TITLE
Beta-amyloid associates directly with PDE4D5 to activate the enzyme and reduce cAMP availability.

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ABSTRACT
Inhibition of phosphodiesterase type 4 (PDE4) in the brains of animal models is protective in Alzheimer's disease (AD). We show for the first time that PDE4 enzymes from the subfamily PDE4D not only colocalize with beta-amyloid plaques in a mouse model of AD but that beta-amyloid directly associates with the catalytic machinery of the enzyme. Mapping suggests that PDE4D may be the preferential PDE4 subfamily for beta-amyloid association as it possess a unique binding site for the peptide. Intriguingly, direct addition of beta-amyloid to cells over-expressing PDE4 caused activation of PDE4D5 and a decrease in cAMP. We suggest a novel mechanism where PDE4 longforms can be activated by beta-amyloid resulting in the attenuation of cAMP signaling.

Keywords: PDE4, Alzheimer's disease, cyclic AMP, peptide array, beta amyloid

Abbreviations

cAMP, Cyclic AMP; AD, Alzheimer's Disease; PDE, phosphodiesterase; mRNA, messenger RNA; Aβ, βeta amyloid; PKA, protein kinase A; UCR, upstream conserved regions; GST, glutathione S-transferase; FRET, fluorescence resonance energy transfer; IBMX, 3-isobutyl-1-methylxanthine

INTRODUCTION

Cyclic-AMP (cAMP) signaling is a crucial pathway for memory formation/cognition and the down-regulation of this second messenger in the brain during Alzheimer's disease (AD) is thought to play a part in the cognitive deficits that are a characteristic of the disease[1,2]. cAMP-specific enzymes from the PDE4 family of phosphodiesterases have been identified as key players in shaping cerebral cAMP gradients that activate CREB via PKA phosphorylation (reviewed in [3]). Several studies involving pharmacological inhibition[4-6], RNA interference[7,8], dominant-negative PDE4 transfection[9], PDE4 knock-out mice[1] and CRISPR-Cas9[2] have indicated that isoforms from the PDE4D subfamily are most influential as targets for therapeutic strategies. Recent evidence also supports the role of PDE4B in this regard[3]. Said strategies counteract the aberrant cAMP signaling that results in a down regulation of CREB transcription factor activity and subsequent loss of synaptic plasticity. These observations have been supported by recent evidence from diseased human brains that show increased PDE4D expression when compared with controls [4]. Although mRNA transcripts[4] and western blotting [5] have been used to demonstrate elevated levels of PDE4D expression in AD models, there have been no attempts to look at the activation state of PDE4D enzymes during neurodegenerative disease. PDE4 enzymes, especially those designated as longforms, have an intrinsic activity that can be enhanced or inhibited by post-translational modification[6-8] or association with peptides[9] or lipids[10]. Here we report that PDE4D enzymes colocalise with beta amyloid (Aβ) plaques in brains of APP mice and that a direct association takes place between Aβ and the PDE4 enzyme. We provide peptide mapping evidence to show why PDE4D isoforms may have more relevance to AD than the other sub-families (PDE4A,B,C) and suggest that the low cAMP concentrations observed in AD brains may be as a result of the activation of PDE4D longforms caused by direct Aβ association.

MATERIALS AND METHODS

Co-localisation of PDE4D and Aβ.

To investigate the potential formation of complexes between PDE4D and Aβ, mouse brain sections obtained from seven month old APP/PS1 Alzheimer's mice (MMRRC strain #034832-JAX) were subjected to dual staining for both markers. Initially, an antigen retrieval step was carried out by incubating the sections with 70% formic acid for 15 minutes, followed by thorough washing in tris-buffered saline (TBS). Subsequently, the sections were incubated overnight at 4°C with a concentration of 2μg/ml rabbit anti-PDE4D (Abcam, Ab14613) diluted in 0.3% TBS-T. Following the primary antibody incubation, sections were treated with a donkey anti-rabbit biotin secondary antibody (1/400 dilution in 0.3% TBS-T) (ThermoFisher) for 1 hour at room temperature. After completing TBS washing steps, sections were subjected to incubation with streptavidin-647 (1/500 in 0.3% TBS-T) (ThermoFisher) for an additional 1 hour at room temperature to visualize PDE4D. Subsequent to the PDE4D visualization, the sections were further incubated overnight at 4°C with a primary mouse anti-human β-Amyloid, 17-24 antibody (clone 4G8) (BioLegend, Ab800712) at a dilution of 1/500 in 0.3% TBST-T. The following day, sections were washed and subsequently incubated with a secondary anti-mouse AlexaFluor 488 antibody (1/250 in 0.3% TBS-T) (Invitrogen) for 1 hour at room temperature. A Hoechst counterstain was performed to visualize cell nuclei.

To further validate PDE4D and Aβ colocalization within neurons, SHSY5Y cells were plated at 50000 cells on glass coverslips and cultured in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% nonessential [aminoacids], and 1% penicillin/streptamycin mixture until ~80% confluency. Cells were then treated with]5μM FAM-Aβ (Anaspec) for 1h or 24h and fixed in 4% paraformaldehyde. Blocking with1% BSA and 0.1% tween80 was performed, followed by overnight incubation with a primary PDE4D]antibody (Abcam, ab14613) and a 1h incubation with an Alexa fluor 555 secondary antibody (Invitrogen,A31572). Counterstaining was done using DAPI dye and coverslips were mounted using fluoromount (invitrogen). Samples were imaged using the Zeiss LSM900 confocal microscope using a 63x oil objective. Z-stacks were obtained and processed in ZEN 3.4 Lite and FIJI image J software. Colocalisation of PDE4D and FAM was determined using the Image J colocalization threshold plugin.

Fluorescence Polarization
Fluorescence polarization measurements were performed on a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany) with the excitation and emission wavelengths 485 nm and 535 nm, respectively. All FAM-Aβ peptides (1-40) were synthesised by AnaSpec and dissolved in DMSO to a stock concentration of 10 mM. The assay was formatted using 10 μl reaction volume per well in non-binding, black 384-well plates (Cat no.# 262260, Thermo Fisher Scientific Assay buffer (PBS, 1 mM DTT and 0.25% Tween-20) was used to dilute all ingredients. All polarisation values are expressed in millipolarisation units (mP). A fixed concentration of 10μM FAM-Aβ (1-40) was used with increasing amounts of GST and GST-PDE4D5. Reactions were incubated for 30 min at room temperature in the dark. In order to examine the existence of non-specific binding, GST protein was used as a negative control. All the experiments were independently repeated at least thrice.

Peptide array
The PDE4 isoform sequence peptide arrays were synthesized as sequential 25mers shifted by 5 amino acids via SPOT synthesis [11] on continuous <u>cellulose</u> membranes using Fmoc-chemistry with a MultiPep 2 instrument (CEM Corporation). For the alanine scanning arrays, versions of arrays were synthesised to incorporate alanine residues in place of the endogenous amino acid. In the event of alanine being the original residue an aspartic acid or glycine was incorporated. The membranes were blocked with 5% milk/TBST (w/v) for 1 h. The PDE4 arrays were then overlaid with either $A\beta_{1-42}$ or $A\beta_{scr}$ (Anaspec) overnight at 4 °C. The arrays were then analysed utilising a far-western immunoblotting approach. Analogous methods were used to probe overlapping $A\beta_{1-42}$ arrays with GST-fusion PDE4D5 protein in order to determine which domains within $A\beta_{1-42}$ are responsible for binding PDE4s. Specifically, Aβ(1-49) arrays were overlain with PDE4D5-GST and a mouse monoclonal GST-HRP (Sigma, A7340) was used at 1:5000 for 2 hours at room temperature to detect binding.

<u>**Co-immunoprecipitation**</u>
SHSY-5Y cells cultured in 6-well plates were treated with either 10 μM $A\beta_{1-42}$ or $A\beta_{scr}$ for 24 h. The neurotoxic 10 μM $A\beta_{1-42}$ derivatives were created as previously described [12]. Cellular lysates were prepared in lysis buffer [25 mM HEPES, 2.5 mM EDTA, 50 mM NaC1, 50 mM NaF, 30 mM sodium pyrophosphate, 10% (v/v) glycerol, 1% (v/v) Triton X-100, pH 7.5, containing Complete™ EDTA-free protease inhibitor cocktail tablets (Roche)] after the treatment. Protein concentration of lysates was determined using the Bradford assay and all samples were equalised for protein

concentration (400 µg protein/IP reaction). Goat anti-Pan-PDE4D antibody (in-house) was used to immunoprecipitate endogenous β-amyloid. The resulting immunocomplexes were captured using 25 µl of Protein G magnetic beads per sample (Pierce #88847) at 4°C overnight with mixing. Normal goat IgG (Bio-Techne Ltd, #AB-108-C) was used as a mock IP control. The beads were washed three times using lysis buffer. Bound proteins were then eluted in SDS-PAGE sample buffer and subjected to SDS-PAGE for immunoblotting using mouse anti-β-amyloid antibody (Sigma #A8354, 1:5000), followed by goat anti-Pan-PDE4D antibody (in-house, 1:5000) to confirm protein input. Immunoreactive proteins were detected by IRDye 680RD donkey anti-mouse IgG (Li-COR, #926-68072) and Alexa Fluor 790 donkey anti-goat IgG (Abcam, #ab175784), respectively. Images were acquired using Li-Cor Odyssey CLx Imaging System and signals was detected at 700 and 800 nm channels.

Proximity ligation assay.
SH-SY5Y cells cultured in 8-well chamber slides (Falcon #354118) were treated with either 10 µM Aβ1–42 or Aβscr (Anaspec) for 24 h. The cells were fixed with 4% (v/v) paraformaldehyde in PBS for 15 min at room temperature. Cells were counterstained with cell surface marker wheat germ agglutinin (WGA) conjugated to AlexaFluor 488 (Invitrogen #W11261) for 5 min, followed by permeabilization using 0.1% Triton X-100 (Sigma–Aldrich) in PBS for 10 min at room temperature. In situ detection of the exogenous beta-amyloid and PDE4D protein-protein interaction was carried out utilising Duolink® proximity ligation assay [13] as per manufacturer's instructions (Duolink®, Merck). Equal concentrations (1:4000) of immunocytochemistry validated PDE4D (goat) and Aβ (mouse) primary antibodies were used in combination with respective Duolink® PLA anti-goat (PLUS) and anti-mouse (MINUS) probes. Slides were finally mounted under coverslips with Prolong Gold Antifade reagent with DAPI (Invitrogen, P36935) and visualised. Images were acquired using an upright Zeiss LSM 880 confocal laser scanning microscope under a 63x oil immersion objective (excitation 594nm, emission 624nm). In order to detect all PLA signals, a series of Z-stack images were collected and were analyzed by ImageJ software.

PDE4 activity assays
An expression PCDNA3 plasmid encoding human PDE4D5-VSVas used before by us[14] was prepared using the Maxi-prep system (Qiagen, UK). For transient transfections, SH-SY5Y cells were transfected using PolyFect® transfection reagent (Qiagen, UK) in accordance with manufacturer's instructions. Cells (~90–100% confluent) were transfected for 48 h with cDNA encoding PDE4D5 and treated for 6 hours with Aβ$_{1-42}$ or Aβ$_{scr.}$ Cells were then washed with PBS and harvested by using a cell scraper in KHEM buffer (50 mM KCl, 50 mM HEPES; pH 7.2, 10 mM EGTA, 1.92 mM MgCl$_2$, 1 mM dithiothreitol (DTT)) supplemented with protease inhibitor Mini-Complete (Roche, UK). Samples were then frozen on solid CO$_2$, thawed and then manually homogenised, followed by passage through a 26-gauge needle several times to ensure complete cell lysis. Cells were centrifuged at 13,000 rpm for 10 min to remove any unbroken cells, and the resulting supernatant was frozen in solid CO$_2$ and stored at −80 °C until required. For experimentation, the protein concentration of whole-cell lysate from transfected and mock-transfected (vector only) cells was equalised (typically to 1 µg/µl). Protein concentration was determined through

Bradford Assay using bovine serum albumin as standard. PDE activity was determined using a two-step radioassay procedure as described previously[15]. Activities were related to a non-treated sample (100% control) over an increasing dose of the $A\beta_{1-42}$ or $A\beta_{scr.}$ In all cases, the transfected PDE accounted for over 97% of the total PDE activity when compared with the untransfected control lysates.

cAMP FRET reporter assay.
Assays were conducted as previously described [16]. HEK293 cells were seeded onto sterile glass coverslips and incubated for 24 hours. The cells were transiently transfected with a cAMP monitoring FRET sensor based on the structure of EPAC1 (EPAC1-cAMPs)[17] using Lipofectamine LTX (Invitrogen). FRET imaging was performed 24 hours following transfection and 60 minutes following pre-incubation with DMSO, $A\beta_{1-42}$ or $A\beta_{scr.}$ For imaging, the cells were buffered in a solution of 125 mM NaCl, 5mM KCl, 20 mM HEPES, 1 mM Na3PO4, 1 mM MgSO4, 1 mM CaCl2 and 5.5 mM glucose, pH7.4 and stimulated with 1 μM forskolin followed by a cAMP saturating concentration of 25 μM forskolin and the non-specific PDE inhibitor IBMX (100 μM). Analysis was undertaken using an Olympus IX71 inverted microscope with a 60x oil immersion objective (Zeiss) and an optical beam splitter (Photometrics). MetaFluor software (Molecular Devices) allowed image acquisition and real time monitoring. FRET changes were measured by excitation at 440 nm and obtaining a ratio of the intensity of emissions at 480 nm and 545 nm. Data are expressed as the % FRET change normalized to the baseline FRET ratio at t=0.

RESULTS

As previous work has indicated that increases in expression of PDE4B and D enzymes are associated with cognitive impairment in AD[4,5,18,19], we decided to stain hippocampal slices taken from APP/PS1 mice with antibodies raised against PDE4D and an antibody that detects Aβ. Magnification at 10X and 20X allowed clear observation of co-localization between the phosphodiesterase and Aβ in plaques that were surrounded by cells expressing PDE4D at a higher level than those positioned more remotely from plaques (Figure 1A). This was not an artefact of non-specific staining, as plaques formed on coverslips by FAM-Aβ 1-42 were not recognized by the PDE4D antibody (Supplementary Figure 1). We also recreated this experiment in vitro using exogenous addition of Aβ to cultured SH-SY5Y cells (Supplementary Figure 2) and again we detected an intracellular colocalization of PDE4D and Aβ. Co-localisation was seen both at 1 hour when FAM- Aβ was intracellular but diffuse and at 24 hours when intracellular aggregates had formed (Supplementary Figure 2). To further support this notion, we utilized proximity ligation (PLA), a technique which we have used before to visualize co-segregation of PDE4 and binding partners [20]. PDE4D and Aβ(1−42) could be detected in close proximity in the cytoplasm, but not in nucleus of SH-SY5Y cells (Figure 1B) and there was significantly more PLA signal when cells were treated with Aβ(1−42) compared with Aβ(1−42) scrambled (Figure 1C). As this is the first indication that PDE4D and Aβ exists in close proximity inside brain cells, we used a biophysical assay to assess the likelihood that the PDE4 and Aβ could associate directly. Fluorescence polarization experiments using fluorescently labeled 1-42 Aβ peptide indicated a dose dependent association

with GST-tagged PDE4D5 but not with GST alone (Figure 1D). Additionally, using a technique previously used to investigate the interaction of Aβ with VDAC1[21] we were able to co-immunoprecipitate Aβ with PDE4D from SH-SY5Y cells pretreated with the peptide (Supplementary Figure 3). The co-immunoprecipitating Aβ bands were at molecular weights observed in the prior study.[21]

The direct interaction between PDE4 proteins and Aβ detected in Figure 1 allowed us to map the binding sites by peptide array, a technique that we have used on multiple occasions to discover PDE-binding partner docking domains[16,22,23]. Immobilized libraries of PDE4 sequences corresponding to PDE4A4 (Figure 2B), PDE4B1 (Figure 2C), PDE4D5 (Figure 2D) and PDE4D7 (Figure 2E) were constructed as 25mers sequentially shifted by 5 amino acids. Arrays encompassing the whole sequence of each PDE4 were overlain with 1-42 Aβ peptide or a scrambled control version. Arrays were then blotted for Aβ, with dark spots signifying a direct interaction between the immobilized PDE4 sequence 25mer and Aβ. Figure 2A depicts the modular structure of PDE4 enzymes, with the conserved catalytic region following on from the two regulatory regions (UCR1 and 2) and the N-terminal targeting domain that is unique to each isoform. Interestingly, Aβ bound to all four isoforms of PDE4 in a region close to the start of the catalytic unit. The Aβ binding region on PDE4s has three putative binding motifs, one of which is unique to PDE4D isoforms (Site 3 outlined in Figure 2A). Firstly, there is a double lysine ('KK', Site 1) motif that appears in all PDE4s (except PDE4C) and starts only eight amino acids from the start of the catalytic unit (Figure 2A, lower panel). Point alanine substitution of either of the lysines dramatically reduced Aβ binding to PDE4B sequences, whereas a double alanine substitution was required to decrease binding to PDE4D and ablate PDE4B and PDE4A association (Figure 3A). Secondly, the arginine - phenylalanine ('RF', Site 2) motif appears in all PDE4 enzymes and alanine substitution of the "R" ablates Aβ binding to PDE4B and dramatically reduces binding to PDE4A and D (Figure 3A). Substitution of the "F" with alanine slightly reduced PDE4A and D binding and dramatically reduced PDE4B association. Double substitution of the "RF" motif to alanine ablated PDE4B binding and attenuated that of PDE4A and D. Quadruple substitution of all 4 residues in both motifs resulted in a complete loss of Aβ association with the PDE4 sequences (Figure 3A). Site 3 is unique to PDE4D isoforms and appears in a rare region of the catalytic site that is less well conserved than the other parts (Figure 3A, lower panel). Alanine scanning analysis shows that each of the residues depicted in bold at the top of Figure 3B are essential for Aβ binding to PDE4D. The Aβ binding sequence was superimposed onto an existing crystal structure of a PDE4D catalytic domain dimer (PBD: 7XAB) [24]. The binding site is external to the dimerisation interface and is composed primarily of α-helical secondary structure (Figure 3C).

As we have discovered Aβ binding sites on PDE4 sub-family members, we investigated potential complementary PDE4 binding sites on the Aβ peptide sequence using peptide array. Using APP transmembrane domain (D672 – L720) which corresponds (following sequential proteolytic cleavage) to the Aβ peptide sequence, we constructed arrays consisting of 20mers, sequentially shifted by 2 amino acids and overlaid these peptide spots with purified PDE4D5-GST or GST alone as a negative control (Figure 4). In doing so, we identified one 20mer sequence (G696-V715) that

bound strongly to PDE4D5-GST but not GST alone (Figure 4A). Point alanine (Figure 4B) and truncation (Figures 4C,4D) analysis of the Aβ 20mer identified that K699 was essential for the association of PDE4D5 and Aβ. The Aβ mutants K699D and N698E also ablated binding (Figure 4B, 3rd and 2nd last spots) suggesting that a salt bridge may form between Aβ and negatively charged residues on PDE4D5. Visualising the PDE4 binding sequence on an existing 3D structure (solution NMR) of the APP Q686 – K726 dimer (PDB: 2LOH)[25] reveals PDE4D5 binding to the dimerisation interface, suggesting PDE4D5 binding may influence the dimerisation/oligomerisation of Aβ (Figure 4E).

In light of the fact that FP and peptide array have indicated that Aβ may form a complex with PDE4D proteins in the catalytic region, and in cognizance of recent reports that although there is an insignificant change in PDE4D expression in the hippocampus of the APP/PS1 mice compared with WT but there is a highly significant threefold change in PDE4 activity[2] we next wanted to check whether PDE4 activity is altered following Aβ binding. Addition of increasing concentrations of Aβ 1-42 and Aβ 1-42 scrambled to cells overexpressing PDE4D5 isoforms followed by assessment of PDE4 activity in cell lysates showed that Aβ could activate PDE4D5 whereas scrambled Aβ could not (Figure 5A). Using a transfected cytosolic FRET-based cAMP-reporter (Figure 5B) we observed a reduction in cellular levels of cAMP following treatment with a sub-optimal concentration of the adenylate cyclase activator Forskolin [1 μM], where cells were pre-treated with Aβ 1-42 for 2 hours (Figure 5D). This was not observed with Aβ 1-42 scrambled control peptide (Figure 5E) or DMSO control (Figure 5C). Statistics shown in Figure 5F. Although small but significant changes in cAMP were detected, this data once again suggests that PDE4 activity was increased when exposed to Aβ. There was no difference between treatments when the probes were saturated following treatment with IBMX and forskolin (Figure 5F) with the maximal FRET change being approximately 20% (Figure 5F).

DISCUSSION

Many review articles[26,27] have catalogued the variety of benefits afforded by PDE4 and PDE4D selective inhibitors[28] in models of AD. Indeed, other ways of attenuating PDE4D activity (e.g. siRNA [29], dominant negatives[30], genetic silencing[1]) have also provided rescue from maladaptations conferred by a down-regulation of cAMP signaling associated with loss of synaptic plasticity. Recent reports of increased PDE4D expression in human AD brains[4] and animal AD model brains [31] are also consistent with the studies describing decreased phospho-CREB in AD models and may represent one reason why PDE4 inhibition is so effective in this disease context. Robust evidence supporting a similar role for PDE4B has also recently been published [3,18,19]. One point that has not been addressed to date is the possible activation of PDE4 longforms during AD progression.

Previous work using human protein microarrays had shown that Aβ and PDE4 can physically interact[32] and in line with our data, only the PDE4D subfamily showed a robust interaction. We provide evidence that Aβ binds directly to PDE4D in a region

at the start of the catalytic core and that this event activates the longform enzyme PDE4D5 (Figure 5A). Such an action could facilitate a reduction in cAMP (Figure 5F) that results in a loss of Phospho-CREB. It is possible that the Aβ peptide in some way relieves the UCR2 "transcapping" of active sites only observed in PDE4 dimers[33]. A similar mode of activation has recently been described for allosteric PDE4 activator compounds that phenocopy the actions of PKA phosphorylation of UCR1[34]. Similar activations of PDE4 longforms can also be triggered by antibodies against UCR2, peptide fragments of the regulatory regions and phosphatidic acid (summarized in[35 36]). All of these are thought to bind to the PDE4 enzyme to confer stabilization of PDE4 dimers in conformations that relieve the auto-inhibition by UCR2 transcapping. Hence, it is possible that the Aβ activation mechanism of PDE4 longforms is similar, although structural work and experiments with PDE4 phospho-mimic mutants would be required to confirm this.

In converse experiments, PDE4D5 was shown to bind to an Aβ site containing K699 (Figure 4). This region is known to be crucial for self-assembly of toxic oligomers and fibrils[37,38] suggesting that PDE4D5 may prevent or reverse Aβ oligomerization. We have already shown that a PDE4D binding partner, HSP20, can bind to the oligomerization domain of Aβ when HSP20 has been phosphorylated by PKA[12]. This action is facilitated by PDE4 inhibition which promotes HSP20 serine 16 phosphorylation by PKA[16] and promotes HSP20-Aβ interaction in order to prevent Aβ oligomerization[12]. It is possible that HSP20 is maintained in its inactive (non-phospho) form in a three-way PDE4-HSP20-Aβ complex where the activating action of Aβ on PDE4 keeps local cAMP concentrations low.

Finally, we show that PDE4D intimately localizes with Aβ in hippocampal cells from APP/PS1 mice. This is to our knowledge the first report of such a relationship where PDE4D expression seems to be enhanced around the areas where plaques have formed. This could be related to possible anti-aggregation effect of PDE4D5 that sequesters monomeric Aβ. Additionally, as we have also shown the intracellular colocalization of PDE4D and exogenously applied Aβ, albeit in a cultured cell line, we speculate that cells located around plaques may have increased cytoplasmic Aβ and therefore enhanced PDE4 activity. We appreciate that this mechanism is yet to be proven in human brains, however, as it is known that PDE4D selective inhibitors reverse learning and memory deficits in this mouse model via PKA and phospho-CREB (summarized in [39]), the robustness of that effect may be down to the fact that concentrated areas of Aβ-activated PDE4D enzymes are depressing local cAMP concentrations.

In summation, our data suggests a new molecular mechanism by which Aβ can down-regulate cAMP in order to promote cognitive deficits associated with AD.

Acknowledgements

Data Availability

All data sets that were used to construct this manuscript are available on request.

Figure Legends

Figure 1 PDE4D and Aβ form complexes. A. Naive APP/PS1 mouse brain sections from mice aged seven months were stained for PDE4D (647, red) and Aβ (488, green) to determine colocalization. Pictures taken at 10x, 20x and 40x magnification show that PDE4D and Aβ co-localize with each other in plaques. Sections were counterstained with Hoechst to visualize cell nuclei. B. Proximity ligation assays were done on SH-SY5Y cells following treatment with 10 μM Aβ1–42 or Aβscr for 24 h. Antibodies against PDE4D pan and Aβ were used in PLA assay. Cells were counterstained with AlexaFluor488-conjugated wheat germ agglutinin to improve cell segmentation. C. Quantification of mean fluorescence PLA signal per cell (n=3, 60 cells per condition). Evaluated with student's T-test, **** = $p < 0.0001$. D. Fluorescence polarization determination of GST or GST-PDE4D5 binding to increasing concentrations of FAM- Aβ(1–40). Results n=3. Evaluated with student's T-test, ***= $p < 0.001$ **** = $p < 0.0001$

Figure 2 Peptide mapping of Aβ binding domains on PDE4. A. Upper panel depicts the modular structure of PDE4 longform enzymes showing the N-terminal unique region, Upstream Conserved Region 1 (UCR1) and Upstream Conserved Region 2 (UCR2), core catalytic region and sub-family specific C-terminal region. Lower panel depicts the amino acid sequences of three Aβ binding sites at the start of the catalytic unit for each of the different sub-families (PDE4A,PDE4B, PDE4C PDE4D). B. Sequential 5 amino shift of PDE4A4 sequence with site 1 and site 2 depicted in bold/underlined. Control is overlain with scrambled Aβ. C. Sequential 5 amino shift of PDE4B1 sequence with site 1 and site 2 depicted in bold/underlined. Control is overlain with scrambled Aβ. D. Sequential 5 amino shift of PDE4D5 sequence with site 1, site 2 and site 3 depicted in bold/underlined. Control is overlain with scrambled Aβ. E. Sequential 5 amino shift of PDE4D7sequence with site 1, site 2 and site3 depicted in bold/underlined. Control is overlain with scrambled Aβ.   All peptide array experiments were repeated n=2.

Figure 3 Delineation of essential binding residues in Aβ docking motif. A. Amino acids implicated in Aβ binding sites 1 and 2 (bold font) from PDE4A, B and D were substituted to alanine (red font) and the binding of Aβ assessed. Control is overlain with scrambled Aβ. Results typical of n=2. B. Amino acids implicated in Aβ binding site 3 (red bold font) from PDE4D were substituted to alanine (red font) and the binding of Aβ assessed. Control is overlain with scrambled Aβ. Results typical of n=2 C structural representation of binding site on PDE4 longform dimer. Protein sequence as per Uniprot Q08499-1. Light Grey, PDE4D catalytic domain monomer 1. Dark Grey, PDE4D catalytic domain monomer 2. Red line, dimer interface. Green, compound 22d. Blue, Zinc molecule. Red, Magnesium molecule. Orange, Q390 – V422. Magenta, PDE4D specific amyloid β binding site residues H407, F409, R410, A412 and R418. PDB: 7XAB – Liu, et al. (2022) Eur J Med Chem. 242:114631.

Figure 4 Mapping the PDE4 binding sites on Aβ. A. Sequences from Aβ were sequentially shifted by 2 amino acids and overlain with either GST or GST-PDE4D5. B. The binding region detected in figure 4A was alanine scanned and overlain with either GST or GST-PDE4D. C. The binding region detected in 4A was sequentially truncated from the C-terminal and overlain with either GST or GST-PDE4D. D. The binding region detected in 4A was sequentially truncated from the N-terminal and overlain with either GST or GST-PDE4D. Results typical of n=3. E. Structural visualization of PDE4D5 binding site on Aβ. Structure based on PBD: 2LOHAPP Q686-K726, Nadezhdin, et al. (2012) FEBS Lett. 586: 1687 1692

Figure 5 PDE4 longforms are activated by Aβ. A SH-SY5Y cells transfected with PDE4D5 were treated for 6 hours with indicated concentrations of $Aβ_{1-42}$ or $Aβ_{scr.}$ before lysates were harvested and evaluated for rolipram inhibited PDE4 activity. Activity is normalized to lysate from non-treated transfected cells. n=3 B. HEK293 cells were transefected with the cAMP reporter EPAC1-cAMPs and treated as indicated. The images show representative traces that allow visualization of reporter. C. Transfected HEK293 cells were pre-treated with DMSO and the reaction to 1uM forskolin monitored. D Transfected HEK293 cells were pre-treated with 10μM $Aβ_{1-42}$ and the reaction to 1uM forskolin monitored. n=3, 15 cells per treatment. E. Transfected HEK293 cells were pre-treated with 10uM $Aβ_{scr}$ and the reaction to 1uM forskolin monitored. n=3, 15 cells per treatment. F. Quantification of relative FRET changes produced in response to 1uM Forskolin (left) or 25μM Forskolin plus 100μM IBMX. n=3, 15 cells per treatment. Students t-test * p<0.05.

Supplementary Figure 1
FAM-Aβ was seeded onto glass coverslips for 24 hours. The coverslips were probed with a PDE4D-specific antibody and stained for PDE4D (647, red) and Aβ (488, green). No red signal was detected, however FAM-Aβ could easily be detected on the coverslip surface. Image representative of n=2.

Supplementary Figure 2
SH-SY5Y cells were treated with FAM-Aβ for 1h or 24h and stained for PDE4D. Z-stacks were obtained, and the orthogonal planes were indicated by white arrows. Colocalization analysis (ImageJ) shows colocalization of PDE4D and FAM-Aβ after both 1h and 24h of exposure. FAM- Aβ appears intracellular but diffuse after 1h of exposure and appears intracellular and aggregate-like after 24h of exposure. Image representative of n=2.

Supplementary figure 3
PDE4D was pulled down from lysates extracted from SH-SY5Y cells treated with $Aβ_{1-42}$ for 24h (right panel, black arrow). The immunoprecipitates of PDE4D were then blotted for Aβ and bands were identified in the IP but not mock IP (left panel, red arrows). Gels are representative of experiments n=3.

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