***Mapping and annotation of novel protein interactors of monoacylglycerols – an upcoming class of signalling lipids***

## Introduction

Lipids are one of the key mediators that drive various biological processes that are involved in normal functioning of cells and their survival as a whole. And given their critical role in influencing these processes, any imbalance/deregulation in the levels of lipids due to defects in their metabolic pathways could potentially lead to various pathophysiological disorders such as neurodegenerative disorders, cardiovascular diseases, autoimmune diseases, and cancer to name a few(1, 2). Although the roles of lipids in cellular signalling and disorders are known, yet there is a lack of knowledge regarding the proteins these signalling lipids interact with and the downstream signalling pathways they trigger. Therefore, it is absolutely pertinent to study these signalling pathways and map the protein interactome of such signalling lipids.

One of classes of the signalling lipids that we intend to map the protein interactome for is the Monoacylglycerol (MAGs). MAGs are made up of a glycerol backbone with a fatty acyl chain attached to either sn-1(3) or sn-2 carbon position of the glycerol via an ester linkage giving rise to 1-MAG or 2-MAG respectively. These constitute a class of signalling lipids, with 2-arachidonylglycerol (2-AG) being one of the most studied MAGs due to its role as one of the most prominent endogenous ligands in the endocannabinoid system. 2-AG along with N-arachidonoylethanolamide (AEA) are termed as endocannabinoids as these acts as agonists for the cannabinoid receptors; CB1 and CB2(3). The activation of these receptors by endocannabinoids has been linked to various (patho)physiological processes like inflammation, pain perception, addiction, locomotor activity, regulation of anxiety and stress, etc. which has further accelerated the research on the endocannabinoid system for newer therapeutic interventions and pharmacologic studies(4), but there are no clear functional roles assigned to other MAG species. In the recent years however, research revolving around MAG species have shed light onto its possible functions as a potential signalling lipid. One of the most recent development being the involvement of MAGs in the glucose stimulated insulin secretion (GSIS) where the MAGs have been shown to bind to an intracellular receptor, Munc13-1 resulting in the release of insulin vesicles from the β-cells of pancreas(5, 6). Yet another instance where a G-protein coupled receptor, GPR119 which was initially annotated to be a receptor for lysophosphatidylcholine (lyso-PC) has been shown to bind to 2-oleoylglycerol with a higher affinity, and is involved in GLP-1 peptide release which mediates GSIS and enhances satiety(7). As the roles of MAG in insulin release and glucose metabolism are being uncovered gradually, this also points out that, it is highly probable that MAGs may

have crucial signalling roles in other organs as well such as in central nervous system (CNS) and immunity where roles of 2-AG have been explored in great detail in comparison to other MAGs in general. Also, research regarding two of the most prominent MAG degrading enzymes namely, monoacylglycerol lipase (MAGL)(8–10) and α/β hydrolysing domain containing protein 6 (ABHD6)(9, 11), have linked them to several biological processes and physiological conditions, which again, is an underlying testament to the roles MAGs play in normal cellular functioning.

We aim to accomplish the mapping of protein interactome of MAGs, using the ligand affinity-based protein profiling (LAPP) approach. LAPP is an upcoming chemoproteomic technique wherein the ligand of interest is appended with two additional functional groups, a photo-crosslinking diazirine group which forms covalent bond with protein interactor upon exposure to UV and an alkyne chemical handle to append the protein – probe complex with reporter tags. Our lab has already standardized the synthesis of PG-DA (sn1-palmitoyl glycerol-diazirine alkyne) (**Fig.1a**), an MAG lipid probe and PA-DA (Palmitic acid-diazirine alkyne), a free fatty acid lipid probe [This probe being a pre-cursor for the synthesis of PG-DA would serve as a competitor for narrowing down on the specific interactors of MAGs] (**Fig. 1b**). Since these lipid probes are almost identical to the natural lipids in all aspects be it, physical, chemical or structural properties, they would mimic the interaction of the lipid with its respective protein interactors in a biological sample which can be identified through proteomics mass spectrometry. This way we will be able to systematically generate the interactome of these signalling lipids. We further plan to validate these lipid – protein interactions through genetic screening and use of various biochemical tools and thus annotate functions of novel proteins or establish new functions of the proteins.

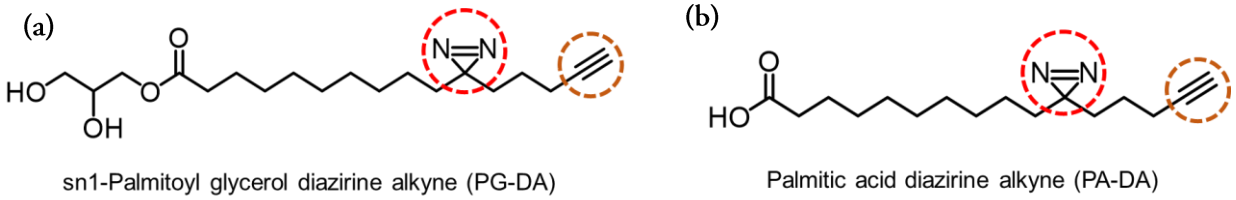


Fig. 1: Structure of synthesised lipid probes (a) sn1-palmitoyl glycerol-diazirine alkyne (PG-DA), an MAG probe and (b) palmitic acid- diazirine alkyne (PA-DA), a free fatty acid probe. The diazirine photo-crosslinking group highlighted in red dotted circle and the alkyne handle is highlighted in brown doted circle.

## Objectives of the Research

The broad goal of the research project is to use the synthetic bifunctional lipid probes, PG-DA and LP-DA to map the protein interactome of these lipids and hence uncover its downstream signalling pathways. The specific aims of the project include;

1. Generate a protein interactome of MAG using PG-DA probe. [*Completed*]
2. Selection and initial biochemical validation of the probable protein interactors of monoacylglycerol (MAG). [*Ongoing*]
3. Establishing the functional role of MAGs and their signalling pathways with respect to their interaction with the selected target proteins. [*Ongoing*]

## Materials, Methods and Experimental Design

*Materials*: All the chemicals and salts were procured from Sigma-Aldrich until stated otherwise. All primary antibodies and the secondary antibodies were bought were bought from Sigma-Aldrich and ThermoFisher Scientific respectively until mentioned otherwise. All tissue culture media, reagents and plasticware were purchased from HiMedia until noted otherwise.

*Mice organ harvesting*: The mice used for the studies belonged to C57BL/6 strain and were 10-12 weeks of age. To harvest the brain from the mice, the mice were anaesthetized by placing them in $CO_2$ chamber. The mice were euthanized by cervical dislocation. The brain was collected in 1.5 mL vial, flash frozen and stored at -80°C until further use. The above-mentioned protocol was performed with a formal approval from Institutional Animal Ethics Committee at IISER Pune (IAEC–IISER Pune) at National Facility for Gene Function in Health and Disease (NFGFHD), IISER Pune.

*Cell culture and harvesting*: Cells used in the study that is Neuro2A (N2A), RAW264.7 and BV2 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum and 1X penicillin streptomycin (MP Biomedicals) at 37 °C, 5% (v/v) $CO_2$. Once the cells reached a desired confluency (70-80%), it was harvested using cold PBS, flash frozen and stored at -80°C until further use.

*Proteome lysate preparation*: The mouse whole brain was homogenized in cold PBS using a Dounce homogenizer. The cell pellets obtained from tissue culture, were resuspended in cold PBS. The sample were then subjected to probe sonication with 70% amplitude, 2s on/ 2s off cycle for 10 cycles on ice. The cell lysate can be used for further experiments as is whereas the mouse brain homogenate was separated into soluble and membrane fractions by ultracentrifugation at 100,000xg for 1hr at 4°C. The supernatant was collected as the soluble fraction. The membrane pellet was resuspended in PBS and probe sonicated under same conditions.

*CLICK reaction*: The probe labelled lysates are subjected to CLICK reagent mixture containing 0.9 mM TBTA (1.7 mM in 4:1 DMSO: tert-Butanol), 9 mM $CuSO_4$ (50 mM in MilliQ water), 9 mM TCEP (50 mM in DPBS) and 0.9 mM Alexa 488 (or Biotin) azide probe (10 mM in DMSO). Typically for a 100 µL protein lysate, 6 µL of TBTA, 2 µL $CuSO_4$, 2 µL TCEP and 1 µL of azide probe are added. This volume is scaled up or down depending on the lysate volume taken.

*Sample preparation for in-gel fluorescence-based studies*: The cell or the brain tissue lysates were firstly estimated by Bradford estimation and aliquots of 1 mL containing 2 mg/mL total protein concentration were prepared. These aliquots were treated with the either the vehicle (DMSO) or the lipid probes [in case of PG-DA, the samples were pretreated with 2 mM PMSF for 45 min at 37°C to inhibit the activity of inherent serine hydrolases (especially lipases) that could potentially hydrolyse the lipid probe]. These samples were then incubated at 37°C for 30 min with constant shaking to allow the proteins to interact with the probe, and then transferred to a 96-well plate. The samples were directly exposed to UV light (365 nm) on ice to allow formation of covalent adducts of probe and the proteins. Alternatively, the samples which were not exposed to UV were also incubated on ice but were kept in dark. The samples were then transferred back to the 1.5 mL tube and CLICK reaction is carried out to tag the protein-probe complex with fluorophore, Alexa 488. The reaction is incubated at 25°C for 1 hr with constant shaking. Lastly, the reaction is quenched by the addition of 6X SDS-PAGE loading buffer and run on an SDS-PAGE (10% Acrylamide) gel to resolve the proteins in the sample. The gel is later visualized by in-gel fluorescence imaging on G-Box Chemi XRQ gel documentation system (Syngene).

*Proteomic sample preparation for mass spectrometry-based analysis*: The amount of protein in the lysate was estimated by Bradford estimation and aliquots of 1 mL containing 2 mg/mL total protein concentration were prepared. These aliquots were treated with 2 mM PMSF prior to the addition of the lipid probes in all experiments involving PG-DA. The aliquots were then separated into two sets of samples; a control sample set which were either not exposed to UV or were treated with PA-DA probe, and, the test sample set were either subjected to UV irradiation or were treated with PG-DA depending on the experiment described in the results section. Both the control and the test samples were subjected to CLICK reaction with Biotin-azide to tag the protein-probe complex with biotin handle. After 1 hr of CLICK reaction, ice-cold chloroform (2 mL), methanol (500 µL) and PBS (1 mL) were added consecutively and the cloudy white solution formed was vortexed and centrifuged at 5000 x g for 15 min. The aqueous and organic phase were aspirated leaving the protein disc intact. The protein disc was additionally washed thrice with 1:1 chloroform: methanol. The protein disc was redissolved in 500 µL of 6 M urea (prepared freshly in PBS) which contained 20 µL of 10 % (w/v) SDS (in MilliQ water) and 50 µL of TCEP-$K_2CO_3$ solution (100 mM TCEP in PBS and

300 mM K2CO3 in water). This mixture containing the protein disc is subjected to water bath sonication at room temperature till the protein disc dissolved completely. To these samples, 200 mM iodoacetamide (prepared freshly in PBS) was added and incubated in dark at room temperature for 30 min. The proteome samples were then avidin-agarose beads [pre-washed thrice in 0.2% SDS in PBS] for 1.5 hr at room temperature with constant shaking. The beads were consecutively washed with 0.2% SDS (in PBS), PBS and MilliQ water thrice to get rid of any protein that were not bound to the beads. The beads were then transferred to 1.5 mL LoBind Eppie tubes and 200 µL 2M urea (in 100 mM TEAB buffer) containing 1.5 µg trypsin is added. After 16 hr digestion at 37°C, the supernatant was transferred to a new 1.5 mL tube and was subjected to reductive demethylation labelling. In brief, the control set of samples were treated with 0.1% light formaldehyde, $CH_2O$ whereas the test set of samples were treated with 0.1% heavy formaldehyde, $CD_2O$ in addition to 0.02 M sodium cyanoborohydride treatment to both the sets of samples. The reaction was quenched after a 1 hr incubation at 37°C with addition of 1% $NH_4OH$ (32 µL) and was later acidified with 20% trifluoroacetic acid (4 µL). The test sample is mixed with its respective control sample and were desalted using C18 column. The eluate from the column was dried and stored at -20°C until its submission to mass spectrometer (SCIEX TripleTOF 6600) for analysis. The data files obtained from the mass spectrometer were searched against the mouse proteome database (available from RefSeq: NCBI Reference Sequences) using Sciex ProteinPilot software as per the recommended criteria from Sciex.

*Mammalian cell transfection*: The mouse cDNA (purchased from Horizon Discovery) of the target genes were cloned into mammalian expression vectors, pcDNA3.1(+)/Myc-His A [6x His and Myc tag at C-terminus] and p3xFLAG-CMV-10 [3x FLAG tag at N-terminus] depending on the domain architecture (available from Uniprot) and previous literature. The sequence verified plasmids with respective tags were then transfected into HEK293T cell line using polyethylenimine 40,000 (PEI40) (Manufacturer) following the manufacturer's instructions. The overexpression of the target genes was checked by western blotting.

*Western blot analysis*: The proteome was resolved by SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare) at 90V for 3 h at 4°C. Post transfer, membrane was blocked with 5% (w/v) skimmed milk in PBST and subsequently incubated with primary antibody (1:1000 dilution in 5% (w/v) skimmed milk in PBST) overnight at 4°C. Membrane was washed with PBST (thrice) and incubated with secondary antibody (1:10,000 dilution in 5% (w/v) skimmed milk in PBST) for 90 mins. Thereafter, membrane was washed in PBST (thrice) and developed using Immobilon western HRP substrate (Merck) and visualized using G-Box Chemi XRQ gel documentation system (Syngene).

*Competition of PG-DA interaction with the parent lipids*: The amount of protein in the cell lysates obtained from the mock transfected (transfection protocol carried out in absence of the target protein encoding plasmid) and target protein transfected was estimated by Bradford estimation and aliquots of 100 µL containing 2 mg/mL total protein concentration were prepared. The lysates were pre-treated with 2 mM PMSF. 50 µM PG-DA along with the parent lipid palmitic acid, PA or palmitoyl glycerol, PG) at 10x concentrations to that of the PG-DA probe was added to the lysates. Rest of the protocol remained the same as described previously.

*Live cell feeding and microscopy-based imaging*: The cell line N2A, RAW264.7 and BV2 were grown on coverslips to 70-80% confluency. The spent culture media was aspirated and the cells were rinsed with Dulbecco's PBS (DPBS) (HiMedia). The cells were treated with THL (tetrahydrolipstatin, a broad-spectrum lipase inhibitor) in serum-free media and incubated at 37°C, 5% (v/v) $CO_2$. The cells were then treated with the lipid probes and incubated at same conditions for an additional 30 min. Thereafter, the media was aspirated and cells were rinsed thrice with PBS to remove any traces of the media. Cold PBS was again added to the cells and was exposed to UV for 10 min. Further, the cells were fixed in methanol at -20°C for 10 min and subjected to stepwise washing with isopropanol and 10: 55: 0.75 chloroform: methanol: acetic acid (v/v) for 2 min at RT to remove excess unbound lipid probe. The coverslip was then incubated with the CLICK mixture (100 µM TBTA, 1 mM $CuSO_4$, 1 mM TCEP and 2 µM Alexa 488-azide) at RT for 1 hr at RT. Once the incubation was complete, the coverslip was rinsed again with PBS and DAPI staining was performed to stain the nucleus. Finally, the coverslip is mounted using fluoromount (ThermoFisher Scientific) and imaged at 63x (oil immersion) on Leica DM6 upright epifluorescence microscope.

## Results

*In-vitro validation of lipid probes by in-gel fluorescence imaging*

The synthesised probes firstly had to be optimised for its labelling efficiency in mouse brain and different cell (N2A, BV2 and RAW264.7) lysates. Since the most extensively studied monoacylglycerol, 2-AG has been shown to exert its effects on the cells involved in central nervous system (CNS) and macrophages, we wanted to map the protein interactors of MAGs in these systems as well. The mouse brain was taken as a representative of CNS which was complemented with two additional cell lines, N2A (murine neuronal cell line) and BV2 (murine microglial cell line) and RAW264.7 (murine macrophage cell line) as a representative for macrophages. The mouse brain membrane and soluble fractions were firstly treated with varying concentrations of the lipid probes with a constant UV cross-linking time, 10 min. We

could observe a dose-dependent increase in the intensity of protein bands and that the probes label the proteins in a UV-dependent manner i.e., the protein-probe complexes form only on exposure to UV (**Fig. 2a and c**). An optimal labelling of proteins was observed with 500 µM lipid probes for both PA-DA and PG-DA. Next, we sought to optimise UV exposure time for the probes, wherein the fractions were treated with 500 µM lipid probes and exposed to UV for varying amounts of time. We could again observe a UV exposure time-dependent increase in the intensity of protein bands (**Fig. 2b and d**) and an optimal labelling was observed with 6 min of UV exposure for both the probes. It is also important to note that PG-DA in general show a higher affinity binding in the membrane fractions, indicated by the increased intensities of the protein bands in the brain membrane fractions than the soluble fractions which is understandable as membrane proteins usually are lipid interacting proteins. A similar trend was observed in other cell lysates as well with both PA-DA and PG-DA probes (data not shown). Therefore, we concluded that the probe concentration of 500 µM with 6 min of exposure to UV would be the optimal conditions for labelling proteins for further chemoproteomic experiments.

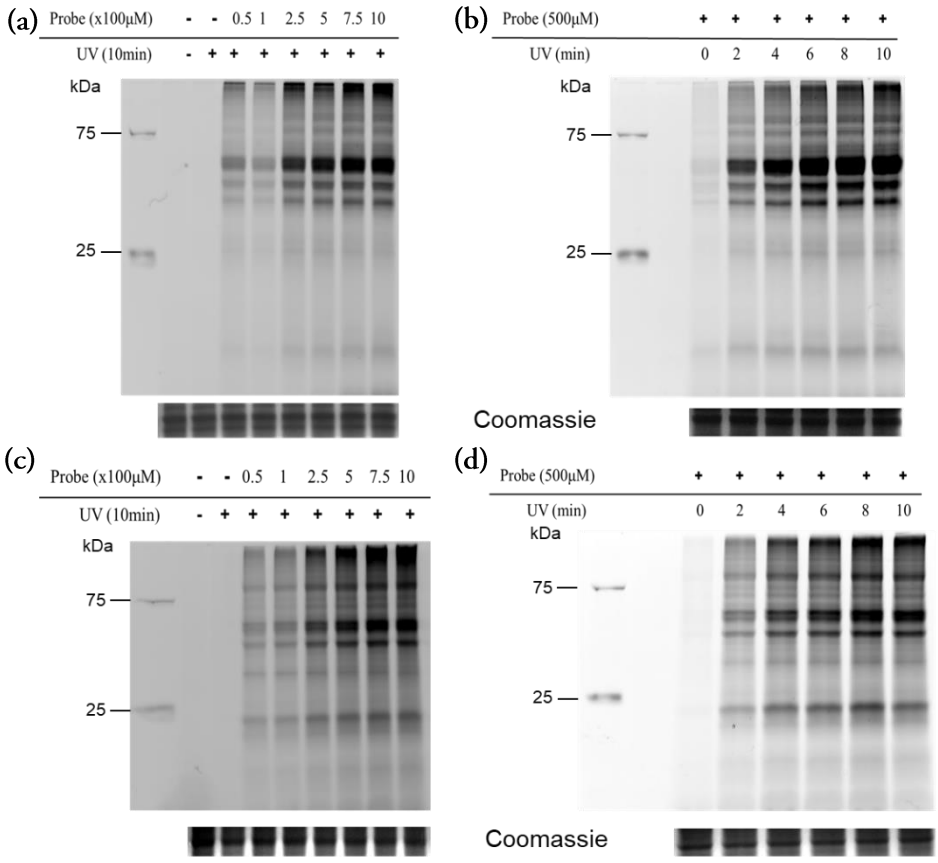

Fig. 2: In-gel fluorescence imaging shows that PG-DA probe shows dose and UV exposure time-dependent labelling of proteins. (a) and (c) PG-DA probe concentration optimization with concentration varying from 0 µM to 1 mM keeping UV exposure constant at 10 min in mouse brain soluble and membrane fractions respectively. (b) and (d)PG-DA probe UV exposure time optimization with exposure time varying from 0 to 10 min keeping the probe concentration constant at 500 µM in mouse brain soluble and membrane fractions respectively.

## *Chemoproteomic profiling of MAG-protein interactions*

With the optimised conditions for both the lipid probes, we next sought to map the protein interactome of MAGs using two different paradigms (A) UV exposed (ReDiMe heavy labelled) vs unexposed (ReDiMe light labelled) (**Fig. 3a**) and (B) PG-DA (ReDiMe heavy labelled) vs PA-DA (ReDiMe light labelled) (**Fig. 3b**). The paradigm (A) would be helpful in determining if the synthesised PG-DA was indeed functional in enriching known lipid interactors. The paradigm (B) on the other hand, would be helpful in filtering out those proteins that would interact with the any fatty acid part of PG-DA rather than PG-DA as a whole and obtain a set of proteins that interact with MAGs specifically. These experimental paradigms conducted in mouse brain fractions (**Fig. 3c and d)** and various cell lysates (data not shown) provided us with an exhaustive list of probable MAG interacting proteins. We were able to enrich several

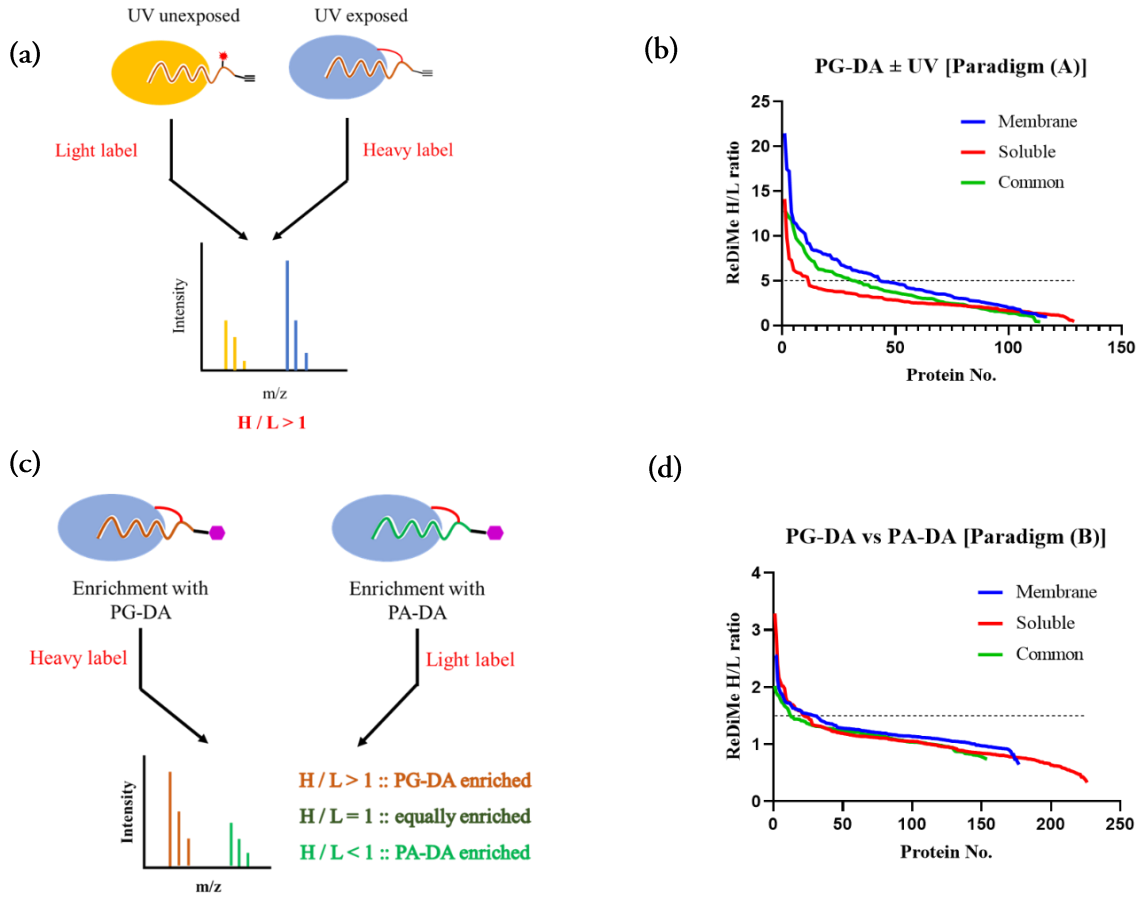

Fig. 3: Chemoproteomic profiling with PG-DA probe following two experimental paradigms. (a) is representation of the experimental paradigm (A) wherein the two sets of samples used for ReDiMe labelling were either exposed or not exposed to UV irradiation (b) is representation of the experimental paradigm (B) wherein the two sets of samples used for ReDiMe labelling were either treated with PG-DA or PA-DA (c) and (d) ReDiMe H/L ratio for all proteins identified in mouse brain fractions [blue line-brain membrane fractions, red line-brain soluble fractions and green line-proteins common in both the fractions] following the experimental paradigm (A) and (B) respectively with 6 min of UV exposure and probe concentrations of 500 μM [the dashed line indicates the H/L ratio cutoff in each paradigm].

lipid interacting proteins from paradigm (A) such as transporters (SLC25A20), metabolic enzymes (ACADM, ACADL, ACSBG1, ACSF2, ALDH2, ECHS1, PTGR1) apolipoproteins (ApoE, ApoA1) suggesting that the synthesised MAG probe was functional and mimicked the properties of the parent lipid. We were also able to observe that several proteins showed a preferential enrichment with PG-DA as a result of competition in the paradigm (B). Based on the preferential enrichment of proteins (ReDiMe Heavy/Light ratio > 1.5) with PG-DA probe in paradigm (B) across various lysates, we selected 4 proteins HPCA (Hippocalcin), NAP1L1 (nucleosome assembly protein 1-like 1), SAMM50 (sorting and assembly machinery component 50) and PCYOX1L (prenylcysteine oxidase 1-like precursor) for further biochemical validation.

*Validation and characterization of Hippocalcin, a potential interactor of MAG*

HPCA is a neuron-specific calcium-binding protein expressed only in the brain of mammals indicating its importance in normal functioning of the CNS. HPCA also consists of the 4 EF hand domains that bind to $Ca^{2+}$ with high affinity, undergo myristoylation and translocate to the membrane in response to increased cytosolic $Ca^{2+}$. HPCA till date, has not yet been shown to interact with MAGs or any other lipids classes in general. But since, we were able to enrich this particular protein in both the paradigms, we were interested in figuring out if it indeed has any affinity towards lipids and if so, how does its interaction with the lipids affect its overall function in neuronal cells. To this end, we transfected FLAG-tagged HPCA construct into HEK293T to observe the lipid probe-HPCA interaction. As observed in (**Fig. 4a**) HPCA shows a higher affinity/binding to PG-DA in comparison to PA-DA which is in agreement from the proteomic studies described. The competition profiles of HPCA labelling by PG-DA in the presence of the palmitoyl glycerol (PG) and palmitic acid (PA) show that the binding of HPCA to PG-DA diminishes in presence PG but the same is not observed with PA (**Fig. 4b**). Although, further biochemical validation for confirming the interaction of HPCA with MAG needs to be carried out, it is safe to assume that HPCA is indeed an MAG interacting protein at this point.

We are also carrying out similar preliminary experiments with the selected proteins as mentioned in the previous section.

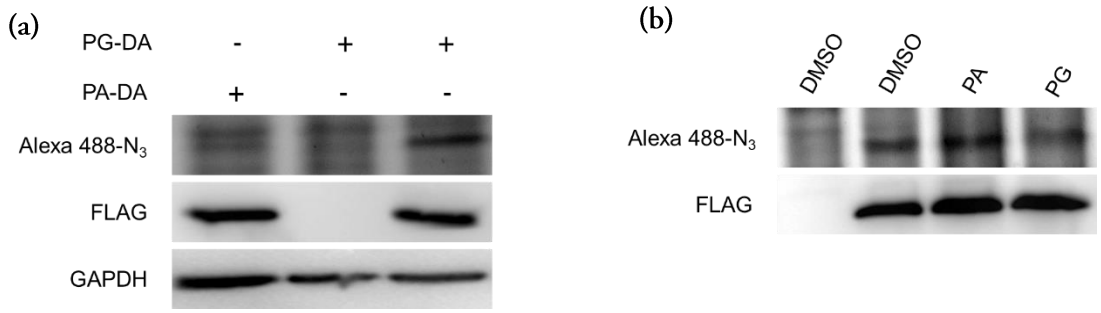


Fig. 4: Experimental validation of HPCA as a MAG interactor. (a) Lipid probe labelling of FLAG-tagged HPCA construct overexpressed by transient transfection in HEK293T. The top panel indicates the cell lysate labelling profiles by the lipid probes, PA-DA and PG-DA [lane 1 and 3 are HPCA transfected and lane 2 is mock transfected]. The middle panel is the anti-FLAG western blot and the lower panel is the anti-GAPDH western blot as loading control. (b) Labelling of HPCA (obtained from HPCA transfected HEK293T lysates) by PG-DA (50 μM) in presence of the competitors (10x the concentration of PG-DA).

*MAG localization in cells*

Since, we hypothesise that MAGs act as signalling lipid in cells, we were also curious to find out the intracellular localization of the lipid in the cells. To this end, we fed the live cells with the PG-DA probe followed by microscopy-based imaging (as described previously), to observe the localization and dynamics of the lipid probe within the cells. As shown in the (**Fig. 5**), PG-DA fed UV exposed N2A cells displayed a higher fluorescence around the nucleus, reminiscent of the endoplasmic reticulum followed by a slightly lower fluorescence diffused throughout the cytoplasm, however the experiments need to be carried out with appropriate organelle markers to further confirm the claim. A similar result was obtained with both the probes in all the three cell lines namely RAW264.7, BV2 and N2A (data not shown). This microscopy-based imaging platform would further enable us to perform co-localization experiments of the selected target proteins with PG-DA and also check if the localization of the lipid probe changes as a function of overexpression of the selected target protein in cells.

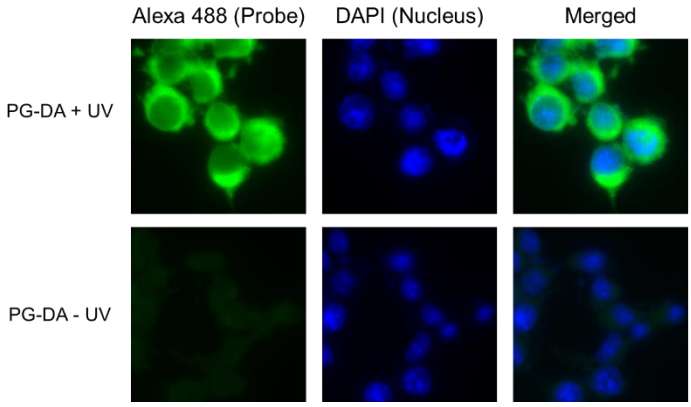


Fig. 5: Imaging protein-lipid interactions in N2A cells with PG-DA probe via microscopy. N2A cells pre-treated with THL for 2 hr were incubated for 30 min with 50 μM probe followed by 10 min of UV irradiation. The cells were then fixed with methanol, and excess lipid probe was washed out. Further a CLICK reaction with Alexa 488-azide followed by DAPI staining was performed and visualized by fluorescence microscopy. The cells in the top panel were exposed to UV whereas cells in the bottom panel were not.

## Statistical Analysis

For the chemoproteomic profiling based experiment, three technical replicates of each lysate (and mouse brain fractions) were submitted for analysis to the mass spectrometer. The data obtained after the searching, was refined to obtain MAG interacting proteins, using the following criteria (1) proteins should have at least 3 unique quantifiable peptides in two of the three technical replicates (2) proteins should have a ReDiMe heavy/light, H/L ratio ≥ 3 in paradigm (A) indicating the proteins that were enriched as a function of crosslinking with the lipid probe and with H/L ratio ≥ 1.5 in paradigm (B) indicating the proteins that were preferentially enriched by PG-DA in comparison to PA-DA. The H/L ratios across the replicates were averaged to visualise the data in a graphical format (plotted using GraphPad Prism 8.0.2). All the other experiments mentioned above were carried out in triplicates to obtain reproducible results.

## Discussion

Monoacylglycerols had been for the longest of time considered as breakdown products of tri- and diacylglycerols. Even though diacylglycerols have been known to be secondary messengers in various signalling pathways(12–14), the potential of MAGs as signalling lipid has never been explored (with 2-arachidonylglycerol being the only exception due to its involvement in endocannabinoid signalling). A recent finding, suggesting that MAGs (other than 2-AG) could may as well be a signalling lipid, piqued our interest in mapping MAG-protein

interactions in the nervous system and understand the functions this lipid plays in the CNS. Therefore, with the use of ligand affinity-based protein profiling (LAPP) approach, we have been successful in accomplishing the same. One of the putative protein interactors that we are currently working on is neuron-specific calcium-binding protein, Hippocalcin (HPCA). Although, this protein has not been shown to interact with lipids in the existing literature, it seems to be a putative interactor of MAGs based on our preliminary experiments. The previous studies have shown that HPCA, regulates the externalization of the AMPA receptors in the pyramidal cells of the hippocampus in response to cytosolic $Ca^{2+}$ levels, wherein increased $Ca^{2+}$ levels in the cytoplasm coupled with NMDA receptor activation trigger the endocytosis of AMPA receptors(15). And yet another independent study, shows that ABHD6, a prominent MAG hydrolysing enzyme negatively regulates the cell surface expression/externalisation of AMPA receptors in hippocampal neurons(16). This has led us to believe that there exists an as of yet unknown ABHD6-MAG-HPCA axis which plays a significant role in the functioning of AMPA receptors in neurons. And since AMPA receptors have already been known to regulate synaptic plasticity in hippocampal and hypothalamic neurons(17, 18) which in turn affects their functions, we are yet to discover how MAGs play a central role in these regions of the brain.

**Impact of research in the advancement of knowledge**

Our studies provide a variety of strategies for studying lipid-protein interactions with the use of lipid probes, for instance, in-gel fluorescence-based imaging, mass spectrometry-based chemoproteomic profiling and microscopy imaging-based lipid localization. The exhaustive list of putative protein interactors of MAGs that we have generated would be highly beneficial for deducing and/or annotating novel proteins whose functions are not yet known or establish new functions of the proteins, like HPCA for example, which was not known to bind lipids. This approach can also be used for developing inhibitors against such protein-lipid interactions via small molecule ligand library-based chemical screening. The synthesized lipid probes can itself further be extended to delineate protein interactome of various other signalling lipid classes. From past few decades, several lipid-protein interactions have been targeted as druggable candidates for treating various symptoms and conditions, with many drugs already approved by FDA and several under various steps of clinical trials. Such a study for delineating protein interactome of under-researched signalling lipids (such as MAGs, Lysophospholipids and oxidized lipids) will direct us towards understanding the functional roles of such lipids *in vivo* and additionally, open up new avenues for developing novel drugs and modern therapeutic interventions.

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