H., Kamat, S. S. & Cravatt, B. F. A calcium-dependent acyltransferase that produces N-acyl phosphatidylethanolamines. *Nat Chem Biol* **12**, 669–671 (2016).
- Zhou, J. *et al.* Structure–Activity Relationship Studies of α -Ketoamides as Inhibitors of the Phospholipase A and Acyltransferase Enzyme Family. *J. Med. Chem.* **63**, 9340–9359 (2020).
- Cravatt, B. F. *et al.* Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384**, 83–87 (1996).
- Piomelli, D. *et al.* N-Acylethanolamine Acid Amidase (NAAA): Structure, Function, and Inhibition. *J. Med. Chem.* **63**, 7475–7490 (2020).
- van Egmond, N., Straub, V. M. & van der Stelt, M. Targeting Endocannabinoid Signaling: FAAH and MAG Lipase Inhibitors. *Annu. Rev. Pharmacol. Toxicol.* **61**, 441–463 (2021).
- Cristino, L., Bisogno, T. & Di Marzo, V. Cannabinoids and the expanded endocannabinoid system in neurological disorders. *Nat Rev Neurol* **16**, 9–29 (2020).
- Maccarrone, M. *et al.* Goods and bads of endocannabinoid system as a therapeutic target: Lessons learned after 30 years. *Pharmacol Rev* (2023) doi:10.1124/pharmrev.122.000600.
- Mock, E. D., Gagestein, B. & van der Stelt, M. Anandamide and other N-acylethanolamines: A class of signaling lipids with therapeutic opportunities. *Progress in Lipid Research* **89**, 101194 (2023).
- Bachovchin, D. A. & Cravatt, B. F. The pharmacological landscape and therapeutic potential of serine hydrolases. *Nat Rev Drug Discov* **11**, 52–68 (2012).
- Liu, Y., Patricelli, M. P. & Cravatt, B. F. Activity-based protein profiling: The serine hydrolases. *Proceedings of the National Academy of Sciences* **96**, 14694–14699 (1999).
- Zhou, J. *et al.* Activity-Based Protein Profiling Identifies α -Ketoamides as Inhibitors for Phospholipase A2 Group XVI. *ACS Chem. Biol.* **14**, 164–169 (2019).
- Petracca, R. *et al.* Novel activity-based probes for N-acylethanolamine acid amidase. *Chem. Commun.* **53**, 11810–11813 (2017).
- Baggelaar, M. P. *et al.* Development of an Activity-Based Probe and In Silico Design Reveal Highly Selective Inhibitors for Diacylglycerol Lipase- α in Brain. *Angew. Chem. Int. Ed.* **52**, 12081–12085 (2013).
- Baggelaar, M. P. *et al.* Chemical Proteomics Maps Brain Region Specific Activity of Endocannabinoid Hydrolases. *ACS Chem. Biol.* **12**, 852–861 (2017).
- Viader, A. *et al.* A chemical proteomic atlas of brain serine hydrolases identifies cell type-specific pathways regulating neuroinflammation. *eLife* **5**, e12345 (2016).
- Baggelaar, M. P., Maccarrone, M. & van der Stelt, M. 2-Arachidonoylglycerol: A signaling lipid with manifold actions in the brain. *Progress in Lipid Research* **71**, 1–17 (2018).
- Schlosburg, J. E. *et al.* Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat Neurosci* **13**, 1113–1119 (2010).
- Chanda, P. K. *et al.* Monoacylglycerol Lipase Activity Is a Critical Modulator of the Tone and Integrity of the Endocannabinoid System. *Mol Pharmacol* **78**, 996–1003 (2010).
- Leung, D., Saghatelian, A., Simon, G. M. & Cravatt, B. F. Inactivation of N-Acyl Phosphatidylethanolamine Phospholipase D Reveals Multiple Mechanisms for the Biosynthesis of Endocannabinoids. *Biochemistry* **45**, 4720–4726 (2006).
- van Rooden, E. J. *et al.* Mapping in vivo target interaction profiles of covalent inhibitors using chemical proteomics with label-free quantification. *Nat Protoc* **13**, 752–767 (2018).
- Janssen, A. P. A. *et al.* Development of a Multiplexed Activity-Based Protein Profiling Assay to Evaluate Activity of Endocannabinoid Hydrolase Inhibitors. *ACS Chem. Biol.* **13**, 2406–2413 (2018).

29. Baggelaar, M. P. *et al.* Highly Selective, Reversible Inhibitor Identified by Comparative Chemoproteomics Modulates Diacylglycerol Lipase Activity in Neurons. *J. Am. Chem. Soc.* **137**, 8851–8857 (2015).
30. Ogasawara, D. *et al.* Rapid and profound rewiring of brain lipid signaling networks by acute diacylglycerol lipase inhibition. *Proc Natl Acad Sci USA* **113**, 26–33 (2016).
31. Deng, H. *et al.* Triazole Ureas Act as Diacylglycerol Lipase Inhibitors and Prevent Fasting-Induced Refeeding. *J. Med. Chem.* **60**, 428–440 (2017).
32. Long, J. Z. *et al.* Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol* **5**, 37–44 (2009).
33. Cisar, J. S. *et al.* Identification of ABX-1431, a Selective Inhibitor of Monoacylglycerol Lipase and Clinical Candidate for Treatment of Neurological Disorders. *J. Med. Chem.* **61**, 9062–9084 (2018).
34. Johnson, D. S. *et al.* Discovery of PF-04457845: A Highly Potent, Orally Bioavailable, and Selective Urea FAAH Inhibitor. *ACS Med. Chem. Lett.* **2**, 91–96 (2011).
35. Ahn, K. *et al.* Mechanistic and Pharmacological Characterization of PF-04457845: A Highly Potent and Selective Fatty Acid Amide Hydrolase Inhibitor That Reduces Inflammatory and Noninflammatory Pain. *J Pharmacol Exp Ther* **338**, 114–124 (2011).
36. Long, J. Z. *et al.* Dual blockade of FAAH and MAGL identifies behavioral processes regulated by endocannabinoid crosstalk in vivo. *Proceedings of the National Academy of Sciences* **106**, 20270–20275 (2009).
37. van Esbroeck, A. C. M. *et al.* Activity-based protein profiling reveals off-target proteins of the FAAH inhibitor BIA 10-2474. *Science* **356**, 1084–1087 (2017).
38. Kerbrat, A. *et al.* Acute Neurologic Disorder from an Inhibitor of Fatty Acid Amide Hydrolase. *New England Journal of Medicine* **375**, 1717–1725 (2016).
39. Soethoudt, M. *et al.* Selective Photoaffinity Probe That Enables Assessment of Cannabinoid CB₂ Receptor Expression and Ligand Engagement in Human Cells. *J. Am. Chem. Soc.* **140**, 6067–6075 (2018).
40. Mock, E. D. *et al.* Discovery of a NAPE-PLD inhibitor that modulates emotional behavior in mice. *Nat Chem Biol* **16**, 667–675 (2020).
41. Speers, A. E., Adam, G. C. & Cravatt, B. F. Activity-Based Protein Profiling in Vivo Using a Copper(I)-Catalyzed Azide-Alkyne [3 + 2] Cycloaddition. *J. Am. Chem. Soc.* **125**, 4686–4687 (2003).
42. Witte, M. D. *et al.* Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat Chem Biol* **6**, 907–913 (2010).
43. Sarott, R. C. *et al.* Development of High-Specificity Fluorescent Probes to Enable Cannabinoid Type 2 Receptor Studies in Living Cells. *J. Am. Chem. Soc.* **142**, 16953–16964 (2020).
44. Pang, Z. *et al.* In situ identification of cellular drug targets in mammalian tissue. *Cell* **185**, 1793–1805.e17 (2022).
45. Chao, D. H. M. *et al.* Visualization of Active Glucocerebrosidase in Rodent Brain with High Spatial Resolution following In Situ Labeling with Fluorescent Activity Based Probes. *PLOS ONE* **10**, e0138107 (2015).
46. Hsu, K.-L. *et al.* DAGLβ inhibition perturbs a lipid network involved in macrophage inflammatory responses. *Nat Chem Biol* **8**, 999–1007 (2012).
47. Chang, J. W., Cognetta, A. B., Niphakis, M. J. & Cravatt, B. F. Proteome-Wide Reactivity Profiling Identifies Diverse Carbamate Chemotypes Tuned for Serine Hydrolase Inhibition. *ACS Chem. Biol.* **8**, 1590–1599 (2013).
48. Prokop, S. *et al.* PharmacoS^{TORM} nanoscale pharmacology reveals cariprazine binding on Islands of Calleja granule cells. *Nat Commun* **12**, 6505 (2021).
49. Gulyas, A. I. *et al.* Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. *Eur J Neurosci* **20**, 441–458 (2004).
50. McFarland, M. J. *et al.* A Role for Caveolae/Lipid Rafts in the Uptake and Recycling of the Endogenous Cannabinoid Anandamide*. *Journal of Biological Chemistry* **279**, 41991–41997 (2004).
51. Oddi, S. *et al.* Molecular Identification of Albumin and Hsp70 as Cytosolic Anandamide-Binding Proteins. *Chemistry & Biology* **16**, 624–632 (2009).
52. Kuerschner, L. & Thiele, C. Tracing Lipid Metabolism by Alkyne Lipids and Mass Spectrometry: The State of the Art. *Front. Mol. Biosci.* **9**, 880559 (2022).
53. Niphakis, M. J. *et al.* A Global Map of Lipid-Binding Proteins and Their Ligandability in Cells. *Cell* **161**, 1668–1680 (2015).
54. Gagestein, B. *et al.* Chemical Proteomics Reveals Off-Targets of the Anandamide Reuptake Inhibitor WOBE437. *ACS Chem. Biol.* *acschembio.2c00122* (2022) doi:10.1021/acschembio.2c00122.
55. Robichaud, P. P. *et al.* On the cellular metabolism of the click chemistry probe 19-alkyne arachidonic acid. *Journal of Lipid Research* **57**, 1821–1830 (2016).
56. Gaebler, A., Penno, A., Kuerschner, L. & Thiele, C. A highly sensitive protocol for microscopy of alkyne lipids and fluorescently tagged or immunostained proteins. *Journal of Lipid Research* **57**, 1934–1947 (2016).
57. Tamura, T. *et al.* Organelle membrane-specific chemical labeling and dynamic imaging in living cells. *Nat Chem Biol* **16**, 1361–1367 (2020).
58. Liang, D., Cheloha, R. W., Watanabe, T., Gardella, T. J. & Baskin, J. M. Activity-based, bioorthogonal imaging of phospholipase D reveals spatiotemporal dynamics of GPCR-Gq signaling. *Cell Chemical Biology* **29**, 67–73.e3 (2022).

59. Bertheussen, K. *et al.* Live-Cell Imaging of Sterculic Acid—a Naturally Occurring 1,2-Cyclopropene Fatty Acid—by Bioorthogonal Reaction with Turn-On Tetrazine-Fluorophore Conjugates**. *Angewandte Chemie International Edition* **61**, e202207640 (2022).
60. Heinbockel, T. Endocannabinoid Signaling Dynamics Probed with Optical Tools. *Journal of Neuroscience* **25**, 9449–9459 (2005).
61. Laguerre, A., Hauke, S., Qiu, J., Kelly, M. J. & Schultz, C. Photorelease of 2-Arachidonoylglycerol in Live Cells. *J. Am. Chem. Soc.* **141**, 16544–16547 (2019).
62. Nadler, A. *et al.* The Fatty Acid Composition of Diacylglycerols Determines Local Signaling Patterns. *Angew. Chem. Int. Ed.* **52**, 6330–6334 (2013).
63. Höglinger, D. *et al.* Trifunctional lipid probes for comprehensive studies of single lipid species in living cells. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 1566–1571 (2017).
64. Frank, J. A. *et al.* Photoswitchable fatty acids enable optical control of TRPV1. *Nat Commun* **6**, 7118 (2015).
65. Lichtenegger, M. *et al.* An optically controlled probe identifies lipid-gating fenestrations within the TRPC3 channel. *Nat Chem Biol* **14**, 396–404 (2018).
66. Westphal, M. V. *et al.* Synthesis of Photoswitchable Δ^9 -Tetrahydrocannabinol Derivatives Enables Optical Control of Cannabinoid Receptor 1 Signaling. *J. Am. Chem. Soc.* **139**, 18206–18212 (2017).
67. Sarott, R. C. *et al.* Optical Control of Cannabinoid Receptor 2-Mediated Ca^{2+} Release Enabled by Synthesis of Photoswitchable Probes. *J. Am. Chem. Soc.* **143**, 736–743 (2021).
68. Wagner, N., Stephan, M., Höglinger, D. & Nadler, A. A Click Cage: Organelle-Specific Uncaging of Lipid Messengers. *Angew. Chem. Int. Ed.* **57**, 13339–13343 (2018).
69. Tobias, J. M., Rajic, G., Viray, A. E. G., Ická-Araki, D. & Frank, J. A. Genetically-targeted photorelease of endocannabinoids enables optical control of GPR55 in pancreatic β -cells. *Chem. Sci.* **12**, 13506–13512 (2021).
70. Gazzi, T. *et al.* Detection of cannabinoid receptor type 2 in native cells and zebrafish with a highly potent, cell-permeable fluorescent probe. *Chem. Sci.* **13**, 5539–5545 (2022).
71. Dong, A. *et al.* A fluorescent sensor for spatiotemporally resolved imaging of endocannabinoid dynamics in vivo. *Nat Biotechnol* (2021) doi:10.1038/s41587-021-01074-4.
72. Dudok, B. *et al.* Retrograde endocannabinoid signaling at inhibitory synapses in vivo. *Science* **383**, 967–970 (2024).
73. Farrell, J. S. *et al.* In vivo endocannabinoid dynamics at the timescale of physiological and pathological neural activity. *Neuron* **109**, 2398–2403.e4 (2021).