

Regulation of Amyloid Precursor Protein Processing by Serotonin Signaling

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Abstract

Proteolytic processing of the amyloid precursor protein (APP) by the β - and γ -secretases releases the amyloid- β peptide (A β), which deposits in senile plaques and contributes to the etiology of Alzheimer's disease (AD). The α -secretase cleaves APP in the A β peptide sequence to generate soluble APP α (sAPP α). Upregulation of α -secretase activity through the 5-hydroxytryptamine 4 (5-HT₄) receptor has been shown to reduce A β production, amyloid plaque load and to improve cognitive impairment in transgenic mouse models of AD. Consequently, activation of 5-HT₄ receptors following agonist stimulation is considered to be a therapeutic strategy for AD treatment; however, the signaling cascade involved in 5-HT₄ receptor-stimulated proteolysis of APP remains to be determined. Here we used chemical and siRNA inhibition to identify the proteins which mediate 5-HT₄ receptor-stimulated α -secretase activity in the SH-SY5Y human neuronal cell line. We show that G protein and Src dependent activation of phospholipase C are required for α -secretase activity, while, unexpectedly, adenylyl cyclase and cAMP are not involved. Further elucidation of the signaling pathway indicates that inositol triphosphate phosphorylation and casein kinase 2 activation is also a prerequisite for α -secretase activity. Our findings provide a novel route to explore the treatment of AD through 5-HT₄ receptor-induced α -secretase activation.

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Introduction

The most common form of dementia in elderly people is Alzheimer's disease (AD), which is pathologically characterized by progressive neuronal loss and deposition of the amyloid- β peptide (A β) in amyloid plaques. Current therapeutic targets are the β - and γ -secretases, which generate A β from amyloid precursor protein (APP). Several drugs inhibiting or modulating the activity of these secretases have failed in clinical trials due to severe side effects or to difficulty in delivery through the blood brain barrier [1]. APP is also cleaved by a third secretase called α -secretase. The α -secretase cleaves APP within the A β peptide sequence, producing a soluble APP α fragment (sAPP α), which precludes A β generation. Indeed, *in vivo* overexpression or upregulation of α -secretase activity in mice indicate that α -secretase activation leads to a decrease in A β production and a reduction in the amyloid plaque load in AD mouse models [2,3]. These effects were accompanied by an improvement in the cognitive deficits, providing considerable support for modulation of α -secretase activity as a viable strategy in the fight against AD [2,3].

To specifically target the non-amyloidogenic pathway of APP processing, a fundamental consideration would be to understand the mechanism of α -secretase activation and to determine the signaling cascade of kinases and second messengers that directly regulate α -secretase-mediated proteolysis of APP. These molecules

can be directly targeted pharmacologically, but also indirectly via G protein-coupled receptors (GPCR), such as the muscarinic, glutamatergic and serotonergic receptors. In particular, the G protein coupled 5-hydroxytryptamine 4 (5-HT₄) receptor is gaining considerable interest as a modulator of α -secretase activity due to its role in memory and learning and regulation of APP processing [4]. Activation of the 5-HT₄ receptor leads to an increase in the population spike amplitude in the hippocampal CA1 region, and this effect persists in a transgenic mouse model of AD [5,6], suggesting that 5-HT₄ receptor-mediated signaling remains functional under these pathological conditions. On the other hand, agonist stimulation of the 5-HT₄ receptor results in increased sAPP α secretion with a concomitant decrease in A β peptide levels in primary neuronal cultures and an alleviation of amyloid plaque load in AD mouse models [7–9]. Such amelioration of disease pathology is correlated with improvements in memory and learning in behavioral paradigms and scopolamine-induced models of cognitive deficit [10–12]. Additionally, an increase in acetylcholine release is observed after 5-HT₄ receptor agonist application *in vivo* [13]. This could be a valuable property when considering 5-HT₄ receptor agonists for AD treatment, which could complement the currently licensed therapy of cholinesterase inhibition for partial symptomatic relief [14].

Despite numerous reports on 5-HT₄ receptor function in memory and learning and its effect on APP processing, the

downstream signaling pathway responsible for this 5-HT₄ receptor-mediated effect is still poorly understood. 5-HT₄ receptor stimulation results in an accumulation of cAMP, a second messenger required for protein kinase A (PKA) and exchange protein activated by cAMP (Epac) activation. However, 5-HT₄ receptor-mediated non-amyloidogenic processing of APP occurs independently of PKA activation, but can be regulated by Epac1 activation of Rac1 and Rap signaling in cell lines and primary neurons [15]. The 5-HT₄ receptor is constitutively bound to the Src non-receptor tyrosine kinase, which is required for ERK activation [16]. In addition, 5-HT₄ receptor stimulation in adrenocortical cells and cardiomyocytes results in an increase of calcium influx, which results in activation of voltage-gated calcium channels through PKA [17,18]. It is unknown whether these latter pathways also contribute to α -secretase activation downstream of the 5-HT₄ receptor. Altogether, these studies suggest a complicated picture of the downstream signaling pathways involved in 5-HT₄ receptor stimulation and reveal the importance of delineation of the mechanism of 5-HT₄ receptor-mediated APP proteolysis.

Finally, several metalloproteinases have been proposed as α -secretase; however, the identity of 5-HT₄ receptor-induced α -secretase activity has not been fully addressed. The disintegrin and metalloprotease ADAM10, a major constitutive α -secretase of APP [19,20], is a feasible candidate [21]. However, ADAM17 is more likely to be the inducible APP α -secretase based on studies which have investigated the regulated ectodomain shedding of other ADAM substrates after protein kinase C (PKC) activation [22]. In support of this is the observation that M1 receptor induced sAPP α release correlates with increased ADAM17 expression levels [23]. Nevertheless, additional metalloproteinases, such as meprin β and membrane-type matrix metalloproteinases, were shown to mediate α -cleavage of APP [24,25].

Here, we specifically determined the intracellular signaling cascade involved in 5-HT_{4d} receptor stimulation and inducible α -secretase activity. We used human SH-SY5Y neuroblastoma cells to analyze APP processing for practical reasons and experimental consistency. Human SH-SY5Y cells can generate sustainable cells with characteristics that resemble the morphology and biochemistry of mature neurons [26]. We present evidence that the G protein-dependent pathway activating Src, phospholipase C (PLC) and casein kinase 2 (CK2) is responsible for the 5-HT_{4d} receptor-stimulated induction of α -secretase activity. Interestingly and in contrast to previous publications, we find that adenylyl cyclase (AC) and cAMP signaling are not required for 5-HT_{4d} receptor-mediated α -secretase activity [15]. Furthermore, we analyzed the reported α -secretases as putative mediators of the 5-HT_{4d} receptor effect on APP shedding using RNAi studies.

Materials and Methods

Reagents and Antibodies

Tissue culture reagents were purchased from Invitrogen. GF109203X, SQ22536, D609, ionomycin, 4,5,6,7-tetrabromo-1H-benzotriazole (TBB), GR113808, NF449, gallein and batimastat were obtained from Tocris. 5-hydroxytryptamine (5-HT), cholera toxin B (CTB) and chlorogenic acid (CGA) were from Sigma-Aldrich. Phorbol 12-myristate 13-acetate (PMA), 2,5-dideoxyadenosine (DDA), IP3K inhibitor, TAPI-1 and GM6001 were obtained from Calbiochem/VWR. Bosutinib was from Selleck. Prucalopride was kindly provided by Movetis (NV, Turnhout, Belgium). Table 1 summarizes the known potencies of the used agonists and antagonists. Following antibodies were purchased: CK2 α (H-286) from Santa Cruz, MMP-9 (G657) and ADAM9 (2099) from Cell Signaling, ADAM17 (T5442) and β -

Actin (A5441) from Sigma-Aldrich. ADAM10 (B42) and APP (B63) antibodies were made in house and previously described [27]. G α_s dominant negative construct was previously described (pcDNAI-Amp-G α_s DN, [28]).

Inhibitor treatment and soluble APP analysis (SEAP assay)

SH-SY5Y human neuroblastoma cells (CRL-2266, ATCC) were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). For analysis of soluble APP secretion, a mix of 1,5 μ g plasmid encoding human wild type APP695 linked to Alkaline Phosphatase (AP-APP) at the N-terminus (pEAK12-AP-APP, [29]), 1,35 μ g of 5-HT_{4d} receptor isoform in pcDNA3.1 (pcDNA3.1-5-HT_{4d}, kindly provided by Joris De Mayer and Jan Schuurkes, Movetis, Turnhout, Belgium) and 0,15 μ g of GFP (pmaxFP-Green-N, Amaxa) was prepared in OPTI-MEM and combined with 20 μ l of Lipofectamine 2000 (Invitrogen). After 20 minutes incubation at room temperature transfection mix was combined with a trypsinized cell suspension in growth medium containing 10% FBS. After another 15 minutes incubation at room temperature cells were seeded in a 96-well plate at 80.000 cells/well. The next day medium was changed to DMEM/F12 supplemented with 5% dialyzed FBS (10,000 molecular weight cutoff), which is devoid of serotonin otherwise present in undialyzed FBS that causes 5-HT₄ receptor desensitization. After three days, cells were washed and incubated in serum free medium (SFM) for another 24 hours. Next cells were stimulated with 1 μ M of the following compounds: prucalopride, 5-HT, PMA and GR113808 in SFM for 24 hours and the conditioned medium was analyzed for secreted AP (SEAP) activity with Great EscAPe SEAP Chemiluminescence Kit 2.0 (Clontech) according to manufacturer's instructions. Luminescence was measured with the EnVision[®] multilabel reader (PerkinElmer). For signaling studies dilution curves of inhibitory compounds were made in combination with induction by 1 μ M prucalopride or 5-HT. DMSO incubation was used as a control in all experiments carried out. The ratio of individual luminescence counts from the tested conditions to the mean value of DMSO treated cells was plotted as SEAP fold induction. Cells were used for the MTS proliferation assay (CellTiter 96[®] AQ₁ Non-Radioactive Cell Proliferation Assay) and the LDH cytotoxicity assay (CytoTox 96[®] Non-Radioactive Cytotoxicity Assay) according to manufacturer's instructions (Promega). Compound dilution curves were performed in the range of the reported effective concentrations (Table 1) and working concentrations were determined in the SEAP assay as those giving significant inhibition of 5-HT_{4d} receptor-stimulated sAPP α secretion. MTS and LDH assays were used to define working concentrations of the different compounds that were non-toxic to the cells.

cAMP assay

cAMP levels were assessed using the Alphascreen[®] cAMP assay kit (PerkinElmer Life Sciences). 2,88 \cdot 10⁶ or 1 \cdot 10⁶ SH-SY5Y cells were seeded in T75 or T25 flasks, respectively. Adherent cells were transfected after 4 hours with Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instructions. A mix of 7,5 μ g pEAK12-AP-APP, 6 μ g pcDNA3.1-5-HT_{4d} and 1,5 μ g pmaxFP-Green-N plasmids (ratio of 5:4:1) was used for transfection in T75 flasks. A mix of 1,25 μ g pEAK12-AP-APP, 0,65 μ g pcDNA3.1-5-HT_{4d} receptor and 2,6 μ g pcDNAI-Amp-G α_s DN or pcDNA3.1 as an empty vector control (ratio of 2:1:4) was used for transfection in T25 flasks. 3 hours later transfection mixes were replaced with growth medium for 16 hours and cells were treated with medium supplemented with 5% dialyzed FBS and SFM as described under "inhibitor treatment and soluble APP analysis".

Table 1. Overview of agonists, antagonists and inhibitors used to investigate the proteins contributing to the induction of sAPP α after 5-HT_{4d} receptor stimulation.

Compound	Target	Ag/antag/inh	Potency	Experimental system	Citation
Prucalopride	5-HT ₄	Ag	EC ₅₀ 10 nM	SH-SY5Y cells	[9]
5-HT	5-HT ₄	Ag	EC ₅₀ 1,1 nM	HEK293 cells	[34]
GR113808	5-HT ₄	Antag	K _i 0,31 nM	Mouse colliculi neurons	[63,64]
Cholera toxin B (CTB)	G α_s	Inh	IC ₅₀ 100 ng/ml	L6 cells	[65,66]
NF449	G α_s	Inh	IC ₅₀ 8 μ M	<i>in vitro</i>	[67]
Gallein	G $\beta\gamma$	Inh	IC ₅₀ 5 μ M	HL60 cells	[68]
SQ 22536	Adenylyl cyclase	Inh	IC ₅₀ 1 μ M	Human blood platelets	[69,70]
2,5-dideoxyadenosine (DDA)	Adenylyl cyclase	Inh	IC ₅₀ 100 μ M	<i>in vitro</i>	[71,72]
Bosutinib	Src	Inh	IC ₅₀ 300 nM	MDA-MB-468 cells	[73,74]
D609	Phospholipase C	Inh	K _i 6,4 μ M	<i>in vitro</i>	[75,76]
GF109203X	Protein kinase C	Inh	IC ₅₀ \approx 5,8 μ M	<i>in vitro</i>	[77,78]
IP3K inhibitor	IP6K, IP3K	Inh	IC ₅₀ 18 μ M	<i>in vitro</i>	[79]
Chlorogenic acid (CGA)	IPMK	Inh	IC ₅₀ 1,15 μ M	<i>in vitro</i>	[80]
4,5,6,7-tetrabromo-1H-benzotriazole (TBB)	Casein kinase 2	Inh	IC ₅₀ 1,6 μ M	<i>in vitro</i>	[81–83]
GM6001	MMP1, 2, 3, 8, 9; ADAM10 and 17	Inh	K _i 0,1–110 nM	<i>in vitro</i>	[84,85]

Ag = agonist; antag = antagonist; inh = inhibitor.
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Then cells were gently dissociated with Versene solution (Invitrogen) to obtain a single cell suspension. Next cells were counted to determine the exact cell number. Equal numbers of cells were combined with the acceptor beads coupled to an anti-cAMP antibody and biotinylated cAMP, both provided in the Alphascreen[®] cAMP assay kit (PerkinElmer Life Sciences), and a serial dilution of compound. After incubating the cells for one hour, streptavidin-donor beads were added and the cells were permeabilized with 0,3% Tween-20 for 30 minutes, which released intracellular cAMP. The assay is based on competition between endogenously produced cAMP by the stimulated cells and exogenously added biotinylated cAMP. The electron transfer between donor and acceptor beads was measured with the EnVision[®] multilabel reader (PerkinElmer). DMSO was diluted to a final concentration of 0,1% and kept equal in all samples to avoid differential effects of different DMSO concentrations on the cells. cAMP concentrations were determined using a standard curve.

Calcium measurements

Calcium imaging was assessed using the Fluo-4 NW calcium assay kit (Invitrogen). SH-SY5Y cells were transfected with pEAK12-AP-APP, pcDNA3.1-5-HT_{4d} receptor and pmaxFP-Green-N in Optilux black wall clear bottom plates (BD Biosciences) and treated as described under “inhibitor treatment and soluble APP analysis”. Next cells were loaded with Fluo-4 NW dye mix according to manufacturer’s instructions. Binding with calcium ions increases fluorescence of the dye. Baseline fluorescence of the dye was recorded at the steady state, while stimulated calcium release was assessed after automated addition of the compounds at different time points using IN Cell Analyzer 2000 (GE Healthcare). Calcium images were analyzed using the “Plot Z-axis Profile” function of ImageJ (NIH). Data are presented as a ratio of fluorescence intensity of Fluo-4 NW at any given time to baseline fluorescence (F/F₀).

Construction of mutated cDNA

Mutations in the cDNA of the 5-HT_{4d} receptor were introduced using the QuickChange II XL site-directed mutagenesis kit from Stratagene. All vector modifications were validated with sequencing using BigDye[®] Terminator v3.1 Cycle Sequencing and the ABI Prism[®] 3100 Genetic Analyzer (Applied Biosystems). Obtained data were analyzed with the Sequence Scanner program and LALIGN tool from ch.embnet.org.

siRNA-mediated knockdown and immunoblotting

Knockdown of the proteins of interest was performed 4 hours after SH-SY5Y cells were transfected with pEAK12-AP-APP, pcDNA3.1-5-HT_{4d} receptor and pmax-FP-Green-N plasmids. Half of the medium was replaced with transfection mix containing 3 nM target protein siRNA and Lipofectamine RNAiMAX (Invitrogen) and left on the cells overnight. Next we proceeded with the protocol as described under “inhibitor treatment and soluble APP analysis”. The following siRNAs were used: Stealth RNAi[™] siRNAs were used for GNAS HSS104240, GNAQ HSS104237, GNA13 HSS173827, PLCG1 HSS108094, CSNK2A1 HSS175396, ADAM9 HSS189548, MMP9 HSS181135 and BLOCK-iT[™] Alexa Fluor[®] Red Fluorescent Oligo as a control (Invitrogen). The siGENOME SMARTpool was used for ADAM10 and siGENOME Non-Targeting siRNA Pool #1 as a control (Dharmacon). The FlexiTube GeneSolution GS6868 SI02664501 was used for ADAM17 and AllStars Negative Control siRNA as a control (QIAGEN). Conditioned medium was collected to measure SEAP activity. For detection of G α_s , G α_q , G α_{13} , CK2, ADAM9, 10, APP and β -Actin, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Sodiumdeoxycholate, 0.1% SDS and Complete protease inhibitor tablets (Roche Applied Science)). For detection of MMP9, conditioned medium was cleared from cell debris by centrifugation at 1500 rpm and concentrated with Ultracel-50 centrifugal filter unit (Millipore) according to manufacturer’s instructions. For detection of ADAM17, cells were homogenized in 50 mM Tris-HCl pH 8.0 and 150 mM NaCl, 1 μ M batimastat

and Complete protease inhibitor tablets and centrifuged at 100,000 g for 1 hour at 4°C. Pellets were resuspended in RIPA buffer supplemented with 1 μ M batimastat and centrifuged at 100,000 g for 1 hour at 4°C and supernatants containing the membrane fraction were collected. Protein concentrations were determined in each preparation using the Bradford assay (Bio-Rad). Equal amounts of protein were separated with SDS-PAGE in Novex Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes (Whatman), blocked and probed with antibodies in 3% milk plus 0,1% Tween-20/TBS buffer. Secondary antibody staining was detected with the Renaissance chemiluminescence kit (Perkin Elmer). β -Actin staining was used as a loading control.

Statistical analysis

Differences between multiple means of data with parametric distribution were assessed by ANOVA followed by Tukey-Kramer or Dunnet's post-hoc tests. All experiments were repeated three times. All statistical analysis was performed with GraphPad Prism 5 (GraphPad Software). Optical density of specific immunobands on western blot was quantified using the 1D densitometry package of Aida Image Analyzer v4.27.039.

Results

5-HT_{4d} receptor-stimulated APP shedding requires G protein signaling and is independent of β -arrestin recruitment

In agreement with previous reports which show that activation of the 5-HT_{4d} receptor induces APP shedding in CHO cells [7], we find a significant upregulation of SEAP (secreted alkaline phosphatase) activity in SH-SY5Y human neuroblastoma cells transiently transfected with human 5-HT_{4d} (pcDNA3.1-5-HT_{4d}) and human wild type APP695 coupled to SEAP (pEAK12-AP-APP) following treatment with the 5-HT₄ receptor agonists prucalopride or 5-HT. Co-treatment with the 5-HT₄ receptor antagonist GR113808 abolishes the increase in AP-APP shedding, demonstrating the specificity of the effect (Figure 1A). Using an alphaLISA specific for sAPP α , we previously demonstrated that the increase in SEAP activity reflects an increase in sAPP α release [9].

Signaling pathway activation down-stream of the 5-HT₄ receptor is mediated through assembly and activation of a heterotrimeric G protein complex. Receptor phosphorylation by GPCR-related kinases (GRKs) limits G protein-mediated signaling and facilitates recruitment of β -arrestins, which mediate receptor internalization and turnover and provide a scaffold for the initiation of signals to several kinases [30]. We used 5-HT_{4d} receptor mutants deficient in either G protein or β -arrestin signaling to distinguish which pathway leads to increased sAPP α secretion upon receptor stimulation. We introduced mutations in the DRY conserved motif, which interrupt coupling to G proteins and prohibit the G protein complex from acquiring an active GTP bound state [31,32]. Expression of the alanine substitution DRY mutant of the 5-HT_{4d} receptor (pcDNA3.1-5-HT_{4d}DRY117/118AAY) in pEAK12-AP-APP transfected cells resulted in significant downregulation of sAPP α secretion after 5-HT treatment, indicating the putative involvement of G protein signal transduction in 5-HT_{4d} receptor-stimulated release of sAPP α (Figure 1B). Recruitment of β -arrestins to the receptor requires phosphorylation of the C terminus, allowing further internalization and possible signal transduction through the scaffolding of down-stream kinases. We generated a 5-HT_{4d} receptor mutant truncated at amino acid 346 in the C terminus (pcDNA3.1-5-HT_{4d} Δ 346), which lacks a conserved sequence of serine and

threonine residues required for association of β -arrestins with the receptor after phosphorylation by GRKs. Expression of this mutant in pEAK12-AP-APP transfected cells maintained stimulated induction of sAPP α secretion after prucalopride and 5-HT treatment (Figure 1B). These data suggest that β -arrestins do not contribute to 5-HT_{4d} receptor-induced APP shedding.

In order to confirm that G proteins participate in α -secretase induction, we co-treated pEAK12-AP-APP transfected cells with inhibitors of G α_s , i.e. CTB and NF449. Inhibition of G α_s signaling indeed resulted in significant decrease of induced sAPP α secretion (Figures 1C and D). We also tested a G α_s DN mutant, which abolishes all GPCR-mediated G protein-dependent signaling [28]. We found that co-transfection of pcDNA1-Amp-G α_s DN with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d} inhibited induced sAPP α secretion after prucalopride and 5-HT treatment (Figure 1E), indicating that G α_s activation is involved in 5-HT_{4d} receptor-stimulated α -secretase activity.

The 5-HT₄ receptors promiscuously activate several G proteins, i.e. G α_s , G α_q and G α_{13} , leading to distinct second messenger generation [33–35]. We wondered which G protein subunit is specifically responsible for the effect on sAPP α secretion. Therefore, we performed RNAi mediated knock-down of G α_s , G α_q and G α_{13} in SH-SY5Y cells and analyzed sAPP α secretion upon 5-HT_{4d} receptor stimulation (Figure S1A). Knock-down efficiency and specificity of siRNA oligonucleotides was confirmed by western blotting (Figures S1B and C). Surprisingly, our data show that single knock-down of each individual G α subunit equally abolishes 5-HT_{4d} receptor-mediated sAPP α secretion (Figure S1A), suggesting that sAPP α release can be mediated through G α_s , G α_q and G α_{13} . Such an effect could be explained if there is a requirement for the functional activation of the G $\beta\gamma$ subunits. We used gallein to inhibit G $\beta\gamma$ signaling and found that co-treatment of pEAK12-AP-APP transfected cells with this inhibitor and prucalopride or 5-HT abolished induction of sAPP α secretion (Figure 1F). Altogether, these studies suggest that G α and G $\beta\gamma$ activation is required for 5-HT_{4d} receptor-stimulated sAPP α release.

5-HT_{4d} receptor-stimulated APP shedding does not involve activation of adenylyl cyclase and cAMP

G α_s and cAMP mediate canonical signaling of 5-HT₄ receptors [33]. Therefore, we sought to determine whether, in SH-SY5Y cells, accumulation of cAMP is also necessary for α -secretase activity as previously described for CHO cells [15]. We used the adenylyl cyclase inhibitors SQ22536 and DDA, which potently inhibit increases in cAMP levels (Figure 2C). Interestingly, we found that these inhibitors do not affect prucalopride or 5-HT-stimulated sAPP α release in SH-SY5Y cells (Figures 2A and B). These results suggest that activation of adenylyl cyclase and accumulation of cAMP is not required for 5-HT_{4d} receptor-stimulated APP shedding.

5-HT_{4d} receptor-stimulated APP shedding requires Src and phospholipase C

Given that 5-HT_{4d} receptor-stimulated APP shedding does not require an elevation in cAMP levels, we sought to determine whether generation of inositol triphosphate (IP₃) is involved in 5-HT_{4d} receptor-stimulated sAPP α release. This second messenger is produced by PLC and can be activated either directly downstream of G α_q and G $\beta\gamma$ or through the Src non-receptor tyrosine kinase (reviewed in [36]). To analyze the contribution of PLC and Src, we co-treated pEAK12-AP-APP and pcDNA3.1-5-HT_{4d} transfected SH-SY5Y cells with the Src inhibitor Bosutinib or

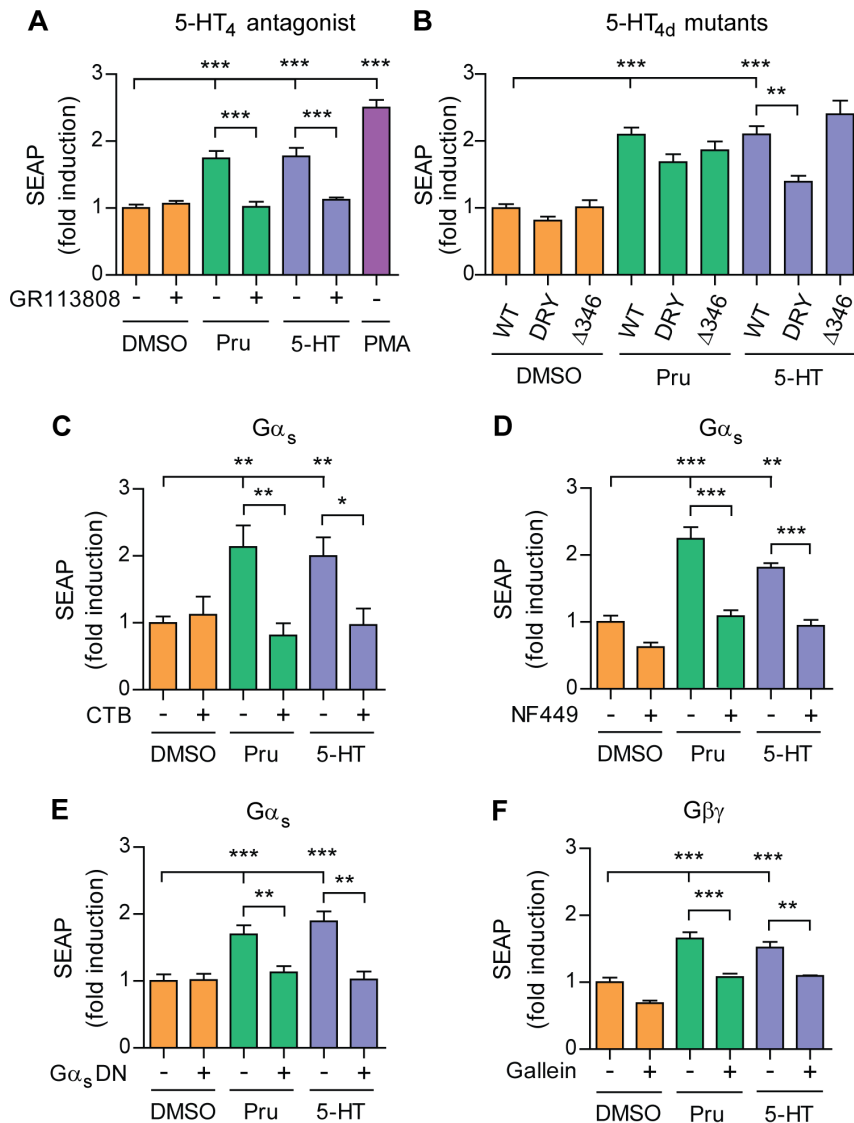


Figure 1. 5-HT_{4d} receptor-stimulated APP shedding requires G protein signaling and is independent of β -arrestin recruitment. (A) Prucalopride induced sAPP α secretion in SH-SY5Y human neuroblastoma cells is specific for the 5-HT₄ receptor. SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d}, were treated with 1 μ M prucalopride and 5-HT (5-HT₄ receptor agonists) in the absence or presence of 1 μ M GR113808 (5-HT₄ receptor antagonist) or PMA and secretion of sAPP α was analyzed via measuring SEAP. (B) SEAP levels were measured in supernatants of SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d} (WT), pcDNA3.1-5-HT_{4d}DRY117/118AA (DRY) or pcDNA3.1-5-HT_{4d} Δ 346 (Δ 346) mutants and stimulated with 1 μ M prucalopride or 5-HT. (C), (D) and (F) SEAP levels were measured in supernatants of SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d} and treated with 1 μ M prucalopride or 5-HT in the absence or presence of 100 μ M CTB ($G\alpha_s$ inhibitor) (C), 100 μ M NF449 ($G\alpha_s$ inhibitor) (D) or 100 μ M gallein ($G\beta\gamma$ inhibitor) (F). (E) SEAP levels were measured in SH-SY5Y cells, transfected with pEAK12-AP-APP, pcDNA3.1-5-HT_{4d} and pcDNA1-Amp- $G\alpha_s$ DN or pcDNA3.1 at a ratio of 2:1:4, respectively, and treated with 1 μ M prucalopride or 5-HT. Values shown are mean \pm SEM of 6 individual wells and were normalized to vehicle control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey-Kramer or Dunnett's post-hoc test. doi:10.1371/journal.pone.0087014.g001

the PLC inhibitor D609 and 5-HT₄ receptor agonists. In both cases, we observed that APP shedding was abolished compared to control treatment (Figures 3A and B).

PLC cleaves phosphatidylinositol 4,5-bisphosphate into IP₃ and diacylglycerol, resulting in mobilization of intracellular calcium and activation of several downstream effector proteins including PKC [37]. In addition, several studies suggest that calcium and PKC signaling can activate α -secretase shedding of APP [38,39]. Co-treatment of transiently transfected SH-SY5Y cells with the PKC inhibitor GF109203X did not induce sAPP α secretion after 5-HT_{4d} receptor stimulation. In contrast, direct activation of PKC with PMA induced sAPP α , but this induction was inhibited with GF109203X

showing that the inhibitor was functional (Figure 3C). Similarly, prucalopride did not significantly alter extracellular calcium influx in SH-SY5Y cells, in contrast to ionomycin and ATP; two positive controls that prove assay functionality (Figure 3D). Taken together these data suggest that Src and PLC, but not PKC or calcium signaling, contribute to 5-HT_{4d} receptor-induced APP shedding.

5-HT_{4d} receptor-stimulated APP shedding requires inositol polyphosphates and casein kinase 2

IP₃ can be further phosphorylated by inositol 1,4,5-trisphosphate 3-kinase (IP₃K) and inositol polyphosphate multikinase (IPMK) to

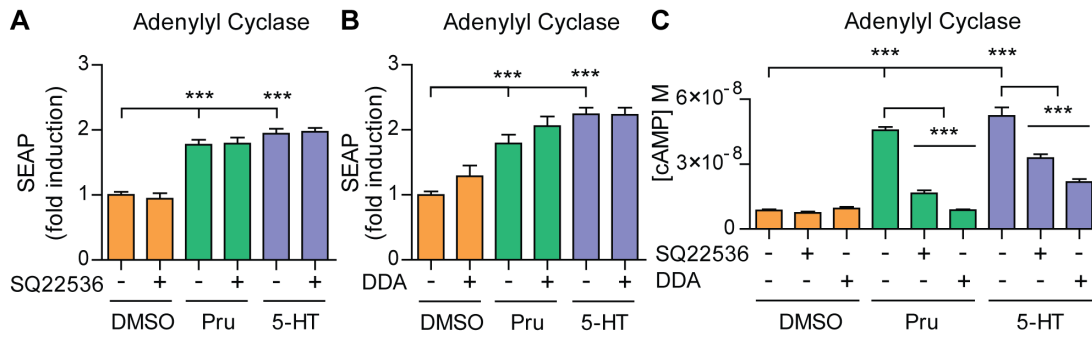


Figure 2. 5-HT_{4d} receptor-stimulated APP shedding does not require accumulation of cAMP. (A) and (B) SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d}, were treated with 1 μ M prucalopride or 5-HT (5-HT₄ receptor agonists) in the absence or presence of 4 μ M SQ22536 (AC inhibitor) (A) or 100 μ M DDA (AC inhibitor) (B) and secretion of sAPP α was analyzed via measuring SEAP. (C) Concentration of cAMP was measured in SH-SY5Y cells, transfected with pEAK12-AP-APP, pcDNA3.1-5-HT_{4d} and pmx-FP-Green-N at a ratio of 5:4:1, were treated with 1 μ M prucalopride or 5-HT in the absence or presence of 4 μ M SQ22536 or 100 μ M DDA. Values shown are mean \pm SEM of 6 individual wells and were normalized to vehicle control. *** $P < 0.001$, one-way ANOVA with Tukey-Kramer's post-hoc test. doi:10.1371/journal.pone.0087014.g002

generate inositol polyphosphates (IP₄, IP₅ and IP₆). These molecules recently emerged as versatile second messengers with an increasing number of cellular functions [40]. We tested the IP3K inhibitor and the IPMK inhibitor CGA in transfected SH-

SY5Y cells and found that prucalopride or 5-HT-stimulated α -secretase activity depends on the generation of these inositol polyphosphates (Figures 4A and B). The reported literature suggests that IP₄ and/or IP₆ can activate CK2 *in vitro* [41]. In

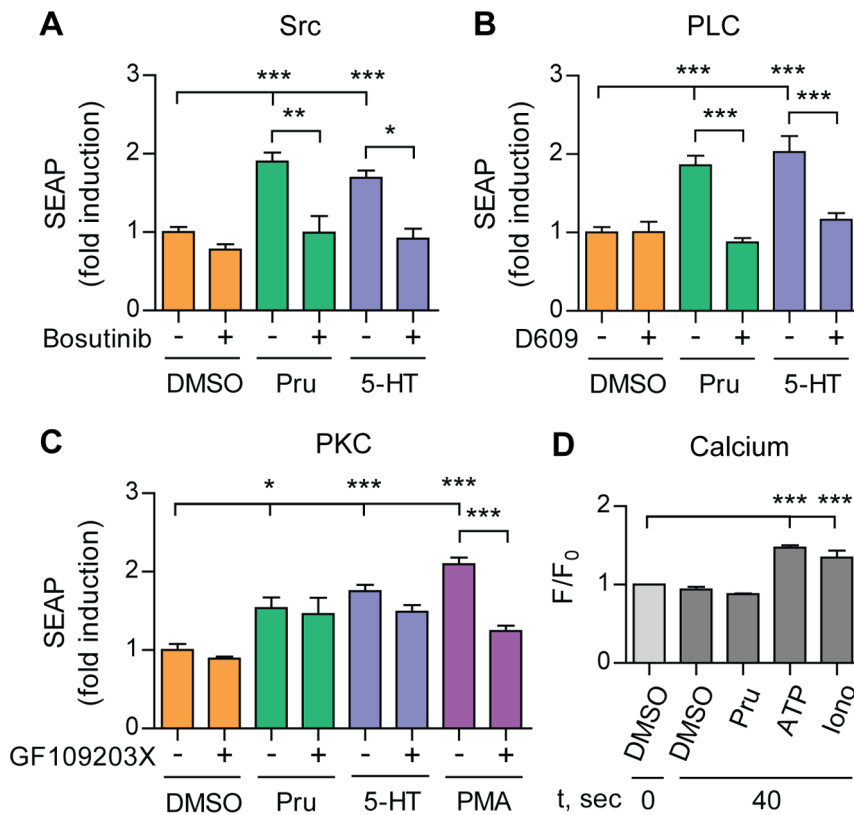


Figure 3. 5-HT_{4d} receptor-stimulated APP shedding requires Src and phospholipase C, but not PKC or calcium. (A), (B) and (C) SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d}, were treated with 1 μ M prucalopride or 5-HT (5-HT₄ receptor agonists) and PMA in the absence or presence of 50 μ M Bosutinib (Src inhibitor) (A), 30 μ M D609 (PLC inhibitor) (B) or 2 μ M GF109203X (PKC inhibitor) (C) and secretion of sAPP α was analyzed via measuring SEAP. Values shown are mean \pm SEM of 6 individual wells and were normalized towards vehicle control. (D) SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d}, were loaded with Fluo-4 NW mix and fluorescence of the calcium-sensitive dye in each well was recorded at the baseline (F₀) and after stimulation with 1 μ M prucalopride, 30 μ M ATP (purinergic ionotropic receptors agonist), 20 μ M Ionomycin (calcium ionophore) or DMSO (F). Calcium response shown is a ratio of maximum fluorescence intensity at 40 sec to baseline fluorescence (F/F₀). Values shown are mean \pm SEM of 2 individual wells and were normalized to vehicle control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey-Kramer's post-hoc test. doi:10.1371/journal.pone.0087014.g003

cells, Wnt3a can induce IP5 generation which then activates CK2 [42]. Inhibition of CK2 activity with TBB in pcDNA3.1-5-HT_{4d} receptor and pEAK12-AP-APP expressing SH-SY5Y cells stimulated with prucalopride or 5-HT led to a decrease in sAPP α down to baseline levels (Figure 4C), suggesting that CK2 is involved in 5-HT_{4d} receptor-stimulated APP shedding. In addition, we found that co-transfection of CK2 siRNA completely abolished stimulated APP shedding in SH-SY5Y cells treated with prucalopride or 5-HT (Figures 4D, E and F). These results demonstrate that 5-HT_{4d} receptor-stimulated APP shedding requires inositol polyphosphates and CK2.

ADAM9, ADAM10, ADAM17 and MMP9 are not responsible for 5-HT_{4d} receptor-stimulated α -secretase activity

Several enzymes of the ADAM and MMP family, such as ADAM9, 10, 17 and MMP9, are suggested candidate proteins responsible for inducible shedding of APP (reviewed in [43]). To determine the relative contribution of the metalloproteinases in 5-HT_{4d} receptor-stimulated sAPP α release, we first analyzed expression levels of ADAM9, 10, 17 and MMP9 in SH-SY5Y cells. Expression of ADAM9, 10, 17 and MMP9 was not changed after prucalopride treatment of pEAK12-AP-APP transfected SH-SY5Y cells (Figures S3A and B). To test whether a metalloproteinase would be responsible for induced α -secretase activity, we

treated the cells with non-toxic concentrations of the broad spectrum metalloproteinase inhibitor GM6001 (dose response curve shown in Figure S4). Treatment with GM6001 abolished induction of sAPP α secretion (Figure 5A), confirming that a metalloproteinase is indeed responsible for 5-HT_{4d} receptor-stimulated sAPP α release. To identify the 5-HT_{4d} receptor-stimulated α -secretase, we performed RNAi knock-down of the candidate α -secretases. We found that induction of sAPP α release was preserved after prucalopride treatment and single knock-down of ADAM9, 10, 17 or MMP9 (Figure 5B). The efficiency of the downregulation was between 85–95% as documented by western blot analysis (Figures 5C and D). These data suggest that ADAM9, 10, 17 or MMP9 are not responsible for 5-HT_{4d} receptor-mediated inducible α -secretase activity in SH-SY5Y cells. We also analyzed constitutive sAPP α secretion upon ADAM10 knock-down in non-treated cells and confirmed that ADAM10 acts as the constitutive α -secretase of APP in our experimental conditions (data not shown).

Metalloproteinases are notorious for their functional redundancy between family members. To test whether more than one candidate metalloproteinase could be responsible for induction of α -secretase activity, we treated transfected SH-SY5Y cells with combinations of RNAi directed at ADAM9 and 10, ADAM9 and 17, ADAM10 and 17 (Figure 6A). We observed no change in sAPP α secretion upon 5-HT_{4d} receptor stimulation under any of these conditions. Moreover, knock-down of all four candidate

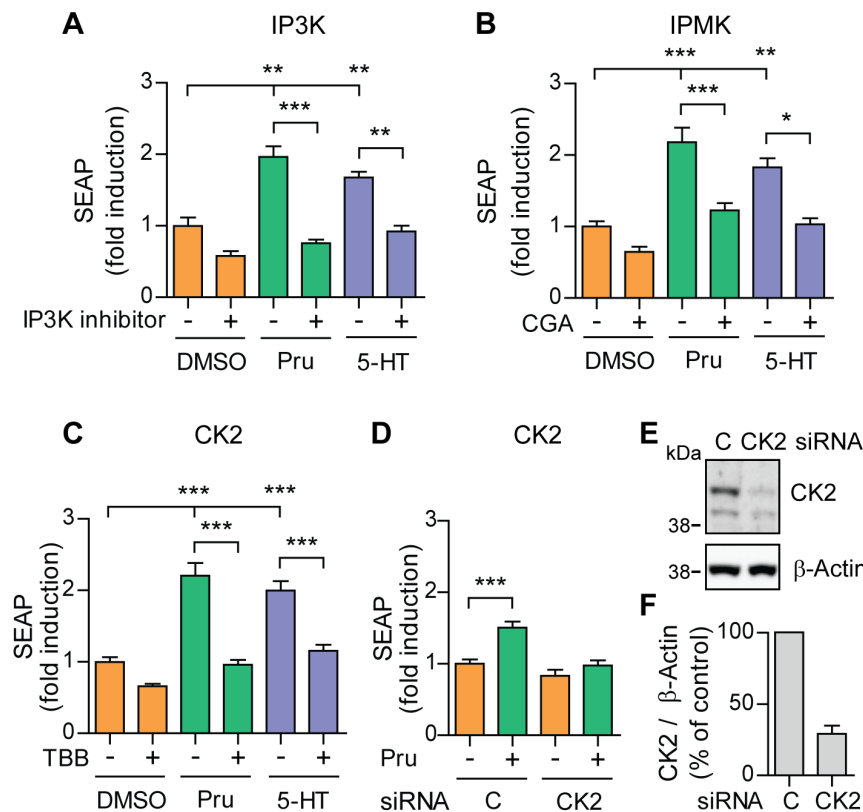


Figure 4. 5-HT_{4d} receptor-stimulated APP shedding requires inositol polyphosphates and casein kinase 2. (A), (B) and (C) SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d}, were treated with 1 μ M prucalopride or 5-HT (5-HT₄ receptor agonists) in the absence or presence of 20 μ M IP3K inhibitor (A), 80 μ M CGA (IPMK inhibitor) (B) or 2.5 μ M TBB (CK2 inhibitor) (C) and secretion of sAPP α was analyzed via measuring SEAP. (D) SEAP levels were measured in supernatants of SH-SY5Y cells, co-transfected with pEAK12-AP-APP, pcDNA3.1-5-HT_{4d} and 3 nM siRNA for knock-down of CK2 and treated with 1 μ M prucalopride. (E) Cell lysates of (D) were analyzed for CK2 expression levels by western blotting. (F) Quantification of experiments in (E). Values shown are mean \pm SEM of 6 individual wells and were normalized to vehicle control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey-Kramer's post-hoc test. doi:10.1371/journal.pone.0087014.g004

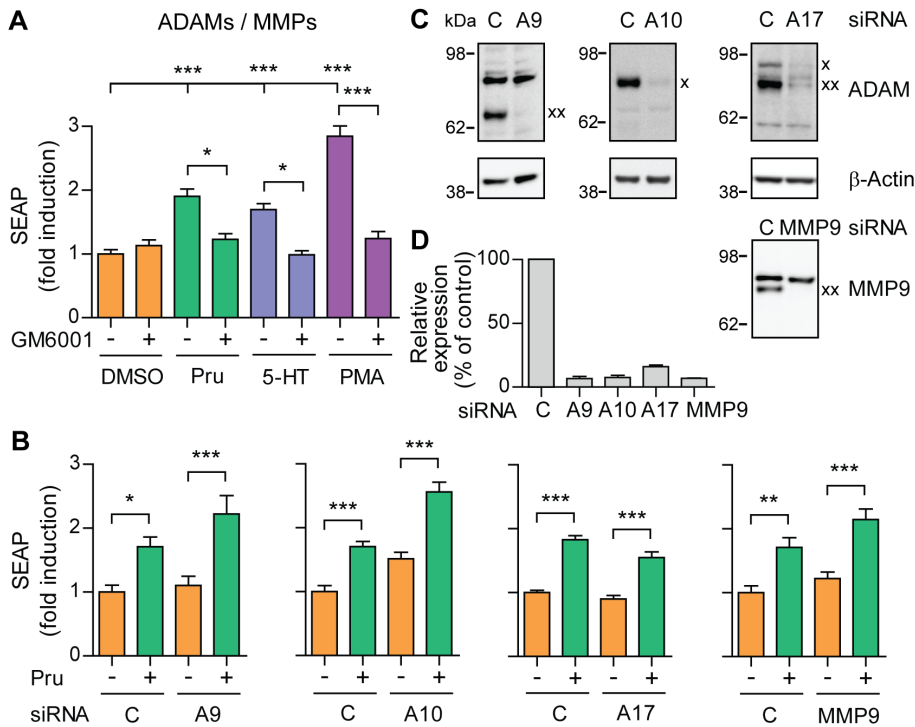


Figure 5. Single knock-down of ADAM9, ADAM10, ADAM17 and MMP9 does not affect 5-HT_{4d} receptor-stimulated APP shedding. (A) SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d}, were treated with 1 μ M prucalopride or 5-HT (5-HT₄ receptor agonists) in the absence or presence of 80 μ M GM6001 (metalloproteinases inhibitor) and secretion of sAPP α was analyzed via measuring SEAP. (B) SEAP levels were measured in supernatants of SH-SY5Y cells, co-transfected with pEAK12-AP-APP, pcDNA3.1-5-HT_{4d} and 3 nM siRNA for knock-down of ADAM9 (A9), ADAM10 (A10), ADAM17 (A17) and MMP9 and treated with 1 μ M prucalopride. (C) Cell lysates of (B) were analyzed for protein expression of ADAM9, 10, 17 and MMP9 by western blotting. The ADAM10 and ADAM17 immature precursor proteins are indicated by an x, whereas the mature catalytically active forms are indicated by an xx for ADAM9, 17 and MMP9. The immature ADAM9 and the mature ADAM10 proteins were not visible. (D) Quantification of experiments in (C). Values shown are mean \pm SEM of 6 individual wells and were normalized to vehicle control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey-Kramer's post-hoc test. doi:10.1371/journal.pone.0087014.g005

metalloproteinases, i.e. ADAM9, 10, 17 and MMP9, still resulted in induction of sAPP α release after 5-HT_{4d} receptor activation (Figure 6B). The levels of C-terminal fragments generated by the cleavage of APP at β - and β' -sites remained unchanged after the knock-down of ADAM9, 10, 17 and MMP9, suggesting that β -secretase activity was not affected by reduced expression levels of these metalloproteinases (Figures S5A and B). We used western blotting to confirm the efficiency and specificity of RNAi mediated downregulation (Figures 6C and D). Notice also the strong upregulation of MMP9 expression when ADAM9, 10 and 17 are downregulated, while single MMP9 knock-down did not affect 5-HT_{4d} receptor-induced sAPP α secretion. Altogether, our data suggest that an unidentified GM6001-sensitive metalloproteinase participates in the regulated cleavage of APP upon 5-HT_{4d} receptor stimulation (Figure 6B).

Discussion

In this report, we examined the signaling pathway that leads to α -secretase induction after 5-HT_{4d} receptor stimulation in the human neuroblastoma SH-SY5Y cell line. We present here a previously uncharacterized signaling pathway involved in the mediation of 5-HT_{4d} receptor-induced α -secretase activity (Figure 7). The characterization of this pathway was based on a combination of pharmacological, siRNA and site-directed mutagenesis experiments. Our data indicate that PLC is essential for α -secretase activation following 5-HT_{4d} stimulation. This effect is

dependent on G α and G $\beta\gamma$ recruitment and signaling downstream of the 5-HT_{4d} receptor. Src tyrosine kinase acts as an intermediate molecule, mediating PLC activation and inositol triphosphate production. The latter is converted by multiple kinases to inositol polyphosphates, which activate CK2. Downstream of CK2, a yet unknown mechanism of α -secretase activation is triggered. The 5-HT_{4d} receptor-induced α -secretase activity could not be ascribed to any known candidate α -secretase (ADAM9, 10, 17 and MMP9) in the SH-SY5Y cells, which has hampered delineation of the final step regulating 5-HT_{4d} receptor-stimulated sAPP α release.

We found also that β -arrestin signaling did not contribute to α -secretase activity upon 5-HT_{4d} receptor stimulation as the mutant receptor deficient in β -arrestin recruitment maintained the ability to stimulate sAPP α secretion after agonist treatment (Figure 1B). Interestingly, β -arrestins have recently emerged as regulators of A β generation downstream of the β 2-adrenergic receptor and GPR3, independently of G protein activation [44,45]. In these studies, β -arrestins appear to bind to the Aph1 subunit of the γ -secretase complex, affecting complex localization and thereby increasing the catalytic activity of the γ -secretase complex. Our work suggests that different signaling pathways regulate α - and γ -secretase activity as we find that G proteins are indispensable for 5-HT_{4d} receptor-stimulated α -secretase activity, while they are not involved in the γ -secretase regulation by GPCRs. Indeed, several molecules that are activated downstream of G proteins are proposed to regulate α -site APP processing, e.g. PKC, PKA, MAPK, ERK and PI3K (reviewed in [46]).

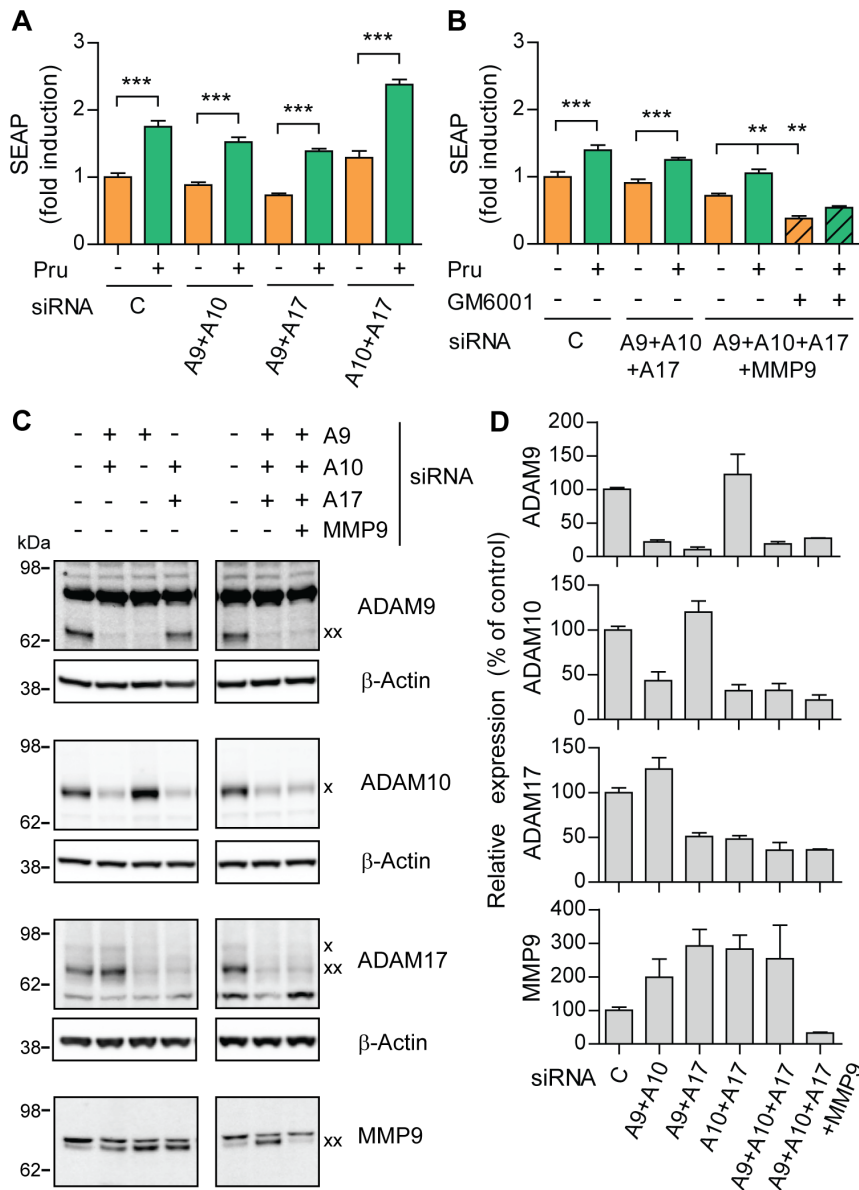


Figure 6. Combined knock-down of ADAM9, ADAM10, ADAM17 and MMP9 does not affect 5-HT_{4d} receptor-stimulated APP shedding. (A) and (B) SH-SY5Y cells, co-transfected with pEAK12-AP-APP, pcDNA3.1-5-HT₄ and combinations of 3 nM siRNA for knock-down of ADAM9 (A9) and ADAM10 (A10), ADAM9 (A9) and ADAM17 (A17), ADAM10 (A10) and ADAM17 (A17) in (A) or ADAM9, 10 and 17 or ADAM9, 10, 17 and MMP9 in (B), were treated with 1 μ M prucalopride (5-HT₄ receptor agonist) in the absence or presence of 80 μ M GM6001 (metalloproteinases inhibitor) (B) and secretion of sAPP α was analyzed via measuring SEAP. (C) Cell lysates of experiments in (A) and (B) were analyzed for protein expression of ADAM9, 10, 17 and MMP9 by western blotting. ADAM10 and ADAM17 immature precursor proteins are indicated by an x, whereas the mature catalytically active forms are indicated by an xx for ADAM9, 17 and MMP9. The immature ADAM9 and the mature ADAM10 proteins were not visible. (D) Quantification of experiments in (C). Values shown are mean \pm SEM of 6 individual wells and were normalized to vehicle control. ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey-Kramer's post-hoc test. doi:10.1371/journal.pone.0087014.g006

Originally the 5-HT₄ receptor was shown to couple to G α_s [17,33–35,47]. We show here that the G α_s , G α_q and G α_{13} subunits are equally required for sustainable α -secretase activity after 5-HT_{4d} receptor stimulation (Figure S1A). We did not observe functional compensation between the different G α subunits, even though protein expression was modulated in a reciprocal manner for G α_s and G α_q (Figure S2). These results were unexpected and we speculate that parallel signaling initiated by the different G α subunits or a certain threshold level of G proteins would be required to transduce the agonist-dependent signal. In

those views reduction of the level of one of subunit could already abolish the signal. Our results also show that the signal relies on the association with the G $\beta\gamma$ subunits as a converging signal transduction mediator (Figure 1F). Similar observations have been previously made for PLC activation by G proteins *in vitro*. IP₃ production was more efficient in the presence of the G protein trimeric complex than in separate preparations of either G α_q or G $\beta\gamma$ alone [48,49]. Interestingly, we also observe a reduction in constitutive sAPP α release after the knock-down of G α_s , G α_q and G α_{13} (Figure S1A). These data suggest a dominant negative effect

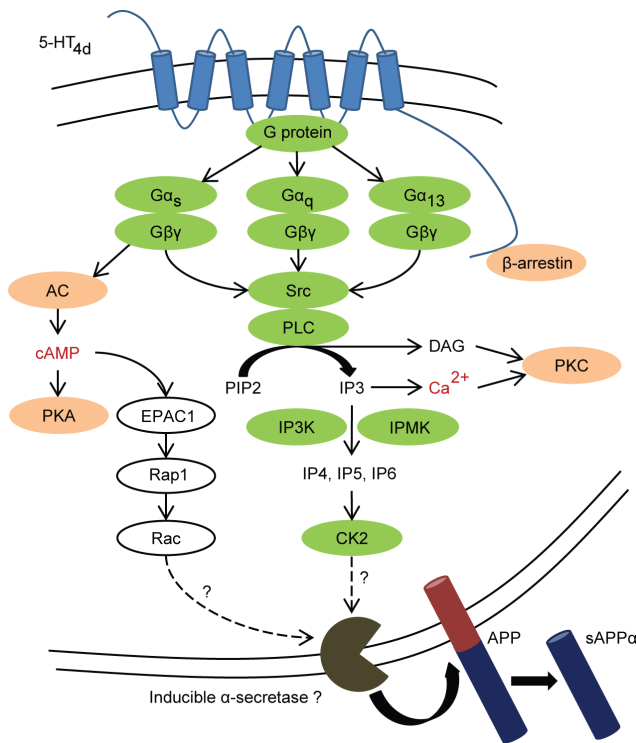


Figure 7. Schematic representation of the proposed 5-HT_{4d} receptor-stimulated signaling pathway leading to increased sAPP α production. The proteins involved in 5-HT_{4d} receptor-mediated non-amyloidogenic APP shedding are shown with green circles while orange circles and red characters indicate proteins or second messengers that were tested but were ineffective in modulating 5-HT_{4d} receptor-stimulated sAPP α release. The dotted lines with the question marks indicate remaining areas of investigation for further elucidation of the molecular mechanism of α -secretase activation. cAMP-dependent pathway of α -secretase induction was previously reported and is depicted as a plausible way for 5-HT_{4d} receptor-mediated sAPP α release [15].
doi:10.1371/journal.pone.0087014.g007

of G proteins inhibition on constitutive α -secretase activity, which may be mediated by additional GPCRs besides the 5-HT_{4d} receptor.

5-HT₄ receptor coupling to different G proteins suggests several possibilities for downstream signal transduction. Several reports describe a PKA-independent and cAMP-dependent α -secretase activation following 5-HT_{4d} receptor stimulation [7,50,51]. In CHO cells, sAPP α release is regulated by Epac1, which promotes small GTPases Rap1 dependent Rac activation [15]. However, we find that AC and cAMP accumulation are not required for 5-HT_{4d} receptor-induced APP shedding under our experimental conditions (Figure 2). Differences in the cellular systems, a human neuronal cell line versus a Chinese hamster ovary cell line, could explain the discrepancy between the studies. We then found that IP₃ generation through Src and PLC activation contributes to 5-HT_{4d} receptor-induced α -secretase activity (Figure 3). PLC is also an important component of the α -secretase activation pathway through G α_q coupled GPCRs, e.g. mGluR1 and mGluR5 [52], M1 and M3 [53], 5-HT_{2a} and 5-HT_{2c} [54] and thus a point of convergence for several transduction pathways activating α -secretase.

Investigations of the cerebral cortex and cerebellum of AD-affected individuals reveal disturbed G protein signal transduction compared to control patients [55]. In accordance, the phospho-

inositide hydrolysis pathway is also altered in AD because of reduced levels of phosphatidylinositol 3-kinase and disturbed agonist and G protein regulation of PLC [56,57]. It is proposed that 5-HT₄ receptor stimulation could counteract such detrimental changes. We show here that the 5-HT_{4d} receptor indeed induces IP₃K and IPMK mediated IP₃ conversion to inositol polyphosphates and that these contribute to the non-amyloidogenic pathway of APP processing (Figure 4). This effect is mediated through the activation of CK2, which was recently identified to be downstream of the cholinergic receptors in a pathway of α -secretase induction [58]. As activation of the 5-HT₄ receptor can increase acetylcholine levels in the brain [6,13] and we need 24 hours to obtain a significant induction of the α -secretase, an indirect mechanism through upregulation of acetylcholine could play a role. As our cells are of the dopaminergic origin, we think this possibility is rather unlikely. However, we cannot rule out that other indirect mechanisms are playing a role in the 5-HT_{4d} receptor-mediated α -secretase induction.

To understand the molecular mechanism of α -secretase activation downstream of the 5-HT_{4d} receptor, we investigated the contribution of ADAM9, 10, 17 and MMP9 in the regulation of APP processing. Previously, regulated α -secretase activity was partially attributed to MMP9, whose expression levels increased after 5-HT_{4d} receptor stimulation in APP-overexpressing H4 human neuroglioma cells [59]. However, in our cellular system, expression levels of the investigated proteinases do not change (Figure S3) and specific protein downregulation suggests that a different metalloproteinase, besides the major candidate α -secretases ADAM9, 10, 17 and MMP9, contributes to 5-HT_{4d} receptor-induced sAPP α release (Figures 5 and 6). Indeed, ADAM10 was not responsible for the 5-HT_{4d} receptor-dependent induction of sAPP α release through the cAMP/Epac pathway [21]. At this moment, we cannot rule out that the remaining protein expression of these four major α -secretases contribute to the preserved inducible α -secretase activity. Our data are consistent with the present view of different proteases contributing to regulated APP processing as previously reported for the M1 receptor, the insulin-like growth factor-1 receptor and the purinergic P2Y2 and P2X7 receptors [23,60–62]. To identify the metalloproteinase(s) responsible for induced α -secretase activity we were reluctant to use differences in susceptibility to GM6001 because we were working with overexpression conditions. This would require a large-scale RNAi knock-down study but is beyond the scope of the current manuscript.

In conclusion, our studies show the complexity of α -secretase regulation upon 5-HT_{4d} receptor stimulation. Taking into consideration that receptor modulation of signaling pathways depends on the cellular context and that recombinant overexpression and RNA interference may reveal cell type specific results, a relevant physiological system should be used for the confirmation of the identified signaling pathway. Clinical trials of agonists targeting 5-HT₄ serotonergic and M1 muscarinic receptors will provide validation of α -secretase activation as a therapeutic approach for the treatment of AD. We report here that PLC dependent production of IP₃ and CK2 activation are important mediators of the 5-HT_{4d} receptor signaling that enhance the non-amyloidogenic processing of APP. These proteins can also participate in signaling downstream of muscarinic receptors, suggesting the possibility of a common pathway for α -secretase activation through GPCRs. Finally, our data will also aid with the development of 5-HT₄ receptor agonists as therapeutics for neurodegenerative or psychiatric disorders and allow for a better understanding of potential risks associated with these drugs.

Supporting Information

Figure S1 5-HT_{4d} receptor-stimulated APP shedding requires the G proteins G α_s , G α_q and G α_{13} . (A) SEAP levels were measured in supernatants of SH-SY5Y cells, co-transfected with pEAK12-AP-APP, pcDNA3.1-5-HT_{4d} and 3 nM siRNA for knock-down of G α_s , G α_q and G α_{13} and treated with 1 μ M prucalopride (5-HT₄ receptor agonist). (B) Cell lysates of (A) were analyzed for protein expression of G α_s , G α_q and G α_{13} by western blotting. (C) Quantification of experiments in (B). Values shown are mean \pm SEM of 6 individual wells and were normalized to vehicle control. ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey-Kramer's post-hoc test. (TIF)

Figure S2 Knock-down of G α_s and G α_q but not G α_{13} alters protein expression of the G protein family. (A) SH-SY5Y cells, transfected with 3 nM siRNA for knock-down of G α_s , G α_q and G α_{13} , were harvested and expression levels of G proteins were analyzed. (B) Quantification of experiments in (A). Values shown are mean \pm SEM of 2 individual wells and were normalized to vehicle control. ** $P < 0.01$, one-way ANOVA with Tukey-Kramer's post-hoc test. (TIF)

Figure S3 Expression levels of major candidate α -secretases and APP do not change upon 5-HT_{4d} receptor stimulation. (A) SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d}, were treated with 1 μ M prucalopride (5-HT₄ receptor agonist) and collected to analyze protein expression of ADAM9, 10, 17, MMP9 and APP by western blotting. ADAM10 and ADAM17 immature precursor proteins are indicated by an x, whereas the mature catalytically active forms are indicated by an xx for ADAM9, 17 and MMP9. The immature ADAM9 and the mature ADAM10 proteins were not visible. (B) Quantification of experiments in (A). Values shown are mean \pm SEM of 2 individual wells and were normalized to vehicle control. (TIF)

Figure S4 The metalloproteinase inhibitor GM6001 can inhibit secretion of sAPP α upon 5-HT_{4d} receptor

stimulation. SH-SY5Y cells, transfected with pEAK12-AP-APP and pcDNA3.1-5-HT_{4d}, were treated with 1 μ M prucalopride or 5-HT in the absence or presence of different concentrations of GM6001 and secretion of sAPP α was analyzed via measuring SEAP. Values shown are mean \pm SEM of 6 individual wells and are normalized towards vehicle control. ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey-Kramer's post-hoc test. (TIF)

Figure S5 Knock-down of ADAM9, 10, 17 and MMP9 does not affect the pattern of CTFs generated by the 5-HT_{4d} receptor-stimulated α -secretase activity. (A) SH-SY5Y cells, co-transfected with pEAK12-AP-APP, pcDNA3.1-5-HT_{4d} and combinations of 3 nM siRNA for knock-down of ADAM9 (A9), ADAM10 (A10) and ADAM17 (A17) or ADAM9, 10, 17 and MMP9, were treated with 1 μ M prucalopride (5-HT₄ receptor agonist) and secretion of sAPP α was analyzed via measuring SEAP. Values shown are mean \pm SEM of 6 individual wells and were normalized to vehicle control. *** $P < 0.001$, one-way ANOVA with Tukey-Kramer's post-hoc test. (B) Cell lysates of the experiment in (A) were analyzed for the levels of different APP C-terminal fragments (CTFs) were analyzed by western blotting using B63 antibody (16% Tricine SDS-PAGE). (TIF)

Table S1
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: AAP AT BDS IT. Performed the experiments: AAP. Analyzed the data: AAP AT BDS IT. Contributed reagents/materials/analysis tools: AAP AT BDS IT. Wrote the paper: AAP AT BDS IT.

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