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Amyloid Precursor Protein Regulation by G Protein-Coupled Receptor 12

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Abstract

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Alzheimer's disease (AD) causes irreversible neurodegeneration and does not have a cure. Its pathology is characterized in part by the production of amyloid-β (Aβ) through deleterious processing of amyloid precursor protein (APP). G protein-coupled receptors (GPCRs) make up the largest and most successful family of drug targets and have been shown to modulate APP processing in AD pathology. G protein-coupled receptor 12 (GPR12) has become a receptor of interest because it has recently been implicated in neurological disease, and it shares >60% homology with G protein-coupled receptor 3 (GPR3), which has been shown to interact with APP and increase Aβ production. Given its significant homology to GPR3, GPR12, which has not been studied before in the context of AD, may regulate APP processing and prove to be a potential drug target for AD treatment. In this project, we sought to explore a potential role for GPR12 in APP processing, with the specific aim of elucidating the mechanisms through which GPR12 may be regulating APP expression. GPR12 was co-expressed with APP in HEK-293T cells, and protein levels of APP were examined via Western blot to determine the effect of GPR12 on overall APP expression levels. Co-immunoprecipitation experiments were also conducted to examine protein-protein interaction between APP and wild-type or truncated versions of GPR12. Lastly, luciferase assays were conducted to examine the effects that co-expression of GPR12 and APP have on GPR12 signaling. We found GPR12 to be a novel regulator of APP levels, as co-expression with APP in HEK-293T cells resulted in a dramatic increase in total APP levels. Furthermore, we also identified GPR12 as a novel APP binding partner, and preliminary data from signaling assays revealed that APP can reciprocally affect GPR12 function and signaling. Taken together, these results suggest that GPR12 plays a significant role in AD pathogenesis and could thus serve as a potential drug target for novel AD therapeutics.

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Table of Contents

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder and the most common form of dementia (1, 2). It is prevalent in almost a third of the population >65 years of age. Most cases of AD (>95%) are sporadic and not inherited in a familial fashion. AD neuropathology is defined by the accumulation of extracellular amyloid-beta (Aβ) peptides in plaques and intraneuronal neurofibrillary tangles composed of the tau protein (3). However, some cases of AD (<1%) are familial, early-onset (mean age of ~45 years) and caused by autosomal dominant mutations in the genes encoding amyloid precursor protein (APP) and presenilins 1 and 2 (3, 4). The clinical and pathophysiological characteristics of the familial and non-familial forms of AD are virtually indistinguishable, making Aβ-based research relevant in treating both forms of AD.

The deposition of Aβ that is associated with an imbalance between its production and clearance (5) leads to the formation of soluble Aβ oligomeric aggregates, which have been shown by various studies to be the main toxic species and cause of AD pathology rather than insoluble Aβ plaques (6-8). Aβ oligomers more than Aβ plaques impair normal synaptic function and long-term potentiation (1), resulting in progressive neurodegeneration, cognitive decline, and eventually death. Almost all clinical trials based on AD drug development programs have failed, so there is a dire need for novel drug targets in AD (9). A logical approach towards identifying novel modulators of Aβ accumulation and oligomerization is to search for potential interactors with APP and regulators of APP processing.

APP is a ubiquitously expressed type I single-pass transmembrane protein with a large extracellular domain and a short intracellular domain (10, 11). The proteolytic cleavage of APP by a complex family of enzymes (α-, β-, and γ-secretases) is crucial to precluding or promoting the production of Aβ in the brain (3). APP processing falls under two pathways: non-

amyloidogenic and amyloidogenic (12). In the non-amyloidogenic pathway, APP is initially cleaved by α-secretase to form the N-terminal soluble fragment of APP (sAPPα) and the C-terminal fragment 89 (C89; α-CTF), the latter of which is subsequently cleaved by γ-secretase to form a p3 peptide. Conversely, in the amyloidogenic pathway, APP is cleaved by β-secretase, resulting in the formation of a different N-terminal soluble fragment (sAPPβ) and C-terminal fragment 99 (C99; β-CTF), the latter of which is subsequently cleaved by γ-secretase to form Aβ (11). CTF type is therefore a useful biomarker for the way in which APP is processed and was examined in this study.

There are various isoforms of APP expressed in the body as a result of alternative splicing, but the most prevalent isoform expressed in the brain is APP 695 (4, 10, 13). In addition, the most well-known and commonly studied gene mutant of APP (APP-swe) is the Swedish mutation (K595N/M596L); it is preferentially cleaved by β-secretase resulting in increased Aβ levels (14-16). APP-swe is therefore useful for studies seeking to measure levels of Aβ production *in vitro*, which at baseline for wild-type APP (APP-WT) may be hard to detect. Thus, the APP 695 isoform containing the Swedish mutation was used in all experiments of this study.

G protein-coupled receptors (GPCRs) make up the largest class of drug targets and have played an important role in drug discovery (17). GPCRs and their endogenous ligands have been linked to numerous diseases including Alzheimer's disease. Specifically, GPCRs are involved in the different stages of APP processing through the regulation of α-, β-, and γ-secretases, and therefore have a significant role in Aβ generation (18). Under the non-amyloidogenic pathway, GPCRs such as muscarinic, metabotropic, and serotonergic receptors regulate α-secretase-mediated proteolysis of APP. On the other hand, the amyloidogenic pathway implicates GPCRs

such as the δ-opioid and adenosine A2A receptors, which modulate β-secretase-mediated proteolysis of APP, and β2 adrenergic and G protein-coupled receptor 3, which modulate γ-secretase-mediated proteolysis (18). Orphan GPCRs are GPCRs for which endogenous ligands are unknown (19). Therefore, understanding their physiological and pathological roles is difficult but crucial in opening up new fields of potential drug targets.

G protein-coupled receptors 3, 6, and 12 (GPR3, GPR6, and GPR12) are members of a small family of orphan GPCRs that share greater than 60% homology and have been implicated in various neuropathological conditions (20). These three orphan GPCRs are constitutively active through the G_s signaling pathway and increase cyclic AMP (cAMP) production in neurons (21). GPR12 has become a receptor of interest for our lab because genetic studies have implicated GPR12 in epilepsy development (22). There have been no reports on the function of GPR12 in the context of AD but, given that the related GPR3 has been shown to form a protein complex with APP (23) and stimulate Aβ plaque formation through γ-secretase modulation (24, 25), we wondered if GPR12 might also regulate APP processing in some way. The localization of GPR12 expression to brain regions such as the cerebral cortex and hippocampus (21, 26) also suggests the receptor may be associated with the cortical atrophy and cognitive decline characteristic of AD in humans (27).

In the present study, we demonstrated that GPR12 does in fact alter APP expression *in vitro*. In further studies, we propose to pinpoint the mechanisms through which GPR12 acts on APP. Our preliminary work has shown that GPR12 can form a protein complex with APP, so we propose to study whether this interaction directly affects APP conformation, leading to preferential cleavage by α- or β-secretases. To explore this possibility, one of our goals in this project was to map the site of interaction between GPR12 and APP. This was done through the

creation of truncated constructs of GPR12 and examination for protein complex formation between these GPR12 mutants and APP. We hypothesized that GPR12 and APP might interact with each other via their N-termini because the N-termini are the largest accessible regions on both proteins. We also hypothesized that GPR12 may regulate APP expression through signaling mechanisms such as the G_s signaling pathway, especially because GPR12 is a constitutively active GPCR. Thus, we explored this possibility as well using specific inhibitors of downstream GPR12 effectors. Our broad goal has been to understand how GPR12 might regulate APP and determine if GPR12 promotes amyloidogenic or non-amyloidogenic APP processing.

Methods

Cell culture

HEK-293T/17 cells were acquired from ATCC (Manassas, VA) and maintained in complete media, which consists of DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humid, 5% CO2, 37 °C incubator. 1 mL of Trypsin-EDTA in Hank's Balanced Salt Solution (Sigma-Aldrich) and 9 mL of complete media were added to a confluent 10 cm dish prior to trituration. Cells were plated 3:10 in 10 cm cell culture plates (VWR) the day before transfection. The day of transfection, cells were at 40-50% confluency and old media was replaced with fresh DMEM. Transfection solutions consisting of 1.5 mL incomplete media (DMEM without FBS and pen/strep), 6 μg total of DNA, and 10 μL of Mirus TransITLT1 (Madison, WI) were vortexed and incubated at room temperature for 15 minutes prior to transfection. Transfection solutions were then added dropwise to the cell culture dishes. At 48 hrs post-transfection, cells were placed on ice and washed with PBS + Ca^{2+} before being harvested.

Western Blotting

Cells were collected in 500 μL of 2x Laemmli Buffer (Bio-Rad) with the addition of 10% HALT Protease and Phosphatase Inhibitor (Thermo Scientific). Cell lysates were sonicated, loaded into 4-20% Tris-Glycine gels (Bio-Rad) for SDS-PAGE electrophoresis, and transferred to nitrocellulose membranes (Bio-Rad). Ponceau Red staining was used to check gel loading and transfer accuracy. Blots were then blocked with 2% milk (in 50mM NaCl, 10mM HEPES, pH 7.3 with 0.1% Tween-20 (Sigma)) for 30 min and incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used (1/1000): anti-amyloid precursor protein antibody Y188 (abcam) anti-amyloid beta N 82E1 (IBL America), anti-HA C29F4 (Cell Signaling Technology), and anti-Actin HRP (Santa Cruz Biotechnology). Blots were washed with milk prior to incubation with horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. The following secondary antibodies were used (1/5000): ECL Anti-Rabbit IgG and ECL Anti-Mouse IgG HRP-linked whole Ab (GE Life Sciences). Blots were then washed with PBS containing 0.1% Tween (PBST) and developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific).

Coimmunoprecipitation

Cells were collected in 500 μL of lysis buffer (1% Triton X-100, 25 mM HEPES, 150 nM NaCl, 1 mM EDTA, and HALT protease inhibitor mix) and lysed by rotating end-over-end for 90 min at 4 °C. Cell lysates were centrifuged and supernatant was collected and incubated with 20 μL of anti-HA agarose beads (Thermo Scientific) by rotating end-over-end for 45 min at 4 °C. After 10

washes with lysis buffer, beads were resuspended in 50 μL of 2X Laemmli buffer to elute protein from beads. Western blots were performed after 24 hrs.

Administration of Compound E

Compound E was used to inhibit γ-secretase and allow for detection of CTF proteins for Western blot analysis. At 24 hrs post-transfection, cells were serum-starved and treated with 100 nM of Compound E (Cayman Chemical) in incomplete media. Duplicate plates were incubated with DMSO to serve as control. Cells were harvested 48 hrs post-transfection following normal Western blot procedure.

cAMP Responsive Element Luciferase Assay

Complete media (75 uL) was loaded into each well of a 96-well plate (VWR). 1 mL of Trypsin-EDTA and 14 mL of complete media were added to a confluent 10 cm plate of HEK-293T/17 prior to trituration. Reconstituted cell solution (50 uL) was then added to each well. At 24 hrs after plating, cells were transfected with appropriate DNA constructs and transfection reagents from the Promega Dual-Glo Luciferase Assay kit. First, a master mix of Luciferase plasmid (50 ng/well), Renilla plasmid (1 ng/well), Mirus TransIT-LT1 (5 uL/well), and incomplete media (50 uL/well) according to the number of wells required. Triplicates were performed for each condition. Next, 50 uL of the master mix was added to DNA constructs (50 ng/well) and transfection solutions incubated at room temperature for 15 minutes. A total of 80 uL of media was removed from each well before 50 uL of transfection solution was added to each well. At 48 hrs post-transfection, 75 uL of firefly substrate was added to each well and plate was incubated for 10 minutes at room temperature prior to plate reading. Renilla reagent was prepped during

the incubation time by diluting the Renilla substrate 1:100 in Stop and Glo buffer. Renilla reagent (75 uL) was pipetted into each well and after another 10-minute incubation period, plate was read. Firefly:Renilla ratio was calculated and data was normalized to control wells.

Statistical Analysis

Western blots were quantified using densitometry performed with ImageJ software. Data were analyzed using GraphPad Prism software by one-way analysis of variance (ANOVA) followed by post hoc analysis using Dunnett's multiple comparison test unless stated otherwise in figure caption. Significance threshold of $p < 0.05$ was used in all analyses. Number of samples (n) for each experimental condition are indicated in figure captions.

Results

Co-expression of GPR12 with APP dramatically increases APP Expression

To determine if GPR12 has an effect on APP expression, *in vitro* overexpression experiments were conducted. Varying concentrations of wild-type HA-tagged GPR12 (HA-GPR12) DNA construct were co-expressed with a constant amount of APP in HEK-293T cells. Western blot analysis and densitometry revealed that APP expression increased about 100-fold when co-expressed with HA-GPR12 (Fig. 1). This robust increase in APP expression with the presence of HA-GPR12 has been consistently observed in subsequent Western blots.

GPR12 induces APP dimerization

APP dimerization as a result of the co-expression of HA-GPR12 with APP was also consistently observed in Western blot analyses. Longer exposures of the blots revealed an APP

band at 200 kDa (Fig. 2A). Since a full-length APP monomer runs at around 100 kDa, the additional band may represent a dimer of APP. Dimerization of APP may be relevant to AD pathology because it affects how APP is trafficked in the cell (28). The localization of APP to different cellular compartments determines its fate in processing because APP is preferentially cleaved by α-secretases at the plasma membrane (29) and by β-secretases in the endosomes (30). Consequently, non-amyloidogenic and amyloidogenic processing of APP occur at the plasma membrane and endosomes respectively.

To rule out the possibility that APP dimerization was simply a result of overexpressing APP *in vitro,* cells were transfected with the largest amount possible of APP DNA construct (6 μg) without exceeding the threshold for cellular toxicity, but APP dimerization was not observed (Fig. 2B). Conversely, the co-expression of HA-GPR12 with only a third of the amount of APP transfected alone led to APP dimerization. Therefore, the formation of APP dimers does not occur due to the sheer amount of APP expressed *in vitro,* but rather as a result of GPR12-mediated regulation.

GPR12 exerts its effects on APP through a different mechanism than GPR3

C83 and C99 are products of γ-secretase cleavage of APP in non-amyloidogenic or amyloidogenic pathways respectively (31). Therefore, their relative amounts can indicate prevalence of either pathway in the cell. Initially, CTFs were not detectable on Western blots when immunoblotting with a non-specific APP antibody. To better visualize the CTFs in order to assess the effect of GPR12 on CTF production, the overexpression experiments from Figure 1 were replicated using HEK-293T cells treated with compound E, a γ-secretase inhibitor. Compound E prevents cleavage of CTFs by γ-secretase and increases total expression levels of

CTFs to allow visualization in Western blot analysis (30). CTFs around 13 kDa became visible after compound E treatment (Fig. 3B). C83 has a molecular weight of ~9 kDa and C99 has a molecular weight of ~11 kDa. However, CTF identity could not be determined without an anti-amyloid beta antibody. These experiments provided valuable insight into the mechanism of APP regulation by HA-GPR12 because even in the presence of a γ-secretase inhibitor, HA-GPR12 still exhibited the same ability to increase APP expression levels by ~50-100-fold as seen in the initial overexpression experiments.

GPR12 alters the ratio of full-length APP to APP CTF production

To harness the potential of CTF identity as a biomarker for whether GPR12 might promote amyloidogenic or non-amyloidogenic processing of APP, Western blots from the compound E experiments were immunoblotted with an anti-amyloid beta antibody that reacts with C99 but not full-length APP after treatment with γ-secretase inhibitor (Fig. 4A). Western blot analyses revealed a significant increase in C99 levels when GPR12 was co-expressed with APP, as compared to the condition with APP transfected alone (Fig. 4B). This result was consistent with the increase in total expression of full-length APP. However, doubling and quadrupling the amount of HA-GPR12 transfected with APP caused a decrease in C99 production opposite to the increasing trend of total full-length APP production (Fig. 3B). Therefore, the ratio of full-length APP to APP CTF increased as the amount of HA-GPR12 increased. These results suggest that HA-GPR12 may play a protective role by decreasing amyloidogenic processing of APP and β-secretase cleavage of APP.

APP co-immunoprecipitated specifically with GPR12 and its truncated versions

To determine if HA-GPR12 regulation of APP might involve direct protein-protein interaction, co-immunoprecipitation experiments were conducted. These studies revealed APP to be co-immunoprecipitated specifically with HA-GPR12 (Fig. 5A-B). To begin mapping the region of protein interaction between HA-GPR12 and APP, HA-tagged GPR12-ΔNT (HA-GPR12-ΔNT) and HA-tagged GPR12-TM5 (HA-GPR12-TM5) constructs were designed and co-expressed with APP before co-immunoprecipitation. The HA-GPR12-ΔNT construct is a truncated version of GPR12 that is missing the entire N-terminus, the region we hypothesized to bind to APP. The HA-GPR12-TM5 construct is HA-GPR12 without its C-terminus and last two transmembrane domains. This truncation removes the receptor's third cytoplasmic loop, which is crucial for G protein coupling and second-messenger-mediated signal transduction for many GPCRs, specifically the rhodopsin class of GPCRs in which GPR12 belongs (32-34). Surprisingly, APP was found to still be co-immunoprecipitated with both truncated forms of HA-GPR12. Although some nonspecific binding was observed in the Western blot for the co-immunoprecipitation of APP with HA-GPR12-ΔNT, HA-GPR12-ΔNT pulled down APP to the same extent as HA-GPR12-WT (Fig. 6A). APP also co-immunoprecipitated with GPR12-TM5 although to a lesser extent than GPR12-WT (Fig. 6B). Taken together, complex formation between GPR12 and APP is neither dependent on the N-terminus nor the C-terminus plus the last two transmembrane domains.

N- and C-termini plus last two transmembrane domains are necessary for GPR12 regulation of APP

To examine the effect that GPR12 mutations may have on APP regulation, HA-GPR12-WT, HA-GPR12-ΔNT, and HA-GPR12-TM5 were co-expressed with APP (Fig. 7A). Co-

transfection with the mutant forms of HA-GPR12 did not result in increased full-length APP expression (Fig. 7B). In addition, the mutant forms of HA-GPR12 did not induce APP dimerization, revealing that the mutant forms of GPR12 were deficient relative to wild-type GPR12 in terms of their ability to regulate APP levels.

Other G_s- coupled GPCRs enhance APP expression, although not to the same extent as GPR12

To determine if the effect on APP expression was specific to GPR12 or instead the result of general G_s-mediated signaling activity, APP was co-expressed with other G_s signaling receptors such as HA-tagged beta-1 adrenergic receptor (HA-β_1AR) and HA-tagged dopamine-1 receptor (HA-D_1R) (Fig. 8A). HA-β_1AR and HA-D_1R were found to increase full-length APP expression level by about 5-fold, a significant effect but not to the same extent and level of significance as HA-GPR12 (Fig. 8B). The observed increase in APP expression cannot therefore be attributed specifically to GPR12, but nonetheless the magnitude of increased APP expression was greater with GPR12 than with the other G_s-coupled receptors.

APP alters GPR12 signaling activity

All experiments described to this point explored the effect of GPR12 on APP, but we also wanted to explore the effect that APP may have on GPR12 signaling (Fig. 9A). Thus, dual-luciferase reporter assays were conducted to compare HA-GPR12 signaling activity in the presence and absence of APP. The signaling activity of HA-GPR12-TM5 with and without APP co-expression was also measured. HA-GPR12-TM5 served as a negative control because without a 3rd cytoplasmic loop, we hypothesized that this GPR12 mutant would have decreased G_s

signaling capability. Interestingly, co-expression of APP with HA-GPR12 significantly increased HA-GPR12 signaling activity (Fig. 9B). As expected, HA-GPR12-TM5 exhibited little signaling activity, although co-expression with APP did induce a modest and statistically significant increase in HA-GPR12-TM5 signaling.

Discussion

In this study, we identified GPR12 as a novel regulator of APP expression. No studies have previously shown GPR12 to be associated with AD, but our data demonstrate that this receptor has the ability to dramatically increase APP expression levels up to 100-fold *in vitro*. This is significant because abnormally high levels of APP in the 5xFAD mouse model of AD are correlated with high burden and accelerated Aβ formation (35). The results we have observed in our overexpression experiments are almost certainly due to the transfection of HEK293T cells with GPR12 and APP, because HEK293T cells express no endogenous levels of GPR12 (36) and very low endogenous levels of APP. Additionally, Western blot analysis revealed no change in the low levels of endogenous APP when GPR12 was transfected alone (Fig. 1-3). Thus, our data support our hypothesis that GPR12 regulates APP based on its significant homology to GPR3.

Despite the fact that both GPR12 and GPR3 regulate APP, the two receptors regulate APP expression in very different ways. Previous studies showed that while GPR3 increases Aβ production via modulation of γ-secretase, it does not appreciably alter levels of full-length APP (23). In contrast to GPR3, we found that GPR12 exerts a dramatic effect on full-length APP, and moreover regulates APP through a completely different mechanism than GPR3 because it continued to cause a robust increase in APP expression levels even in the presence of a γ-secretase inhibitor. This suggests that, unlike GPR3, GPR12 does not modulate APP expression via γ-secretase.

We observed that C99 production decreased with increasing amounts of GPR12, which should not be a result of increased γ-secretase activity through the amyloidogenic pathway because γ-secretase was inhibited. Thus, GPR12 may serve as a mediator of non-amyloidogenic

processing, not only because C99 is the cleavage product of β-secretase, but also because membrane bound C99 forms homodimers, and this homodimerization has been linked to Aβ production (37-39). Another study has demonstrated that intraneuronal accumulation of C99 is directly linked to endosomal-autophagic-lysosomal dysfunction, which could in turn disrupt the proper degradation of Aβ (40). It is possible that the decrease in C99 levels is a result of GPR12 binding to APP or altering its conformation in a way that blocks the β-secretase cleavage site. There is evidence suggesting that some GPCRs such as the δ-opioid receptor (DOR) modulate β-secretase function by forming a complex with β-secretase and γ-secretase and translocating them to the late endosomes and lysosomes for amyloidogenic processing (41). GPR12 could similarly form a complex with β-secretase in a way that prevents proteolysis of APP by β-secretase. On the other hand, GPR12 could be increasing α-secretase activity and the release of sAPPα, which has neuroprotective effects and prevents Aβ generation (42, 43). Several GPCRs such as muscarininc acetylcholine receptors (mAChRs), metabotropic glutamate receptors (mGluR), and certain 5-hydroxytryptamine receptors (5-HT) stimulate sAPPα secretion *in vitro* and *in vivo*, suggesting increased non-amyloidogenic processing by α-secretase (44-46). In our compound E experiments blotting with a general APP antibody, we observed an increase in non-specific CTF expression (Fig. 3B), suggesting that while C99 production decreased, C83 production may have increased. In future studies, it would be useful to study protein-protein interactions between GPR12 and α- and β-secretases as well as the effects that α- and β-secretase inhibitors have on GPR12 regulation of APP expression.

One of the most striking results from the current study was the effect that GPR12 had on full-length APP dimerization. Our experiments repeatedly revealed the presence of APP dimers when GPR12 was co-expressed with APP. Previous work has shown that APP dimerization may

result in internalization of APP into endosomes and increased amyloidogenic processing (28). APP dimerization increases localization of APP to the endoplasmic reticulum and early and late endosomes (30) and alters the conformation of APP to enable more efficient cleavage by β-secretases (47). Therefore, APP dimerization may alter APP processing in favor of Aβ formation. Our discovery that GPR12 causes APP dimerization contradicts the hypothesis that GPR12 facilitates non-amyloidogenic processing over amyloidogenic processing because most of the literature on APP processing suggests that APP dimerization promotes Aβ production (48, 49). However, the effect that APP dimerization has on Aβ production is still inconclusive, with some groups stating that dimerization of APP lacking its intracellular region is linked to increased non-amyloidogenic processing (38). Given that APP can dimerize in a variety of ways with itself and its protein fragments, further studies are necessary to characterize the type of APP dimerization and physiological outcome that GPR12 is inducing.

Our study also identified GPR12 as a novel binding partner of APP. Although we hypothesized that the N-terminus of GPR12 interacts with APP, our co-immunoprecipitation studies have shown that the binding interaction between GPR12 and APP relies on neither the N-terminus nor C-terminus plus sixth and seventh transmembrane domains of GPR12. In future studies, we will continue to map the interaction between GPR12 and APP by conducting co-immunoprecipitation experiments with constructs for each transmembrane domain of GPR12. While the truncated versions of GPR12 we have tested so far have co-immunoprecipitated with APP, they did not have any effect on APP expression levels in overexpression experiments and did not cause APP dimerization like GPR12-WT. This suggests that although the N- or C-termini plus last two transmembrane domains are not required for protein-protein interaction between APP and GPR12, they may be important domains for stabilizing APP dimer conformation if

GPR12-APP complexes are somehow bringing together APP monomers at the cell surface to facilitate APP dimerization. These findings also suggest that downstream signaling mechanisms are necessary for APP dimerization since APP expression, and not complex formation, is affected. If GPR12 promotes APP dimerization at the cell surface, there would be more support for its implication in amyloidogenic processing of APP because APP dimerization decreases localization at the cell surface, promotes APP endocytosis, and results in significantly increased levels of APP in endosomes (28), which facilitates the generation of Aβ (50). To fully examine how GPR12 may be affecting APP trafficking, we propose to conduct surface-biotinylation assays to quantify the ratio of APP localized to the cell surface to APP that is internalized due to the co-expression of GPR12 with APP *in vitro*.

Our overexpression experiments comparing GPR12 to other G_s signaling GPCRs when co-expressed with APP reveal that the regulation of APP may not be specific to GPR12, but rather G_s signaling. However, GPR12 still exerts a larger effect on APP expression than the other G_s signaling GPCRs examined in this study: β_1AR and D_1R. One caveat of this result is the fact that GPR12 is a constitutively active receptor whereas β_1AR and D_1R are not constitutively active. To account for this limitation, the same experimental set-up with the addition of the known ligands of β_1AR and D_1R to their respective experimental conditions may bring the signaling activity of β_1AR and D_1R up to that of GPR12 so that their effect on APP expression can then be compared in a more even-handed manner.

In addition to demonstrating a significant effect of GPR12 on APP expression, we also found that APP reciprocally exerts effects on GPR12 signaling. Preliminary data from a luciferase signaling assay demonstrated that co-expression with APP significantly increased GPR12-mediated G_s signaling activity. We propose that APP may be doing so by (a) increasing

GPR12 localization to the cell surface or by (b) stabilizing or altering GPR12 conformation in a manner that enhances G protein coupling and downstream signaling (Fig. 10). It has been shown that APP can compete for β-arrestin 2 binding of α_{2A}-adrenergic receptor (α_{2A}AR) and as a result, prevent internalization of the GPCR (51). In addition, G protein-coupled signaling is mediated by the membrane-tethered APP intracellular domain (AICD), which can interact with $G\alpha_s$ to elicit greater cAMP dependent signaling in neurons (52). It is possible that APP alters GPR12 signaling through a similar mechanism. Additional luciferase signaling assays can be conducted to determine if there may be differences in G_s signaling activity across different G_s signaling GPCRs when they are co-expressed with APP to determine if other effects of regulation may be specific to GPR12.

One of the limitations of our study is that all of the data we have presented have been taken from *in vitro* experiments performed on HEK-293T cells. To better examine the regulation of APP by GPR12 in their natural environment, SH-SY5Y cells could be used since they are morphologically similar to primary neurons (53). However, HEK-293T cells allowed us to control for endogenous levels of GPR12 and APP, which would be high in primary neurons. *In vivo* experiments should be conducted by measuring levels of GPR12 from brain lysates of 5xFAD mice or post-mortem AD patients and comparing them to GPR12 levels from brain lysates of age-matched WT mice and post-mortem healthy individuals respectively. Eventually, levels of APP and APP dimerization in GPR12 knock-out mice should be compared to those of WT mice. These *in vivo* studies would address the shortcomings of our *in vitro* studies, which may not be expressing true physiological levels of APP in the normal or AD brain. Finally, to definitively identify GPR12 as a non-amyloidogenic or amyloidogenic species in APP

processing, immunoassays should be conducted using an Aβ-42 enzyme-linked immunosorbent assay (ELISA) kit to determine if GPR12 actually alters Aβ production.

Our study has shown for the first time that GPR12 regulates APP expression levels and physically interacts with APP. We have provided evidence for the involvement of GPR12 in both non-amyloidogenic and amyloidogenic processing. Although there is not yet definitive evidence for the implication of GPR12 in one pathway over the other, our findings that GPR12 increases total expression levels by 100-fold and the ability of GPR12 to consistently cause APP dimerization give us reason to believe that GPR12 is a regulator of amyloidogenic processing. These results are fascinating since the highly homologous GPR3 has already been implicated as a regulator of Aβ production, although via a completely different mechanism from GPR12. Our discovery of GPR12 as a novel regulator of APP processing may result in the establishment of GPR12 as a promising drug target for the future treatment of AD.

Tables and Figures

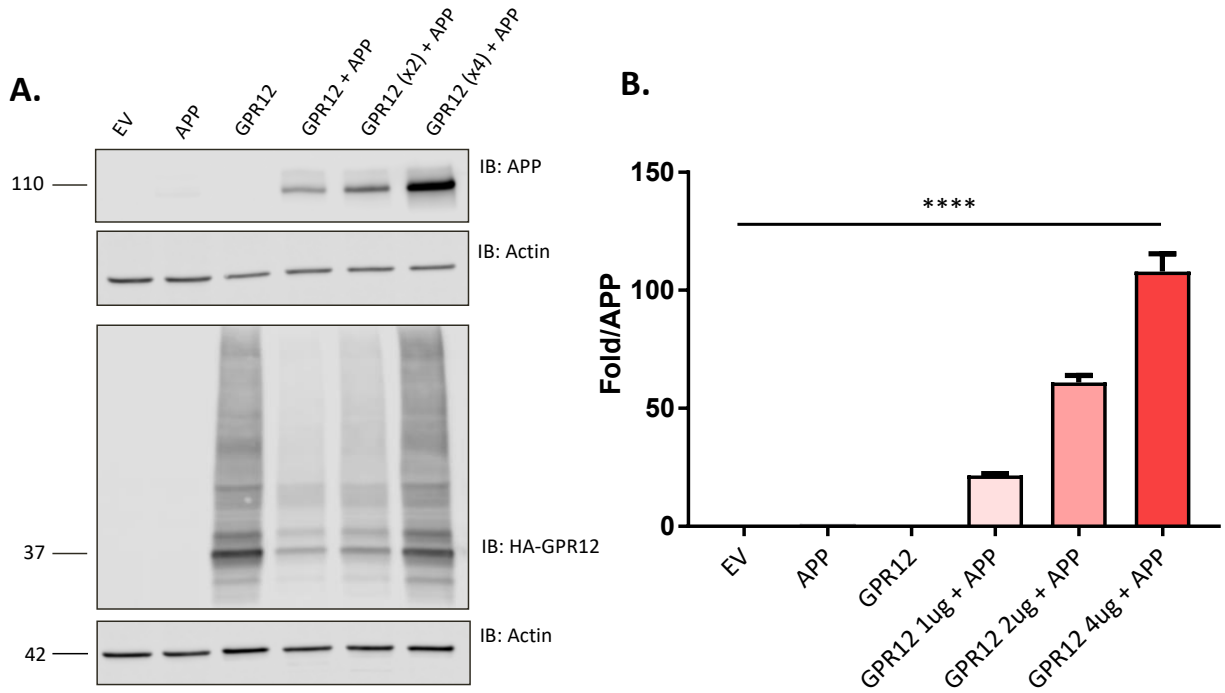

Figure 1. Co-expression of GPR12 with APP causes a robust increase in full-length APP expression. HEK293T cells were transfected with a total of 6 μg of DNA comprised of either Empty Vector, APP, GPR12, or a combination of GPR12 and APP plasmids. Significant effects of increasing the concentration of GPR12 when co-transfected with APP were observed. Densitometric quantification **(A)** of Western blot analyses **(B)** revealed a 100-fold increase in APP expression when APP was co-transfected with 4 μg of GPR12 construct. Data are normalized to actin and expressed as fold over APP, mean ± SEM (n = 3). One-way ANOVA test, ****$p \leq 0.0001$.

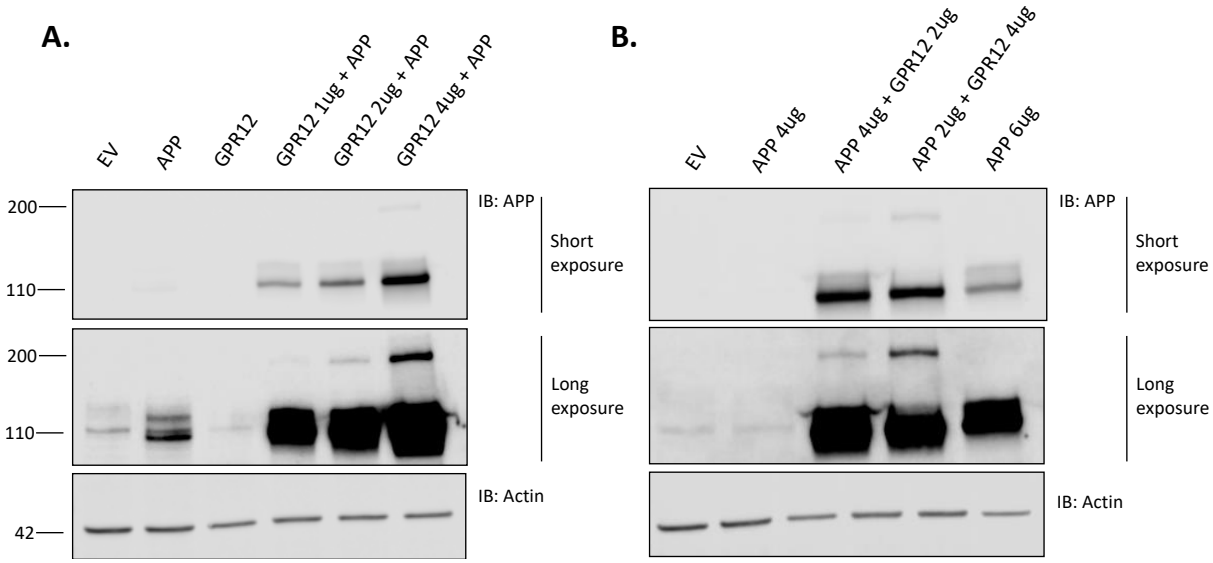

Figure 2. Co-expression of GPR12 with APP leads to APP dimerization. (A) The same Western blot in **Fig. 1** is shown at a longer exposure image. With longer exposure, additional APP bands appear at around 200 kDa exhibiting the same increasing trend in density as the full-length monomer at 110 kDa. The bands at 200 kDa suggest the occurrence of APP dimerization since the molecular weight is approximately double that of the monomer. **(B)** The long exposure image shows that no dimerization occurs when cells are transfected with 6 μg of APP alone, whereas the condition with only 2 μg of APP co-expressed with GPR12 shows dimerization.

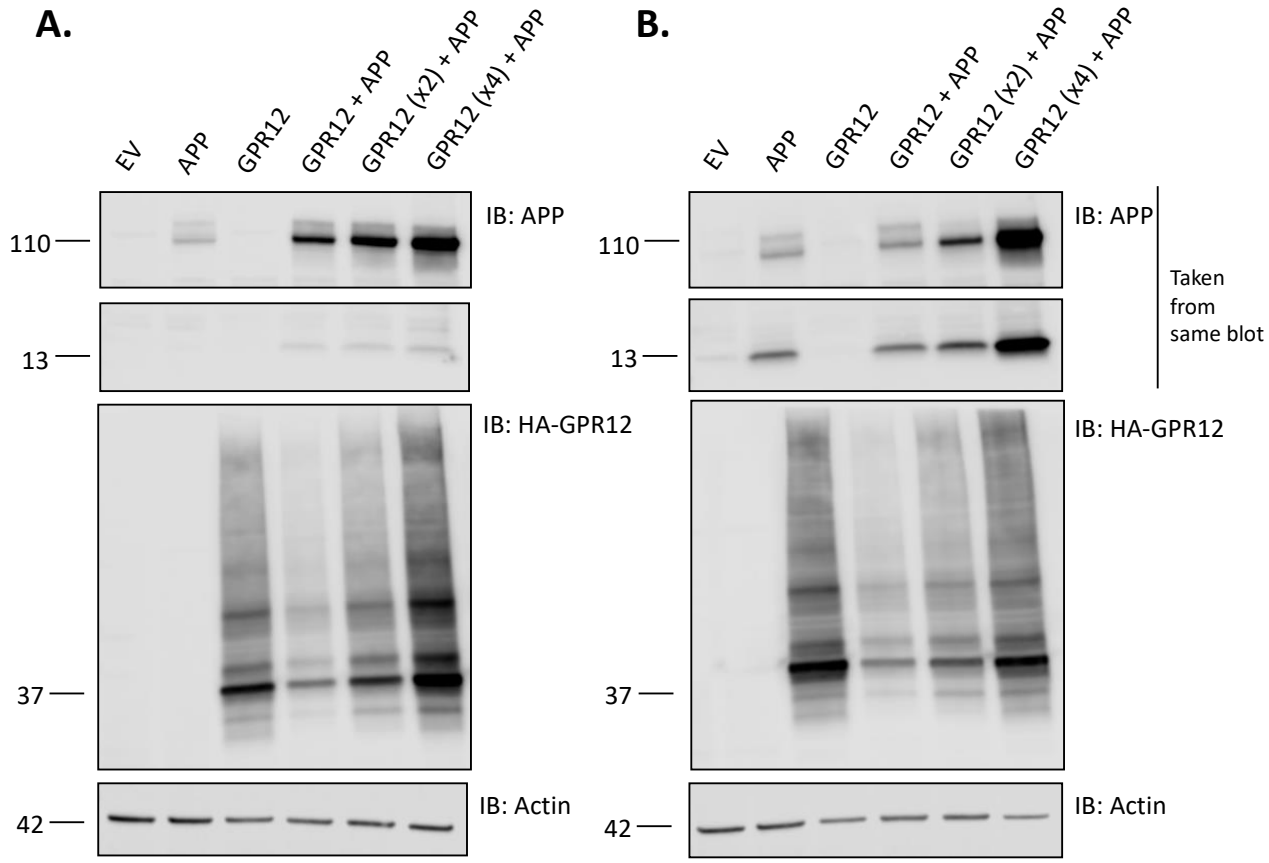

Figure 3. Administration of Compound E successfully enhances expression of CTFs. HEK293T cells were incubated in DMSO for control **(A)** and compound E for γ-secretase inhibition **(B)**. Inhibition of γ-secretase enhances expression of CTFs at ~13 kDa. Full-length APP increases in expression when co-expressed with GPR12 even in the presence of Compound E ($n = 4$).

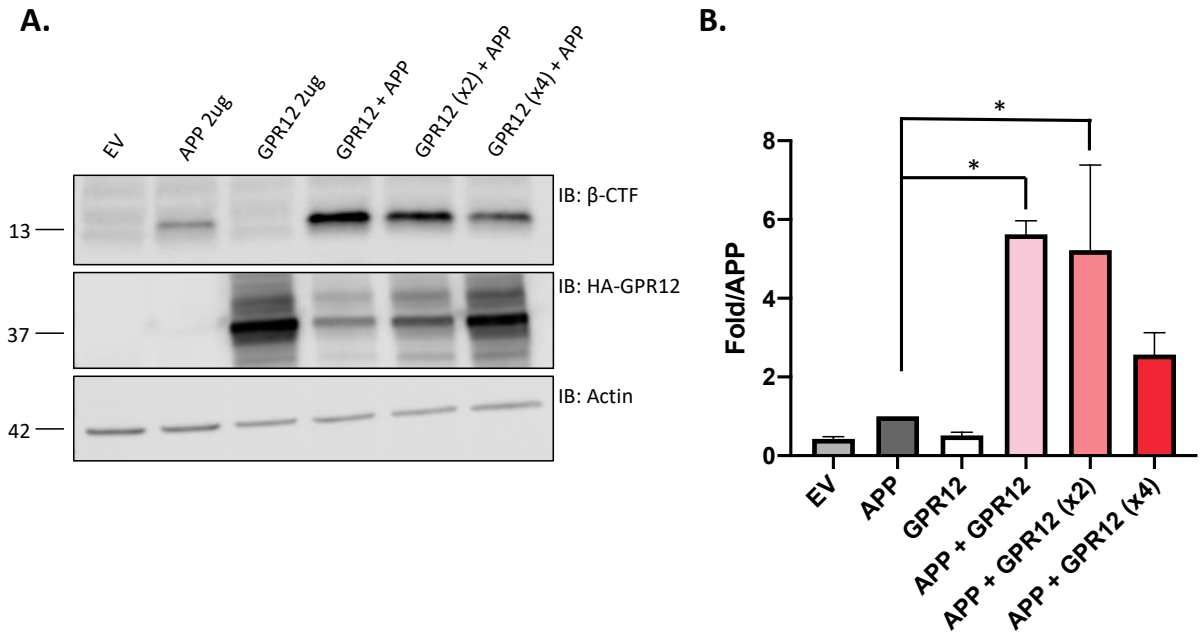

Figure 4. GPR12 alters the ratio of full-length APP to APP CTF production. (**A)** The same samples from the experiment in Fig. 3 were re-run and blotted for the β-secretase derived fragment, β-CTF (C99) with anti-amyloid beta N 82E1 antibody. 82E1 reacts with β-CTF but not full-length APP after treatment with γ-secretase inhibitor. **(B)** Co-expression of GPR12 with APP increases β-CTF production. However, while increasing the amount of GPR12 transfected leads to increased full-length APP expression, it causes decreased β-CTF expression. Data are normalized to actin and expressed as fold over APP, mean ± SEM (n = 3). One-way ANOVA (post hoc: Dunnett's multiple comparison test), *$p \leq 0.05$.

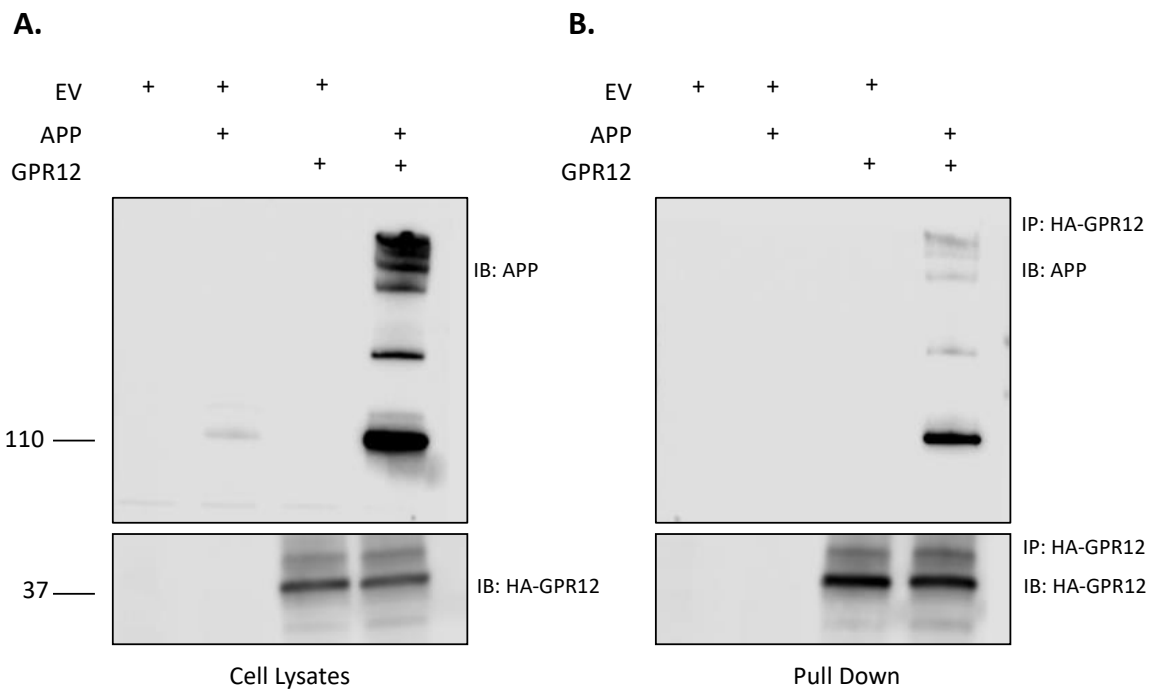

Figure 5. GPR12 forms a protein complex with APP. HEK293T cells were co-transfected with 4 μg of GPR12 and 2 μg of APP for this co-immunoprecipitation assay. **(A)** Western blot of soluble lysate. **(B)** Anti-HA agarose beads were used to pull down HA-tagged GPR12 protein. The presence of full-length APP in Western blot analysis indicates that GPR12 directly interacts with APP and forms a protein complex with it. (n = 4)

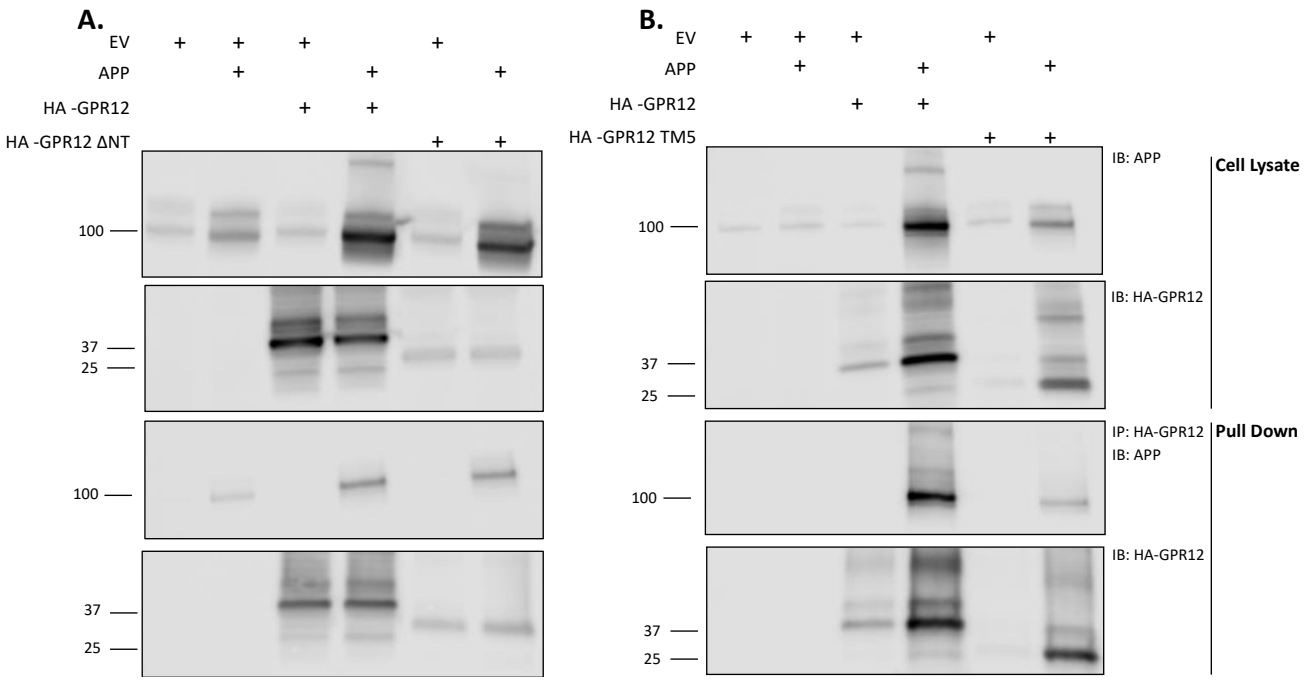

Figure 6. Complex formation between GPR12 and APP is not dependent on the N-terminus or the C-terminus plus the last two transmembrane domains of GPR12. (A) APP co-immunoprecipitated with the GPR12-ΔNT construct. However, there is a lot of non-specific binding of APP to the agarose beads (n = 4). **(B)** APP co-immunoprecipitated specifically with the GPR23-TM5 construct (n = 3).

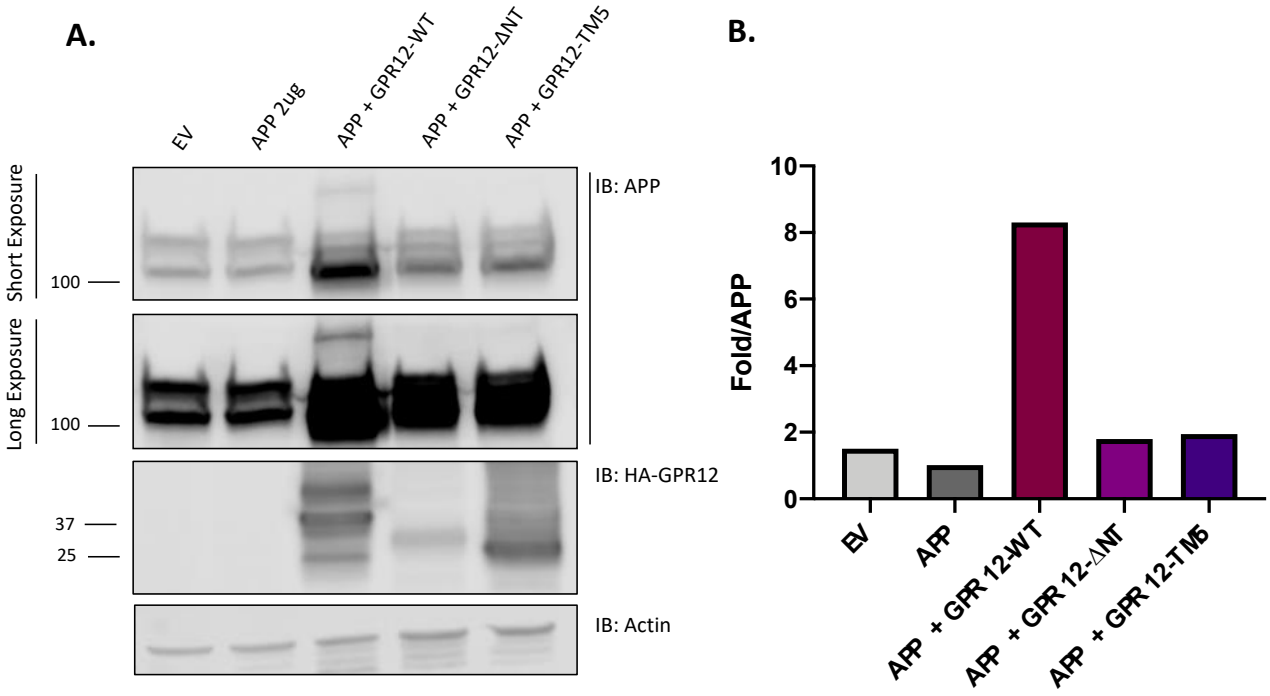

Figure 7. Truncated versions of GPR12 do not alter APP expression significantly nor do they cause APP dimerization. (A) APP co-expressed with wild-type (WT), ΔNT, and TM5 constructs of GPR12 in HEK-293T cells. **(B)** Western blot analysis indicates that only WT GPR12 has the ability to increase APP expression and induce APP dimerization. Data are normalized to actin and expressed as fold over APP (n = 1).

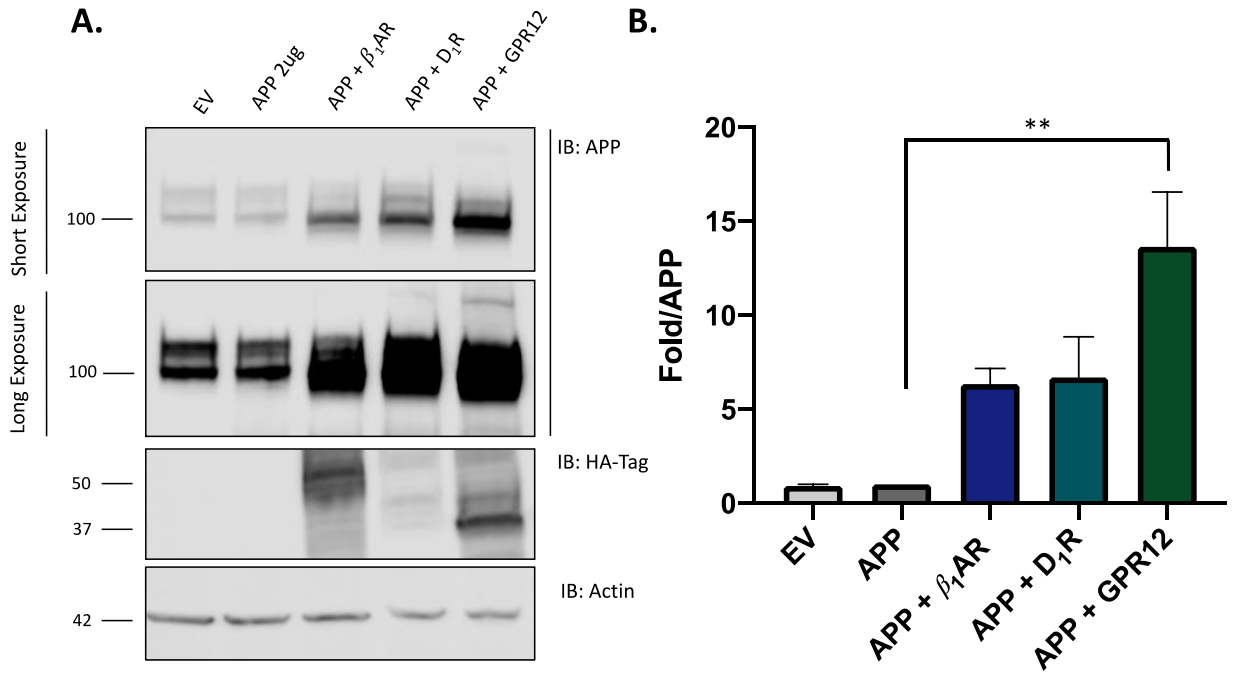

Figure 8. Other G_s signaling GPCRs do not alter APP expression to the same extent as GPR12. (A) APP co-expressed with beta-1 adrenergic receptor (β_1AR) and dopamine-1 receptor (D_1R), both of which are G_s signaling GPCRs and serve as controls. **(B)** Like GPR12, β_1AR and D_1R are able to increase APP expression, but neither increase APP expression to the same extent as GPR12. Data are normalized to actin and expressed as fold over APP, mean ± SEM (n = 3). One-way ANOVA (post hoc: Dunnett's multiple comparison test), **p ≤ 0.01.

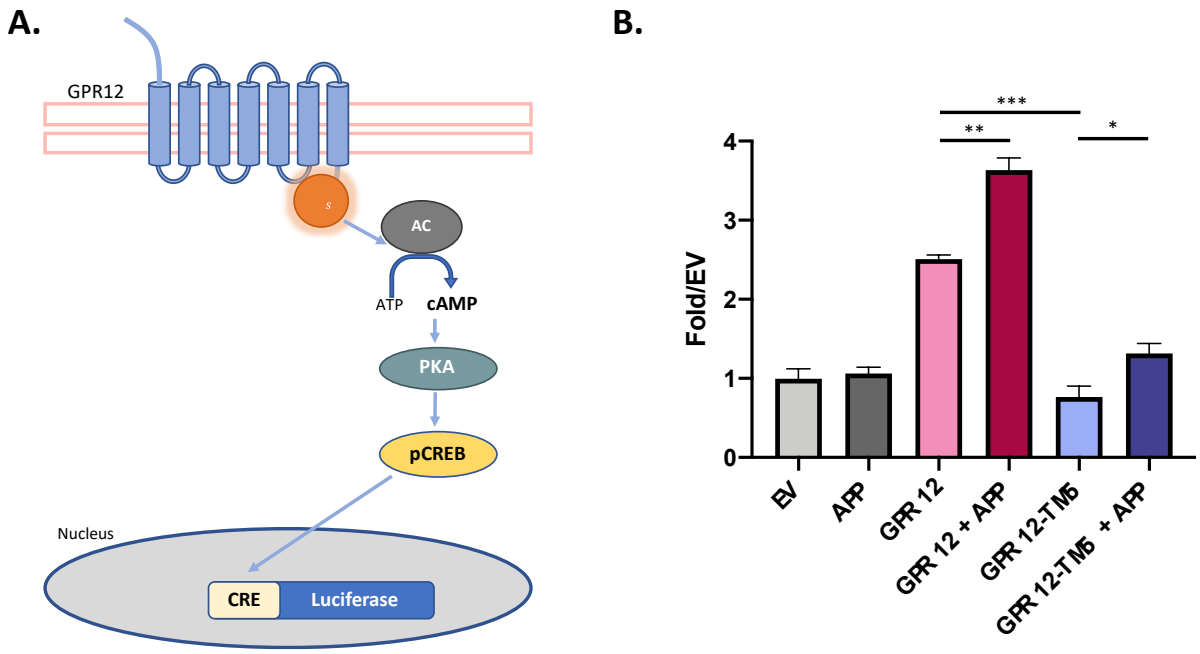

Figure 9. Co-expression of GPR12 with APP leads to increased G_s signaling. (A) Schematic diagram of GPR12 activating cAMP through the G_s signaling pathway. A luciferase gene reporter assay can be used to detect the activity of GPR12 signaling. **(B)** Co-expression of APP with GPR12 increases G_s signaling activity of GPR12 compared to GPR12 overexpresed alone. GPR12-TM5 has lower signaling activty than GPR12-WT, but its signaling activity also increases when coexpreseed with APP. Data are normalized to renilla and expressed as fold over EV (n = 1). Unpaired t test with Welch's correction, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

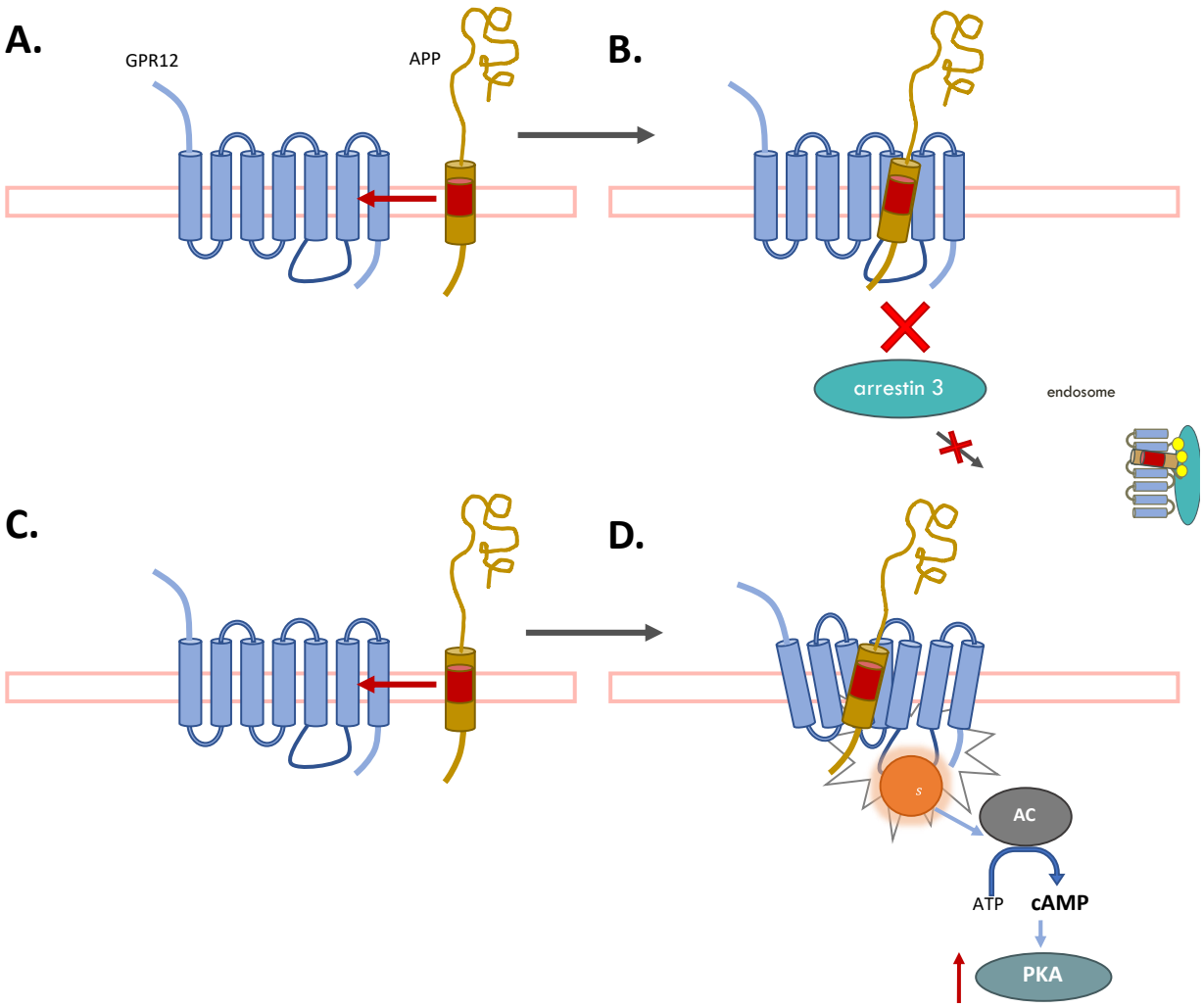

Figure 10. Models for how APP is increasing GPR12 activity through G_s signaling. (A, C) Schematic diagram of GPR12 and APP prior to complex formation. **(B)** Binding of APP to GPR12 at the third cytoplasmic loop prevents recruitment of arrestin 3 (β-arrestin 2) so that GPR12 is not internalized via endosomes. **(D)** Binding of APP to GPR12 causes conformational change of GPR12 and enhances G_s protein coupling and downstream signaling activity.

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