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Summary

The serotonin 5-HT$_4$ receptor has recently gained a lot of attention for its functional roles in central processes such as memory and cognition. In this study, we show that activation of the human 5-HT$_4$ (h5-HT$_4$) receptor stimulates the secretion of the non-amyloidogenic soluble form of the amyloid precursor protein (sAPPα). 5-HT enhanced the level of secreted sAPPα in a time- and dose-dependent manner in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor isoform**.** The increase was inhibited by the selective 5-HT$_4$ receptor antagonist, GR113808. The 5-HT$_4$ selective agonists, prucalopride and renzapride, also increased secreted sAPPα in IMR32 human neuroblastoma cells. The stimulatory effect of 5-HT was mimicked by forskolin, a direct activator of adenylyl cyclase, and 8-Bromo-cAMP, a membrane permeant cAMP analogue. On the contrary, inhibition of protein kinase A (PKA) by H89 potentiated the 5-HT-induced increase in both secreted and cellular sAPPα. This phenomenon involves a novel PKA-independent stimulatory process which overcomes a PKA-dependent inhibitory one. Finally, activation of the h5-HT$_{4(e)}$ receptor did not modify extracellular Aβ in CHO cells transfected with the human APP695**.** Given the neuroprotective and enhancing memory effects of sAPPα, our results may open a new avenue for the treatment of Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is characterised by the presence of senile plaques and tangles in the brains of affected patients (1). Senile plaques are mainly composed of a small insoluble peptide of 40 to 42 amino acids called amyloid β-protein (Aβ) and most evidence gathered over the past decade indicates that accumulation of Aβ is a key event in the pathogenesis of AD (for review see 2). Aβ derives from a larger transmembrane glycoprotein, the amyloid precursor protein (APP), with several isoforms resulting from the alternative splicing of a single primary transcript, and is expressed in all mammalian cells. Mutations in the APP gene result in the abnormal processing of APP which may lead to Aβ deposits and accumulation in the brain of AD patients (3, for review see 4).

APP can be processed via various complex pathways to produce amyloidogenic and non amyloidogenic peptides. In the α-secretase pathway, APP holoprotein is cleaved within the amyloidogenic Aβ domain producing a large amino-terminal non-amyloidogenic soluble APP (sAPPα), which is secreted in the extracellular medium (5). Secreted sAPPα has potent neurotrophic and neuroprotective activities by preventing oxidative damage induced by Aβ (6, 7, 8). Alternatively, the so-called β-, γ-secretase pathway of APP leads to the formation of intact Aβ peptides that can form neurotoxic Aβ aggregates (9). Because of their role in the production of Aβ, the molecular identification of APP secretases and the regulation of their cellular activity are under intense investigations. Recently, an aspartic protease named BACE (β-site APP cleaving enzyme), has been cloned and characterized for its β- secretase activity (for review, see 10). Also, a number of β- and γ-secretase inhibitors have been synthesised and successfully used to lower Aβ secretion (11, 12, 13). However, the β- and γ-secretases may be implicated in the processing of other proteins having specific functions and consequently the use of inhibitors may have unwanted side effects (14).

G-protein coupled receptors (GPCRs) may regulate APP metabolism though a complex network of intracellular second messengers (15, for review, see 16). These include phospholipase C-coupled receptors such as muscarinic acetylcholine m1 and m3 receptor subtypes and metabotropic glutamate receptors (16, 17, 18). More recently, the neuroprotective activity of the corticotropin- releasing hormone receptor type I which is positively coupled to adenylate cyclase has been shown to be accompanied by an increased release of sAPPα (19). Many populations of neurons are affected in AD, especially the cholinergic and serotoninergic neurons of hippocampal and cortical regions. Since neuronal activity may be involved in the regulation of APP metabolism, impaired neuronal signalling may lead to abnormal processing of APP and subsequent Aβ deposition. Consequently, it is important to understand the regulatory mechanism of APP metabolism by neurotransmitter receptors.

The serotonin 5-HT$_4$ receptor has recently gained a lot of attention for its physiological effects in the brain (20). Indeed, with the recent availability of selective 5-HT$_4$ ligands, pharmacological studies have shown that activation of the 5-HT$_4$ receptor increases memory and learning in rats (21). Accordingly, 5-HT$_4$ receptor binding sites have been detected in the hippocampal formation and in neocortical areas (22), and activation of the 5-HT$_4$ receptor stimulates acetylcholine release in rat frontal cortex (23). Moreover, there is a marked loss of 5-HT$_4$ receptor binding sites in cortical and hippocampal regions in the brain of AD patients (24). Altogether these observations suggest that the 5-HT$_4$ receptor may be a potential pharmacological target for the treatment of AD.

Several splice variants of the human 5-HT$_4$ receptor (h5-HT$_4$) have recently been described, particularly at the C-terminus where alternative splicing results in divergent sequences following Leu^{358} (25, 26, 27, 28). These h5-HT$_4$ receptor isoforms belong to the family of seven transmembrane domain G-protein coupled receptors which activate adenylyl

cyclase (25, 26, 27, 28). Most of the h5-HT$_4$ receptor splice variants are expressed in the brain. Despite a report of beneficial effects of 5-HT$_4$ ligands on memory in rats (21), there is no information about the biological significance of the recently cloned h5-HT$_4$ receptor isoforms in the brain. In this study, we show for the first time that activation of the human 5-HT$_4$ (h5-HT$_4$) receptor increases the secretion of the non-amyloidogenic sAPPα. This process is demonstrated in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor, a neuronal h5-HT$_4$ receptor isoform and in IMR32 neuroblastoma cells using 5-HT$_4$ selective ligands. In addition, we analysed the signalling pathways involved in sAPPα release after activation of the h5-HT$_{4(e)}$ receptor isoform.

Experimental Procedures

Materials- All media, sera, and antibiotics used in the cell culture were purchased from Life Technologies (Cergy Pontoise, France). The 5-HT$_4$ receptor antagonist, GR113808 ([1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl1-methyl-1H-indole-3-carboxylate), as well as the 5-HT$_{1B}$ receptor antagonist, GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide), were gifts from Glaxo Research Group (Ware, Hertfordshire, UK). Renzapride ((BRL 24924), (±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride),and prucalopride (4-amino-5-chloro-2,3-dihydro-N-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofurancarboxamide monohydrochloride) were generously given by SmithKline Beecham (Harlow, U.K.) and Janssen Research Foundation (Beerse, Belgium), respectively. 5-HT was from Aldrich (L'Isle d'Abeau Chesnes, France). Forskolin was from Sigma (Saint Quentin Fallavier, France), and 8-Bromo-cAMP and the protein Kinase A inhibitor, H89, were obtained from Calbiochem (France Biochem, Meudon, France).

Cell culture and transient transfection **-** The human neuronal neuroblastoma cell line IMR32 was purchased from ATCC (Rockville, USA) and was grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 1% non essential amino acids and antibiotics. Chinese Hamster Ovary cells (CHO) cells stably expressing the h5-HT$_{4(e)}$ receptor isoform were cultured as previously described (28). For transient transfection experiments, CHO cells stably expressing the h5-HT$_{4(e)}$ receptor isoform were transfected with the cDNA encoding the human APP695 using jetPEI™ (Polyplus-transfection, Illkirch, France) according to the manufacturer instructions. Briefly, 50-60% confluent cells culture were incubated for 5 hours in a mix of a cationic

transfection reagent vector and APP695 cDNA. At the end of the incubation period, media were changed and cells were cultured for an additional period of 60 hours before the determination of sAPPα and Aβ.

PCR study- PCRs for the detection of the expression of h5-HT$_4$ receptors mRNA in IMR32 cells were performed as previously described (28). Briefly, total RNA was extracted from IMR32 cells using the Trizol RNA purification system (Life Technologies Inc., Cergy-Pontoise, France). RNA was then reverse transcribed with oligo(dT) primers and Superscript reverse transcriptase (Life Technologies Inc., Cergy-Pontoise, France). cDNA specific for the common part of the h5-HT$_4$ receptor isoforms was detected using a nested PCR amplification. A first reaction was performed using 50 ng of cDNA together with specific primers, C1F (5'-TAATGCTGGGAGGCTGCTGG-3') and C1R (5'-AGAGGATGATGAGGAAGGCA–3') designed to the 5'- and 3'-end of the h5-HT$_4$ receptor, respectively. Products of this first reaction were used as templates for a nested PCR amplification with specific C2F forward primer (5'-ATGGTCAACAAGCCCTACGC-3') and C2R reverse primer (5'-GCAGAGGCAGAAGCAACCCA -3') which recognise the common part of h5-HT$_4$ receptors. PCR reactions were performed as follows: 30 cycles (30 sec at 94°C, 45 sec at 55°C and 1.5 min at 72°C) and a final elongation (8 min at 72°C). The PCR products were run on a 1.5% agarose gel and visualised under UV.

Antibodies- R1736 (antibody kindly provided by Dr. Dennis Selkoe, Harvard Medical School, Boston) is a rabbit polyclonal antiserum raised to a synthetic peptide of amino-acids 595-611 of APP (29). This corresponds to positions –2 to +15 of human Aβ and it presents 90% (15 amino acid on 17) of sequence homology with the hamster sequence which is identical to other rodents such as mouse (Genebank accession number: AF030413). R1736 is

not only selective for the human APPα but also for the rodent form. Indeed, it has been successfully used to show the endogenous α secreted cleavage of APP molecules with a molecular weight of around 110 kDa in several rodent cellular systems (19, 29). 22C11 is a monoclonal antibody directed against the N-terminal part of APP (5, Boehringer Mannheim, Mannheim, Germany). Monoclonal antibodies 4G8 and 6E10 (Signet pathology systems, Dedham, USA) are reactive to amino acid residue 17-24 and 1-17 of Aβ, respectively (52).

Measurement of sAPPα by Western blot- For determination of secreted and intracellular sAPP**,** confluent CHO and IMR32 cell cultures were cultured in 5% dialysed FCS-containing medium overnight and in serum free medium for 4 h, respectively. At the beginning of the assay, CHO cells were washed with PBS and preincubated for 15 min in serum-free medium supplemented with 1 µM GR127935 to block the activity of endogenous 5-HT$_{1B}$ receptors. Then 5-HT$_4$ ligands and drugs were added for the indicated time periods. Conditioned supernatants containing secreted proteins were collected and cells were lysed for subsequent analyses.

Collected media were centrifuged to remove cell debris, size-selected using Sephadex G-25M columns (Amersham Pharmacia Biotech Inc, Orsay, France) and reconstitued in 70 µl of buffer consisting of 50 mM Tris pH 7.4, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The protein amount in each sample was determined with the bicinchoninic acid assay (Sigma, Saint Quentin Fallavier, France). 50 µg of cell lysates or 100 µg of secreted proteins were run on 8% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Hybond-P, Amersham Pharmacia Biotech, Orsay, France). Membranes were blocked by incubation overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (20 mM Tris pH 7.6, 137 mM NaCl) containing 0.5% Tween-20, then were rinsed in Tris-buffered saline containing 0.5% Tween and incubated with the

monoclonal antibody 22C11 (5) (1:80 dilution), the polyclonal antiserum R1736 (1:3000 dilution) or the monoclonal antibody 6E10 (1:1000 dilution). After incubation at room temperature for 2 h, blots were rinsed in Tris-buffered saline containing 0.5% Tween and were incubated with a horseradish peroxidase-linked sheep anti-mouse or goat anti-rabbit immunoglobulin antibody (Amersham Pharmacia Biotech, Orsay, France) at 1:7500 dilution for 1 h at room temperature. Immunoreactive bands were then visualized by the ECL detection kit (Amersham Pharmacia Biotech, Orsay, France) on Kodak ML lights films. For quantification, films of representative experiments were scanned using a Biorad image acquisition system (Ivry-Sur-Seine, France, gel doc 1000) and fold induction of sAPPα expression level compared to corresponding controls was calculated. Western blot experiments were repeated at least 3 times with identical results.

Determination of Aβ- Secretion media and cell lysates were collected and diluted in a one tenth volume of TNE buffer 10X (NaCl 150 mM, EDTA 1 mM, Tris 10 mM pH 7.5). Total Aβ was immunoprecipited overnight with a 200 fold dilution of 4G8 clone in the presence of protein G-Sepharose. Following immunoprecipitation, samples were electrophoresed on 16.5% Tris-tricine SDS-PAGE gels and transferred onto a 0.2 µM nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany) at 380 mA for 45 min. Filters were boiled in phosphate-buffered saline for 5 min and analysed by Western blot as described above using a 1:1000 dilution of the monoclonal antibody 6E10.

Cyclic AMP radioimmunoassay- For measurement of intracellular cAMP production, IMR32 neuroblastoma cells grown to confluence in 60 mm plates were incubated overnight in serum free DMEM. At the beginning of the assay, IMR32 cells were preincubated for 15 min in serum-free medium supplemented with 5 mM theophylline and 10 µM pargyline. The 5-HT$_4$

antagonist, GR113808, was also included during this preincubation period. 5-HT or other serotoninergic agonists were then added for an additional 10 min period. The reaction was stopped by aspiration of the medium and addition of 500 µl ice-cold perchloric acid (20%). After a 30 min period, neutralisation buffer was added (HEPES 25mM, KOH 2N). cAMP was quantified using a radioimmunoassay kit (cAMP competitive radioimmunoassay, Immunotech, Marseille, France).

Protein kinase A activity assay- This assay was performed as previously described (30). Briefly, CHO cells were grown in 100 mm dishes, then stimulated as described and lysed in a buffer containing 50 mM HEPES pH 7.6, 150 mM NaCl, 10 mM EDTA, 10 mM NaPPi, 2 mM sodium orthovanadate, 100 mM NaF, 100 UI/ml aprotinin, 5 µg/ml leupeptin and 1% Triton X-100. The lysates were centrifuged at 12000 g for 10 min at 4°C and were incubated with the following buffer containing 10 mM HEPES pH 7.2, 68.5 mM NaCl, 2.7 mM KCl, 0.15 mM KH_2PO_4, 0.5 mg/ml glucose, 25 mM ß-glycerophosphate pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA, 1.85 mM $CaCl_2$, 0.1 mM ATP, 100 µM Kemptide (Calbiochem) and 20 µCi of γ-[^{32}P]ATP (3000 Ci/mmol, NEN Life Science products, Paris). The reaction was performed at 37°C for 10 min and then stopped by spotting onto Whatman P81 papers. The papers were washed three times with 1% (v/v) orthophosphoric acid, rinsed twice in ethanol and air dried. The radioactivity was determined by scintillation counting.

Statistical analysis- An unpaired Student´s *t*-test was used to calculate differences between means; differences were considered significant when $p < 0.05$.

Results

5-HT stimulates the cellular release of sAPPα in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor- The effect of the activation of the h5-HT$_4$ receptor on sAPPα release was analysed in CHO cells stably expressing the neuronal h5-HT$_{4(e)}$ receptor isoform (28). This CHO cell line stably transfected with the recombinant h5-HT$_{4(e)}$ receptor displayed a typical 5-HT$_4$ pharmacological profile as previously determined in binding studies and adenylyl cyclase assays (28). In addition, the receptor is expressed at physiological density since the number of specific 5-HT$_4$ binding sites is in accordance with that found in human brain (28).

Using an N-terminal monoclonal antibody against APP (22C11) (5, 15), we showed by Western blot that secreted sAPPα is increased in CHO cell clones following a 30 min incubation with 1 μM 5-HT compared to untreated control cells (Figure 1). Since the 22C11 monoclonal antibody can also recognise APP-like proteins (APLP) such as APLP2 (31), we performed immunoblots with the R1736 antiserum which is specific of sAPPα (29). Similarly, a single band migrating at the expected molecular mass of 110 kDa was also detected with R1736 (Figure 1). These data suggest that secreted sAPP is produced by non-amyloidogenic α-cleavage.

5-HT- induced secretion of sAPPα in CHO cells expressing the h5-HT$_{4(e)}$ receptor was dose dependent (Figure 2A). Figure 2A shows a representative gel depicting an increase in sAPPα secretion with increasing concentrations of 5-HT after 30 min of application. The levels of secreted sAPPα after treatment with micromolar concentrations of 5-HT was significantly different in CHO cells transfected with the h5-HT$_{4(e)}$ receptor from that of non transfected cells (Figure 2A, lower panel). 5-HT- induced increase in sAPPα release reached a plateau and the maximal release of sAPPα obtained at 1 μM 5-HT was about 2.5 fold (range 2.1-3.7) above its basal level (Figure 2A, lower panel). No effect of 5-HT on sAPPα release

could be detected in the non transfected CHO control cells (Figure 2A, lower panel). In addition, the level of intracellular sAPPα was also enhanced after 30 min treatment with increasing concentrations of 5-HT (Figure 2B). Time course analyses showed that 5-HT-induced release of sAPPα was rapid and the amount of secreted sAPPα reached a plateau with a maximum after 15 min incubation with 1 μM 5-HT in CHO cells expressing the h5-HT$_{4(e)}$ receptor (Figure 3).

Effects of a selective 5-HT$_4$ antagonist, GR113808, on basal and stimulated levels of secreted sAPPα in CHO cells- Next, we examined whether the selective 5-HT$_4$ antagonist, GR113808, blocked 5-HT- induced sAPPα secretion. As shown in Figure 4, increasing concentrations of GR113808 antagonised 5-HT- enhanced sAPPα release in CHO cells expressing the h5-HT$_{4(e)}$ receptor. This result indicates that the effects of 5-HT on secreted sAPPα is specifically mediated by the h5-HT$_{4(e)}$ receptor (Figure 4). In addition, a constitutive activity of the h5-HT$_{4(e)}$ receptor on basal sAPPα release was observed in the absence of any 5-HT$_4$ ligand (Figure 5A). Indeed, the relative basal level of secreted sAPPα in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor was around 2 fold higher than in untransfected CHO cells (Figure 5A). This effect was not due to the presence of 5-HT in culture medium since cells were cultured overnight before the assay in dialysed FCS-containing medium. These data clearly indicate that expression of the h5-HT$_{4(e)}$ receptor induced a spontaneously active receptor state which may influence sAPPα release.

Since constitutive activation of G-protein-coupled receptors is often related to inverse agonist properties, we tested the effects of the potent 5-HT$_4$ antagonist, GR113808, on the intrinsic activity of the h5-HT$_{4(e)}$ receptor (Figure 5B). GR113808 (1 μM), which behaved as inverse agonist at the h5-HT$_{4(e)}$ receptor on cAMP production (28), also reduced the constitutive activity of the h5-HT$_{4(e)}$ receptor on sAPPα extracellular release (Figure 5B).

Analysis of the signalling pathways involved in the h5-HT$_{4(e)}$ receptor induced- sAPPα secretion- h5-HT$_4$ receptor isoforms are positively coupled to adenylyl cyclase (25, 26, 27) and we have recently shown that activation of the h5-HT$_{4(e)}$ receptor isoform stably expressed in CHO cells strongly enhances cAMP production (28). To examine the involvement of cAMP in APP metabolism, we treated CHO cells transfected with the h5-HT$_{4(e)}$ receptor isoform with the adenylyl cyclase activator forskolin (1 μM) or the membrane permeant 8-Bromo-cAMP (10 μM) for 15 min. Both drugs mimicked the effects of 5-HT- induced sAPPα secretion since they caused a robust increase in secreted sAPPα as determined by Western blot using the R1736 antiserum (Figure 6A). However, sAPPα secreted form observed in forskolin and cAMP treated cells appears to migrate at a lower molecular weight than in the control (Figure 6A) suggesting that the glycosylation state of secreted sAPPα may be influenced by cAMP. Therefore, these results suggest an effect of cAMP on cellular trafficking by increasing full length APP to compartments involved in α-secretase processing. The increases in secreted sAPPα was accompanied by an increase in the intracellular level of sAPPα (Figure 6A).

It is well known that activation of 5-HT$_4$ receptors induces intracellular production of cAMP which then activates protein kinase A (PKA) (32). Therefore, to test the role of PKA in 5-HT- induced increase in cellular and secreted sAPPα, we treated CHO cells stably expressing the 5-HT$_{4(e)}$ receptor with 10 μM of the PKA inhibitor, H89, and measured intracellular and secreted levels of sAPPα following 5-HT$_{4(e)}$ receptor activation (Figures 6B, 6C). Surprisingly, H89 failed to block the response to 5-HT but it strongly enhanced basal sAPPα and 5-HT-induced sAPPα (Figures 6B, 6C). Indeed, 5-HT (1 μM) increased about 2-fold the cellular level of sAPPα compared to control untreated cells whereas the stimulation reached more than 6-fold (range 2-8.5) the control value in the presence of H89 (10 μM)

(Figure 6C). H89 was effective since it completely inhibited 5-HT-enhanced PKA activity in this cellular system (Figure 6D). Taken together, these results suggest that PKA exerts a negative regulation on 5-HT- induced increase in secreted and cellular sAPPα. Since 5-HT$_4$ receptors activate both cAMP production and PKA, its effect on sAPPα regulation must involve a PKA-independent mechanism.

Selective 5-HT$_4$ ligands regulate sAPPα secretion in human IMR32 neuroblastoma cell line- To further test for the effects of the activation of native h5-HT$_4$ receptors on sAPPα secretion, we used IMR32 cells which express h5-HT$_4$ receptor transcripts as determined by RT-PCR analysis (data not shown). This cell line also expresses additional neurotransmitter receptors such as the serotonin 5-HT$_{2C}$ receptor (33). Therefore, we used the 5-HT$_4$ agonists, prucalopride and renzapride, to activate selectively the h5-HT$_4$ receptor and determined its effect on sAPPα release. As shown in Figure 7A, 5-HT, prucalopride and renzapride used at the concentration of 1 μM produced an increase in secreted sAPPα. Among these 5-HT$_4$ ligands, prucalopride was the most potent agonist in stimulating sAPPα (Figure 7A). As expected, the selective 5-HT$_4$ antagonist, GR113808 (1 μM), blocked the cellular release of sAPPα induced by prucalopride and renzapride. However, it failed to do so with 5-HT, probably because of the presence of other 5-HT receptors such as the 5-HT$_{2C}$ receptor in this cell line. This 5-HT receptor has also been shown to be involved in APP processing (34). We then verified whether the strong effect of prucalopride on sAPPα release would correlate with its effect on cAMP production. Prucalopride (1 μM) induced a robust stimulation of basal cAMP in IMR32 cells and, as expected, it was ineffective in the presence of the prototypic 5-HT$_4$ antagonist, GR113808 (1 μM) (Figure 7B).

Effect of 5-HT$_4$ receptor activation on Aβ production- Many assays are not sensitive enough to detect Aβ peptides in the medium of cells containing low amounts of endogenously expressed APP. CHO cells have been reported to express very low level of Aβ (51) and most of the antibodies directed against Aβ peptides are selective of humans and do not cross react at optimal concentration with the rodent forms. For all these reasons, we have transiently transfected the cDNA encoding the human APP695 in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor isoform and determined the effects of 5-HT$_4$ receptor activation on both secreted APPα (sAPPα) and Aβ peptides. To detect Aβ, we set up a sensitive and specific Immunoprecipitation/Western blot protocol by employing monoclonal antibodies 4G8 for immunoprecipitation and 6E10 for Western blotting (see Experimental procedures). The antibody 6E10 is specific for the α-secretase cleaved form of human APP (52).

As shown in Figure 8A, 5-HT enhanced human sAPPα secretion in CHO cells transfected with APP695. These results indicate that the activation of the h5-HT$_{4(e)}$ receptor not only stimulates the release of endogenous sAPPα but also the human form in CHO cells. In order to elucidate whether the stimulating effect of the 5-HT$_4$ receptor affects Aβ generation, Aβ (1-40 and 1-42) was immunoprecipitated from the corresponding CHO cell culture media (Figure 8B). In fact, we did not observe any effect of 5-HT (1 μM) on Aβ production compared to untreated control CHO cells (Figure 8). We also assessed whether intracellular Aβ was affected by 5-HT cell treatment. Although our immunoprecipitation/Western blot protocol can readily detect as little as 500 pg of synthetic human Aβ, we were not able to detect any clear band representative of intracellular Aβ in CHO cell lysates transiently transfected with APP695 under this experimental condition (data not shown). This finding is in accordance with the fact that Aβ is more abundant in the extracellular medium than in cell lysates (53).

Discussion

In this study, we have examined the effects of 5-HT$_4$ ligands on intra- and extracellular levels of sAPPα. We employed two cellular systems: a CHO cell line stably expressing physiological density of the h5-HT$_{4(e)}$ receptor isoform (28) and a human neuroblastoma cell line, IMR32, which expresses native 5-HT$_4$ receptors. In CHO cell clones, we showed that 5-HT enhanced the level of extracellular sAPPα in a time- and dose-dependent manner. The increase in cellular release of sAPPα was detected within 15 min following application of 5-HT, suggesting that it derives from a pool of preexisting intracellular proteins and not from newly synthetized APP proteins. Furthermore, 5-HT-induced sAPPα secretion was blocked by a selective 5-HT$_4$ antagonist, GR113808. Hence, we showed that 5-HT$_4$ agonists enhanced release of secreted sAPPα in neuroblastoma IMR32 cells. Increased APP secretion was detected by a non-selective monoclonal antibody as well as by the R1736 antiserum which recognizes α-secretase-cleaved APP (29). Altogether, these data indicate that 5-HT and 5-HT$_4$ ligands increase the cellular release of sAPPα through their specific interaction with the h5-HT$_4$ receptor. To the best of our knowledge, this is the first report showing a specific cellular function for the recently cloned h5-HT$_{4(e)}$ receptor.

Recently, evidence has been provided that members of the ADAM family act as α-secretases. α- cleavage of APP can occur at multiple locations within the cell as it has been shown for PKC-induced sAPPα secretion (58). In our study, the cellular mechanism by which the which the activation of the h5-HT$_{4(e)}$ receptor results in the secretion of sAPPα may not be directly linked to an increase in the activity of the α secretases but could also reflect an effect on cellular trafficking of APP. Indeed, one could imagine that activation of the 5-HT$_{4(e)}$ receptor causes an increase in the trafficking of APP to cellular compartments involved in α-secretase processing. This is supported by the results of Figure 6 showing that the intracellular

pool of sAPPα in cells stimulated with cAMP is migrating faster than that of the control. This possibly indicates an effect of this second messenger on the glycosylation state of secreted sAPPα.

Non amyloidogenic secreted sAPPα has potent neuroprotective functions against excitotoxic and oxidative insults in various cellular models (6, 7), and it was recently shown to confer resistance against p53- mediated apoptosis (35). In addition, sAPPα promotes neurite outgrowth and regulates neuronal excitability (36). Intracerebroventricular administration of secreted forms of sAPPα to amnestic mice has potent memory-enhancing effects and blocks learning deficits induced by scopolamine (37). This observation together with our reported data may explain the memory-enhancing effects of 5-HT$_4$ ligands *in vivo* (21). Since loss of synaptic function and deficits in cognition and memory are central pathological events in AD, our data allow us to speculate that 5-HT$_4$ agonists may have beneficial effects for the treatment of AD.

The regulation of sAPPα has been previously shown for other GPCRs (16). For instance, muscarinic m1 and m3 receptor activation results in the activation of phospholipase C (PLC) leading to a rise in intracellular calcium, diacylglycerol and inositol triphosphate which parallels sAPPα secretion. The stimulation of metabotropic glutamate receptors was also shown to increase the release of sAPPα (17, 38). The effects of PLC- coupled receptors on sAPPα secretion are thought to be mainly mediated by protein kinase C (PKC) which has also been shown to decrease Aβ production in various cell lines (39). Serotonin 5-HT$_4$ receptors are positively coupled to adenylate cyclase and consequently they increase cAMP production (26, 28, 40). In our study, forskolin and 8-bromo-cAMP mimicked the stimulation of sAPPα by 5-HT in CHO cells. These findings agree with previous observations showing that cAMP and seven transmembrane receptors which are coupled to the heterotrimeric Gs protein may increase sAPPα (41, 19). However, in C6 cells transfected with the full length

APP751, forskolin and the cAMP analogue, dibutyryl cAMP inhibited both the constitutive and phorbol ester stimulated secretion of sAPPα (42). In astrocytes, both drugs also inhibited the increase in sAPPα secretion caused by phorbol ester, but did not affect basal sAPPα levels (43). Although further experiments are needed to solve this discrepancy, it is conceivable that the balance between the PKA-dependent inhibitory and the PKA-independent stimulatory mechanisms may differ in different cell types.

Interestingly, a constitutive activation on secreted sAPPα was found with the h5-HT$_{4(e)}$ receptor stably expressed in CHO cells. Constitutive coupling to cAMP was already described for the h5-HT$_{4(e)}$ receptor and other 5-HT$_4$ receptor splice variants such as the h5-HT$_{4(d)}$ receptor (40). In addition, we found that GR113808 decreased basal secreted levels of sAPPα indicating that this selective 5-HT$_4$ antagonist behaves as an inverse agonist. This confirms our previous finding that GR113808 reduces the basal cAMP production in CHO cells transfected with the 5-HT$_{4(e)}$ receptor (28). At present, the physiological relevance of inverse agonism is not clear. Inverse agonistic properties are suggested to be of therapeutic relevance in the treatment of disorders related to constitutive receptor activation. Our results suggest that inverse agonists, through their action on GPCRs signalling, may also influence the processing of molecules having important physiological functions such as APP.

Activation of PKA usually results from an increased production of cAMP via Gs protein coupled receptor. Activation of the recombinant h5-HT$_{4(e)}$ receptor isoform in CHO cells as well as that of the native h5-HT$_4$ in IMR32 neuroblastoma cells were accompanied by a net increase in the level of intracellular cAMP. Classicaly, cAMP mediates its action by activation of PKA and phosphorylation of substrate proteins. Surprisingly, the PKA inhibitor H89 strongly boosted the stimulatory effect of 5-HT on intra- and extracellular levels of sAPPα in CHO cells stably expressing the 5-HT$_{4(e)}$ receptor isoform. We also found that H89 increased the constitutive expression of sAPPα. This observation agrees with the previous

study of Marambaud *et al*. (44) who showed that PKA inhibitors selectively lower Aβ production in human embryonic kidney 293 cells expressing wild type or mutated APP. However, these authors did not observe any effect of H89 on the basal constitutive release of sAPPα.

Our results show that the effect of the activation of the h5-HT$_4$ receptor on sAPPα secretion involves cAMP production and a novel PKA-independent stimulatory process which overcomes a PKA-dependent inhibitory one. In most cellular systems, cAMP effects have been considered to be the result of PKA activation. However, PKA-independent signalling pathway has been reported for some ion channels such as the pacemaker channel (45, 46). In addition, several guanine nucleotide exchange factors which regulate the activity of small GTP binding proteins can also be directly activated by cAMP (47, 48). The functions of specific small G proteins are not fully understood and it will be interesting to determine whether they are involved in sAPPα secretion. Finally, to explain the inhibitory effect of PKA on 5-HT-induced intracellular and extracellular content of sAPPα, one could speculate that the 5-HT$_{4(e)}$ receptor isoform is coupled to an additional signal transduction cascade which could influence sAPPα secretion. It is now well accepted that one GPCR is capable of interacting with more than one G protein to result in multifunctional signalling (49). This is illustrated by the work of Namba *et al.* (50) who have shown that the isoforms of the prostaglandin EP3 receptor, which differ only at their C-terminal tails and are produced by alternative splicing, couple to different G proteins to activate different messenger systems.

In contrast to sAPPα, we were unable to detect any change in secreted Aβ levels upon stimulation of the 5-HT$_{4(e)}$ receptor in CHO cells transfected with the human APP695. Our data contrast with several reports showing reduced Aβ formation under conditions of enhanced sAPPα secretion (for review see 16). For instance, in cells overexpressing APP, cholesterol depletion- induced sAPPα secretion is accompanied by a decrease of Aβ

production (54). However, other studies failed to demonstrate any association between sAPPα secretion and Aβ generation both *in vivo* and *in vitro* (55). Rossner *et al*. (2000) (56) showed that constitutive activation of PKC in guinea pig brain increased sAPPα secretion without any effect on secreted Aβ suggesting that the α- and β-secretase pathways may be differentially controlled. Their conclusion is supported by the different cellular sites of sAPPα and Aβ generation. In neurons, Aβ 1–42 and Aβ 1–40 are produced in the endoplasmic reticulum and in the trans-golgi network, respectively (for review see 2). In our experimental conditions, we detected total extracellular Aβ and therefore we cannot exclude that 5-HT may differentially influence Aβ 1–42 and Aβ 1–40 levels. This hypothesis is currently under our investigation using selective antibodies against each peptide. Also, intracellular and extracellular Aβ peptides may be differentially regulated at it has been recently shown for the growth factor, insulin (57). Stable CHO cell lines coexpressing the h5-HT$_4$ receptor and APP695 should allow us to address this question.

In conclusion, in the present study, we provide the molecular basis of the involvement of the h5-HT$_4$ receptor in the regulation of sAPPα secretion. Our results show that selective 5-HT$_4$ agonists increase non amyloidogenic secreted sAPPα in non-neuronal and neuronal cells. Given the neuroprotective activity and memory enhancing effects of sAPPα, 5-HT$_4$ receptors could constitute a potential novel pharmacological target for the treatment of AD.

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Abbreviations

The abbreviations used are : AD, alzheimer's disease, Aβ, amyloid β-protein, APP, amyloid precursor protein, sAPPα, non-amyloidogenic soluble of the amyloid precursor protein , GPCRs, G-protein-coupled receptors, 5-HT, 5-hydroxytryptamine, h5-HT$_4$, human 5-HT$_4$ receptor, GR113808, [1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl1-methyl-1H-indole-3-carboxylate), GR127935, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide, renzapride (BRL 24924), (±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride, prucalopride (4-amino-5-chloro-2,3-dihydro-N-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofurancarboxamide monohydrochloride), DMEM, Dulbecco's modified Eagle's medium, FCS, foetal calf serum, CHO cells, Chinese hamster ovary cells, SDS, sodium dodecyl sulfate

PKA, cAMP-dependent protein kinase, PLC, Phospholipase C, PKC, Protein kinase C

Figure Legends

Figure 1. Characterisation of secreted sAPP derivatives in extracellular medium of CHO cells. CHO cells were treated or not with 1 μM 5-HT as described in Experimental Procedures. 100 μg of secreted proteins were run on 8% sodium SDS gel and immunoblotted with 22C11 antibody or R1736 antiserum. Arrows indicated secreted non amyloidogenic sAPPα migrating at about 110 kDa. CT, control untreated cells. Results are representative of three independent experiments.

Figure 2. Activation of the h5-HT$_{4(e)}$ receptor isoform dose-dependently increases expression levels of non amyloidogenic sAPPα in CHO cells. A, Representative immunoblot (upper panel) showing the effects of increasing concentrations of 5-HT on the cellular release of sAPPα in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor isoform. After incubating the cells with the indicated concentrations of 5-HT for 30 min, the culture medium was collected and treated as described in Experimental Procedures. Secreted sAPPα was measured by Western blot using the R1736 antiserum. A 110 kDa molecular weight marker is indicated at the right. Quantification of specific secreted sAPPα detected in the blots (lower panel). The amounts of secreted sAPPα detected in the media of transfected cells or untransfected parental cells (UT) were expressed as fold activation of the control untreated cells in the same experiment. Results are means ± S.E.M. for 3 to 5 independent experiments. *$p<0.05$, **$p<0.01$ compared with untransfected parental cells (*t*-test). **B,** Representative immunoblot showing the effects of increasing concentrations of 5-HT on intracellular contents of sAPPα in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor isoform. Cellular treatment and immunoblots were performed as in **A.** The quantification of the signal is

presented as relative protein expression. The expression in untreated control cells was defined arbitrarily as 1. CT, untreated control cells; UT, untranfected cells.

Figure 3. The h5-HT$_{4(e)}$ receptor time-dependently enhances the release of secreted sAPPα. A, Representative immunoblot showing at different time points the effects of 5-HT on the cellular release of sAPPα in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor isoform. Cells treated with 1 μM 5-HT were incubated at indicated times. Secreted sAPPα was measured by Western blot using the R1736 antiserum as described in Experimental Procedures. A 110 kDa molecular weight marker is indicated at the right. **B,** Quantification of specific secreted sAPPα detected in the blots. The amounts of secreted sAPPα detected in the media of transfected cells or untransfected parental cells (UT) were expressed as fold activation of the control untreated cells in the same experiment. Results are means ± S.E.M. for 3 independent experiments. **p<0.01, ***p<0.001, compared with untransfected parental cells (*t*-test).

Figure 4. Secreted sAPPα induced by the activation of the h5-HT$_{4(e)}$ receptor is inhibited by the 5-HT$_4$ antagonist, GR113808. CHO cells were preincubated with increasing concentrations of GR113808 10 min prior to treatment with 1 μM 5-HT. After an additional 30 min period, sAPPα was detected in the culture medium by immunoblotting using the R1736 antiserum. The quantification of the signal is presented as relative protein expression. sAPPα expression in untreated control cells (CT) was defined arbitrarily as 1. A 110 kDa molecular weight marker is indicated at the right. Results are representative of four independent experiments.

Figure 5. Effects of a selective 5-HT$_4$ antagonist, GR113808, on basal and stimulated levels of secreted sAPPα in CHO cells stably transfected with the h5-HT$_{4(e)}$ receptor.

A, Constitutive activity of the h5-HT$_{4(e)}$ receptor on basal sAPPα release. Secreted sAPPα was detected in untransfected (UT) and transfected cells. **B,** Effects of GR113808 in the absence or in the presence of 1 μM 5-HT on secreted sAPPα in CHO cells expressing the h5-HT$_{4(e)}$ receptor. Cells were preincubated with 1 μM GR113808 and were stimulated with 1 μM 5-HT for 30 min. In **A** and **B** Western blots were performed using the R1736 antiserum. The quantification of the signal is presented as relative protein expression. Secreted sAPPα expression in untransfected (UT) and control cells was defined arbitrarily as 1. Results are representative of three independent experiments.

Figure 6. Analysis of the signalling pathways involved in 5-HT- induced APP processing in CHO cells stably transfected with the h5-HT$_{4(e)}$ receptor. A, Effects of the adenylyl cyclase activator forskolin (1 μM) and the membrane permeant cAMP analogue, 8-Bromo-cAMP (10 μM) on sAPPα release. Both drugs were applied to the cells for 15 min and levels of sAPPα were determined by Western blot using the R1736 antiserum. **B,** Representative immunoblots showing the effects of the PKA inhibitor, H89, on basal and 5-HT-induced intracellular and extracellular content of sAPPα. H89 (10 μM) was preincubated 10 min before the 20 min treatment with 1 μM 5-HT. Immunoblots were performed with the R1736 antiserum. **C,** Quantification of specific cellular sAPPα detected in the blots. Results are means ± S.E.M. of 3 independent experiments. **D,** PKA activity assay. Cells were treated as in **B** and the PKA assay was performed as described in Experimental Procedures. Results are means **±** S.E.M. of 3 independent experiments. In **B** and **D**, the amounts of sAPPα or the PKA

activity were expressed as percentage of the control untreated cells (CT) in the same experiment. *p<0.05, **p<0.01 compared with untreated control cells (*t*-test).

Figure 7. Selective 5-HT$_4$ ligands regulate APP processing in IMR32 neuroblastoma cells. A, Representative Western blot of secreted sAPPα upon stimulation with 1 μM of 5-HT$_4$ ligands employing the R1736 antiserum followed by ECL is shown. The 5-HT$_4$ receptor antagonist, GR113808 (1 μM), blocked the release of sAPPα induced by renzapride and prucalopride. The intensity of the signals was analyzed by densitometer reading of the autoradiographs and is presented as relative protein expression. The expression in untreated control cells was defined arbitrarily as 1. **B,** Effects of the selective 5-HT$_4$ agonist, prucalopride (1 μM), on cAMP production in IMR32 cells. GR113808 was applied to the cells at the concentration of 1 μM. cAMP measurements were performed as described in Experimental Procedures. Values are expressed as the percentage of control untreated cells. Data are from two independent experiments performed in triplicate and are means ± S.E.M.

Figure 8. Effect of h5-HT$_{4(e)}$ receptor activation on Aβ secretion. CHO cells stably expressing the h5-HT$_{4(e)}$ receptor isoform were transiently transfected with the cDNA encoding the human APP695. Sixty hours after transfection, cells were treated or not with 1 μM 5-HT and secreted sAPPα and Aβ were detected in the media by immunoprecipitation and Western blot as described in Experimental Procedures. Representative immunoblots are shown. Human sAPPα (A) and Aβ (B) were detected on the same extracellular media. Molecular weight markers are indicated on the right. As a standard, 500 ng of synthetic Aβ (1-42) was directly loaded into the gel. CT, untreated control cells. Results are representative of three independent experiments.

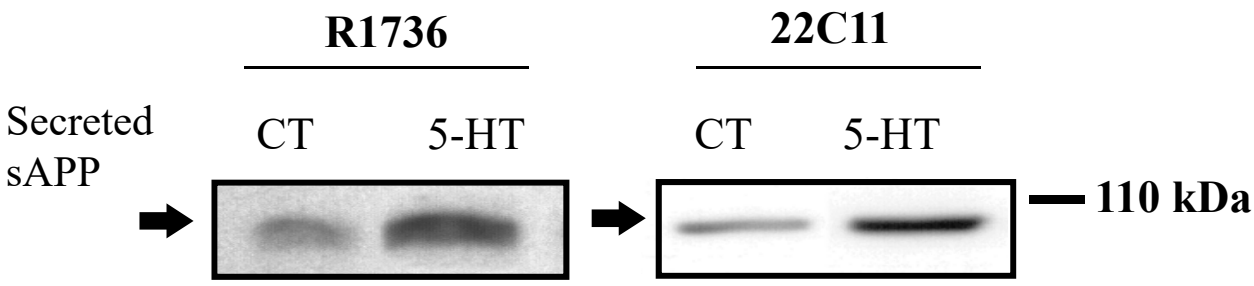


Figure 1

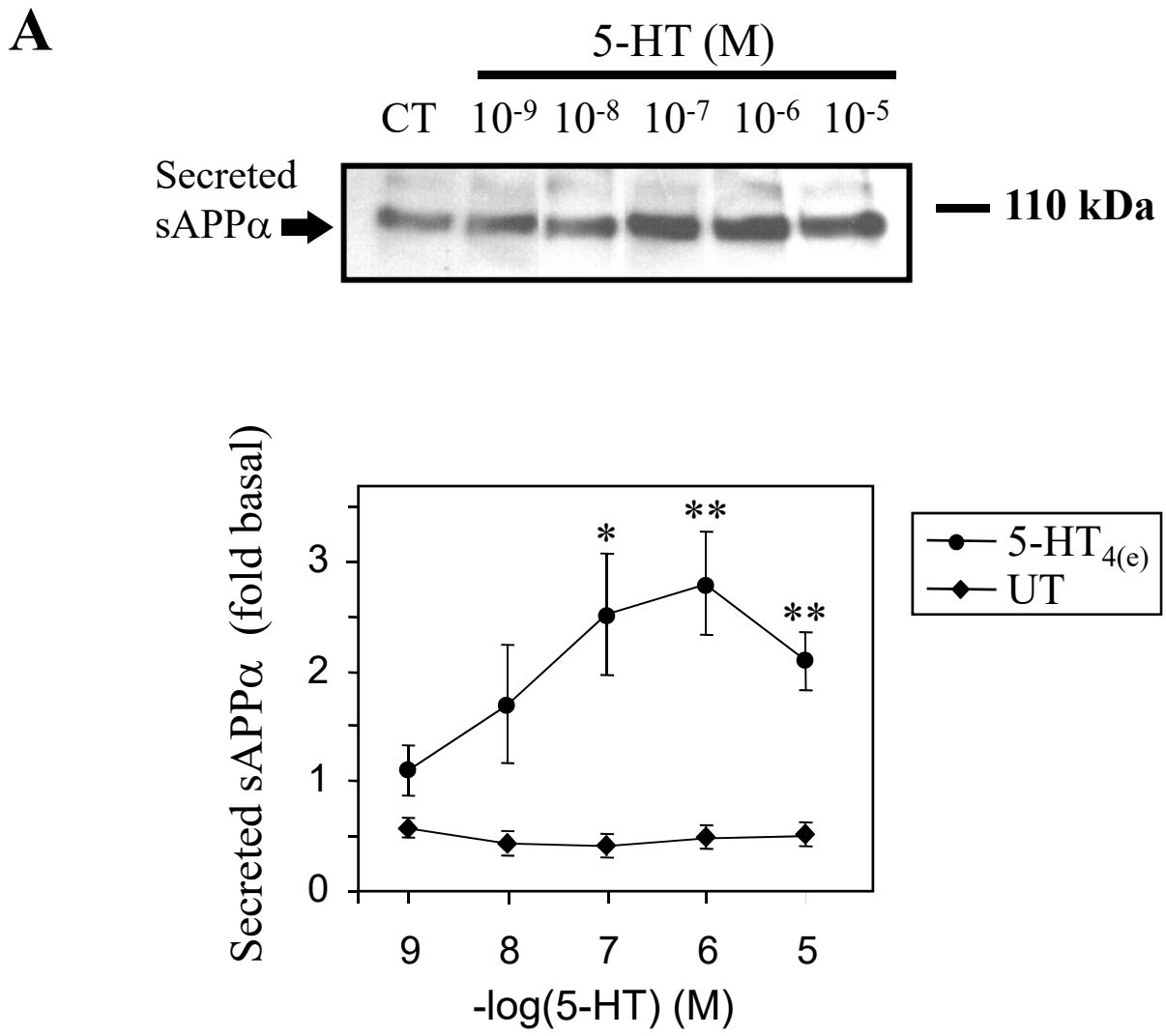
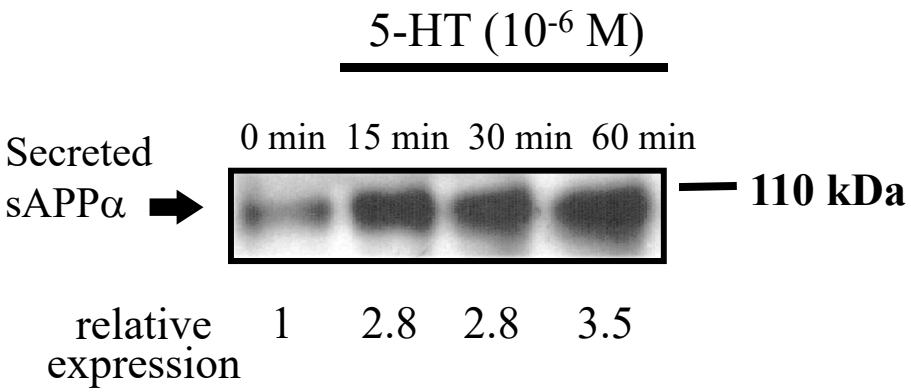

A
5-HT (M)
CT
10-9
10-8
10-7
10-6
10-5
Secreted
sAPPα
110 kDa
Secreted sAPPα (fold basal)
0
1
2
3
9
8
7
6
5
-log(5-HT) (M)
*
**
**
5-HT4(e)
UT
B
5-HT (M)
CT
10-9
10-8
10-7
10-6
10-5
Cellular
sAPPα
110 kDa
relative
expression
1
1.1
1.3
1.6
2.6
2.6

Figure 2

A


5-HT (10^{-6} M)
0 min 15 min 30 min 60 min
Secreted sAPPα
110 kDa
relative expression 1 2.8 2.8 3.5

B

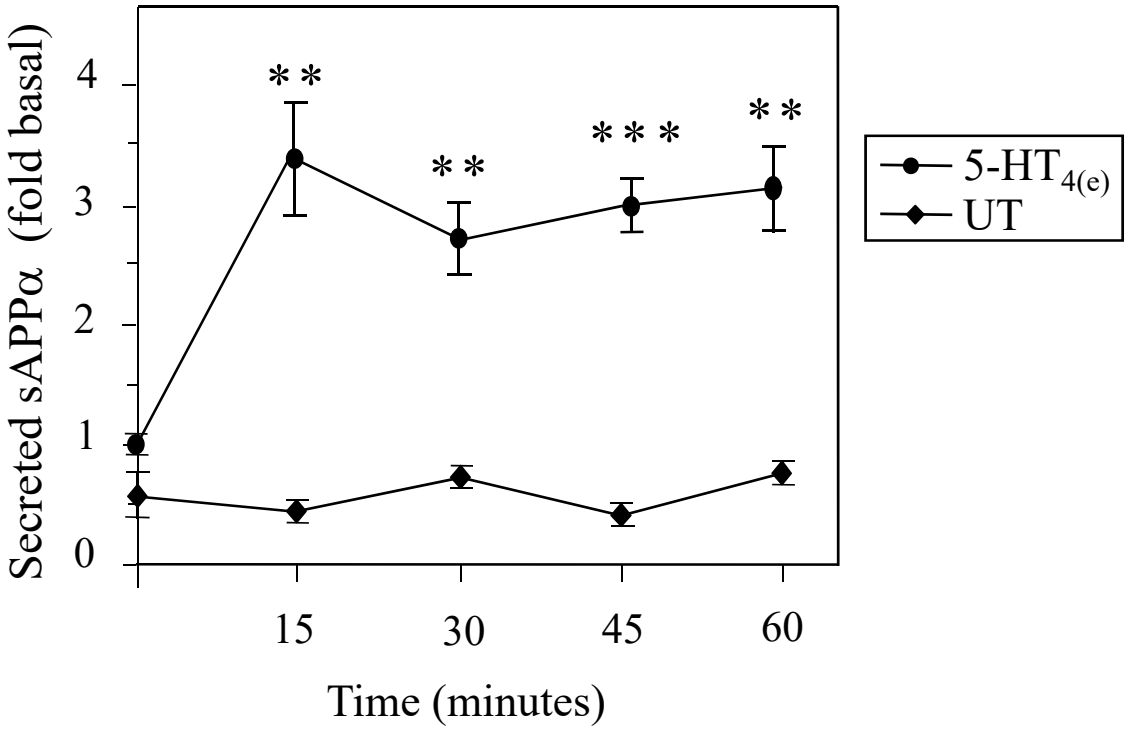

Secreted sAPPα (fold basal)
0
1
2
3
4
**
**
***
**
15
30
45
60
Time (minutes)
5-HT4(e)
UT

Figure 3

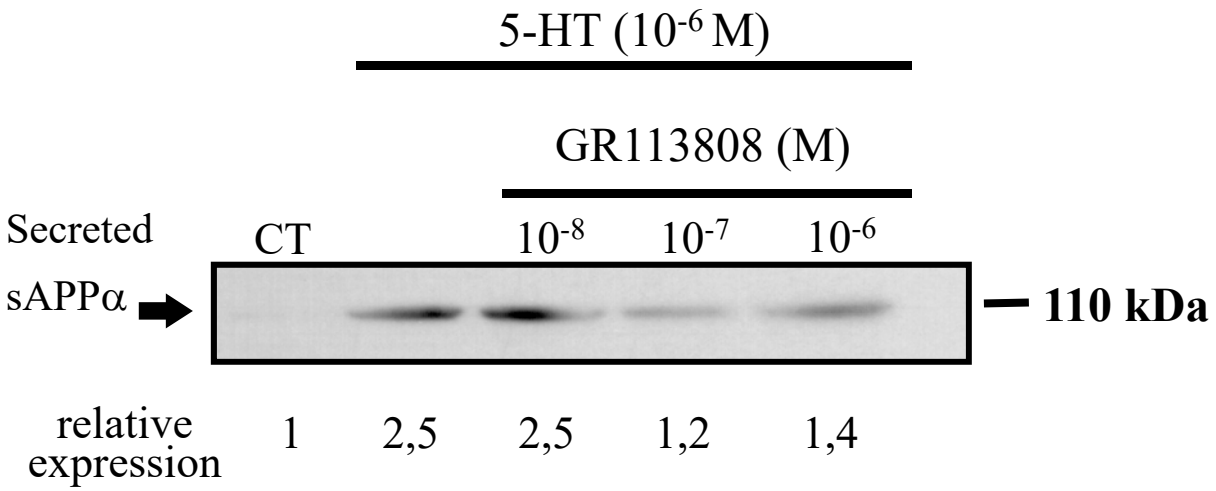
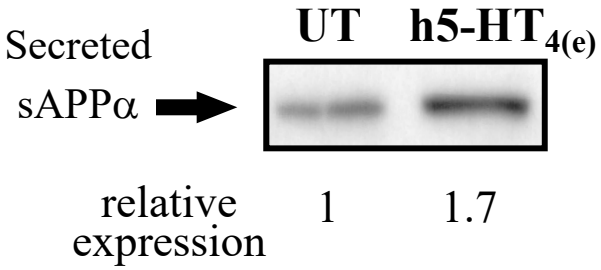

5-HT (10^{-6} M)
GR113808 (M)
Secreted
sAPPα
CT
10^{-8}
10^{-7}
10^{-6}
110 kDa
relative expression
1
2,5
2,5
1,2
1,4

Figure 4

A


UT
h5-HT4(e)
Secreted
sAPPα
relative
expression
1
1.7

B

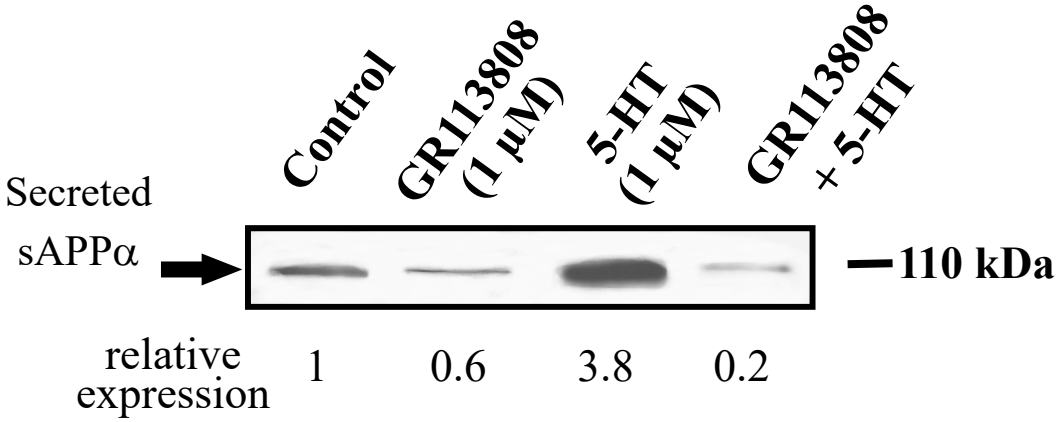

Control
GR113808
(1 μM)
5-HT
(1 μM)
GR113808
+ 5-HT
Secreted
sAPPα
110 kDa
relative
expression
1
0.6
3.8
0.2

Figure 5

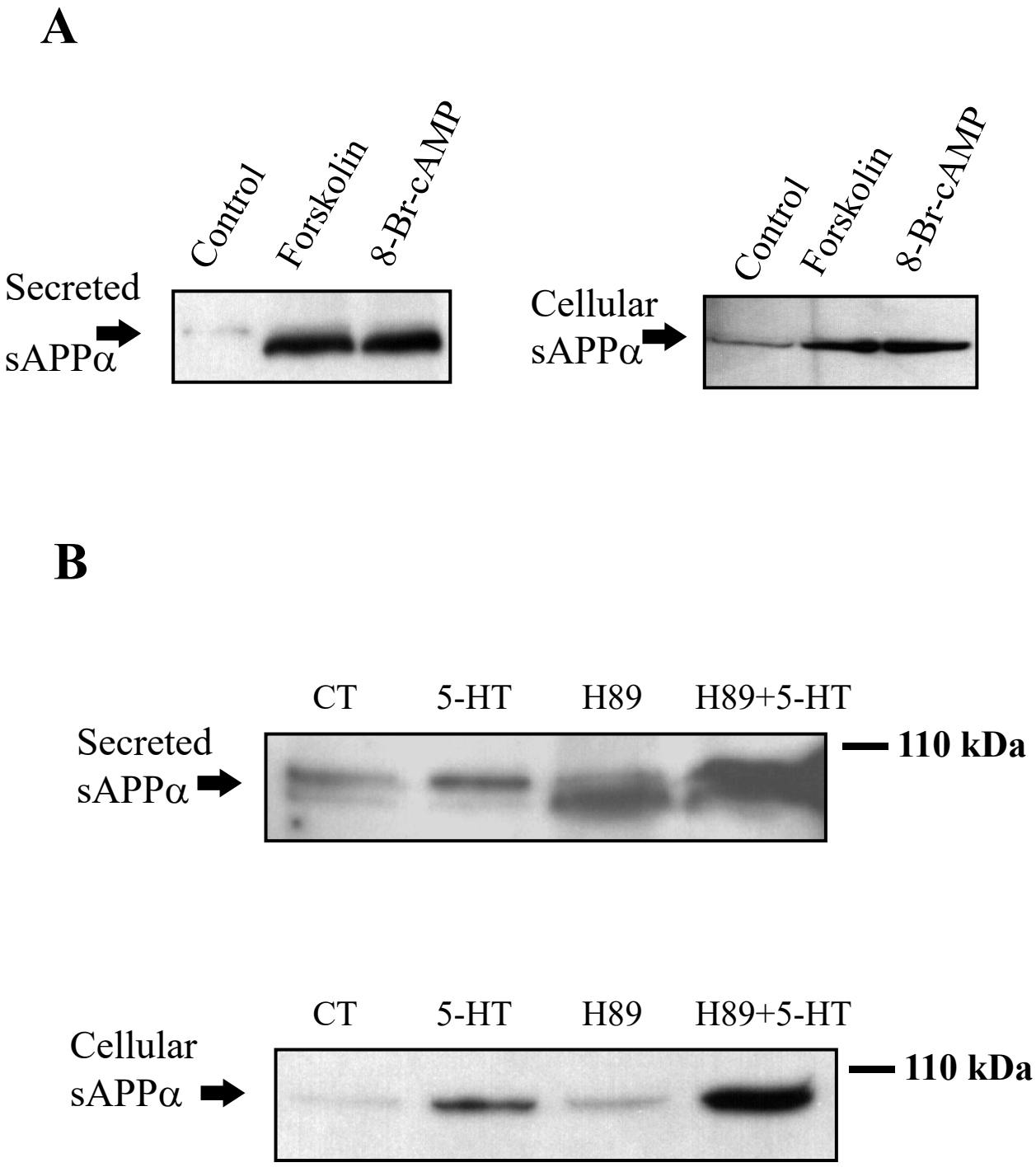
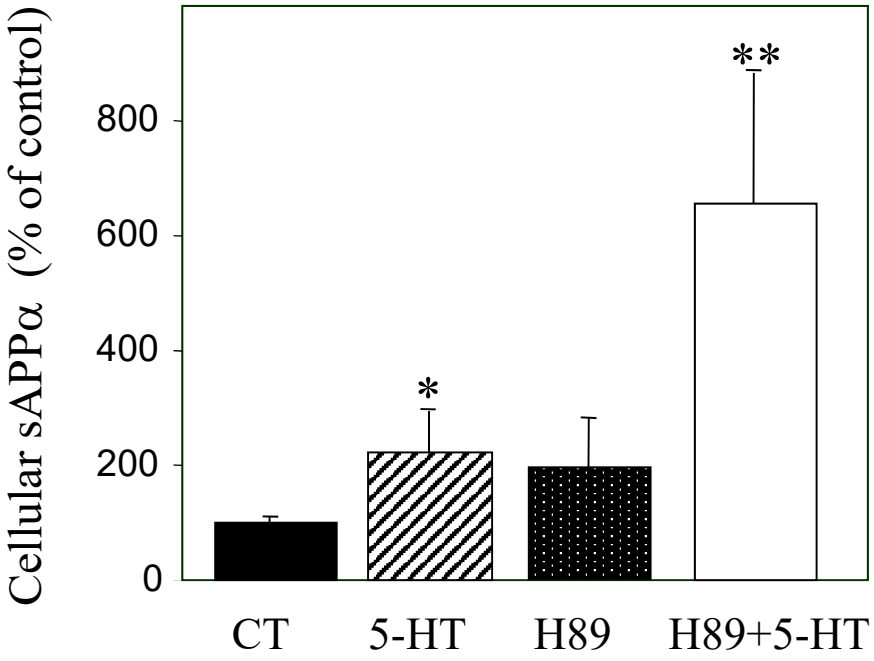

A
Control
Forskolin
8-Br-cAMP
Secreted
sAPPα
Control
Forskolin
8-Br-cAMP
Cellular
sAPPα
B
CT
5-HT
H89
H89+5-HT
Secreted
sAPPα
110 kDa
CT
5-HT
H89
H89+5-HT
Cellular
sAPPα
110 kDa

Figure 6

C


Cellular sAPPα (% of control)
800
600
400
200
0
*
**
CT
5-HT
H89
H89+5-HT

D

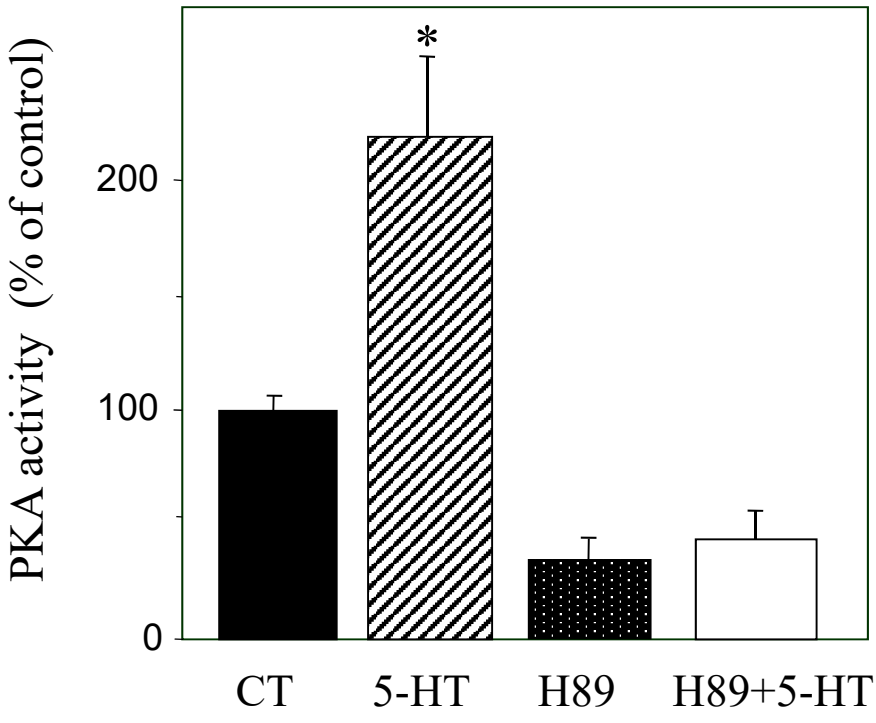
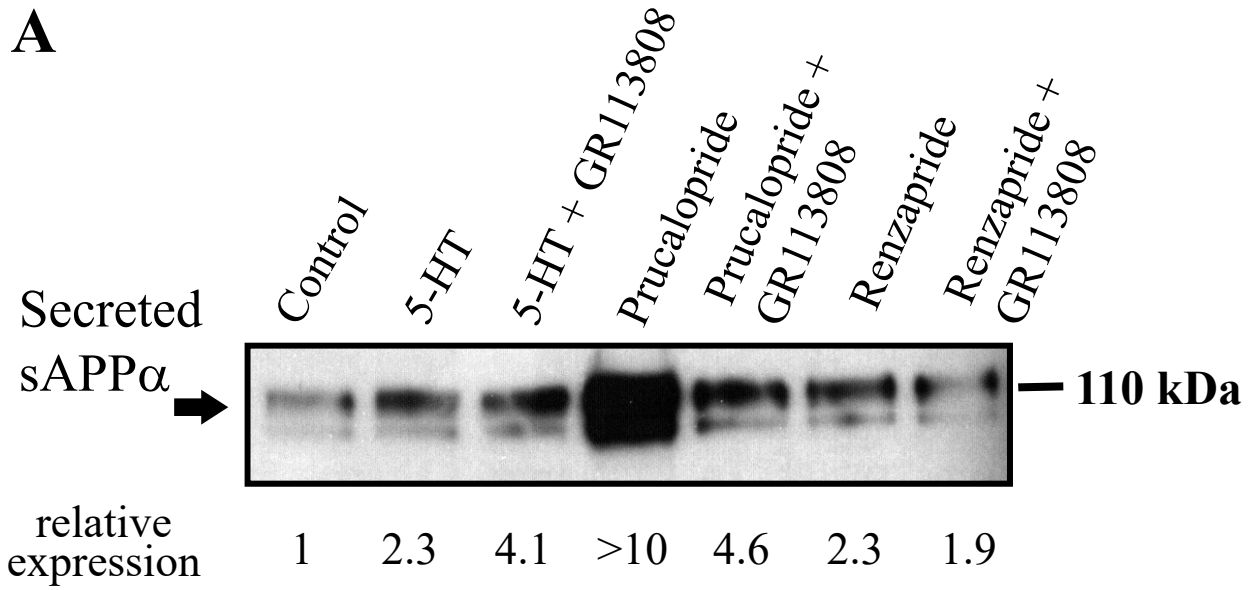

PKA activity (% of control)
200
100
0
*
CT
5-HT
H89
H89+5-HT

Figure 6


A
Control
5-HT
5-HT + GR113808
Prucalopride
Prucalopride + GR113808
Renzapride
Renzapride + GR113808
Secreted sAPPα
110 kDa
relative expression
1
2.3
4.1
>10
4.6
2.3
1.9

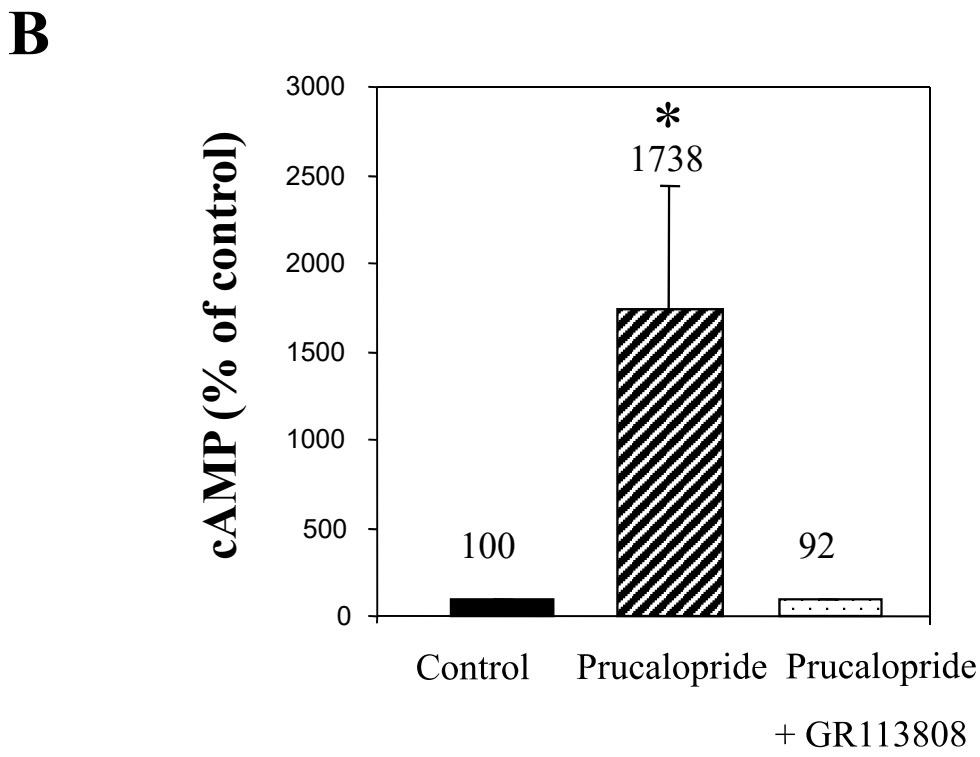

B
cAMP (% of control)
3000
2500
2000
1500
1000
500
0
*
1738
100
92
Control
Prucalopride
Prucalopride + GR113808

Figure 7

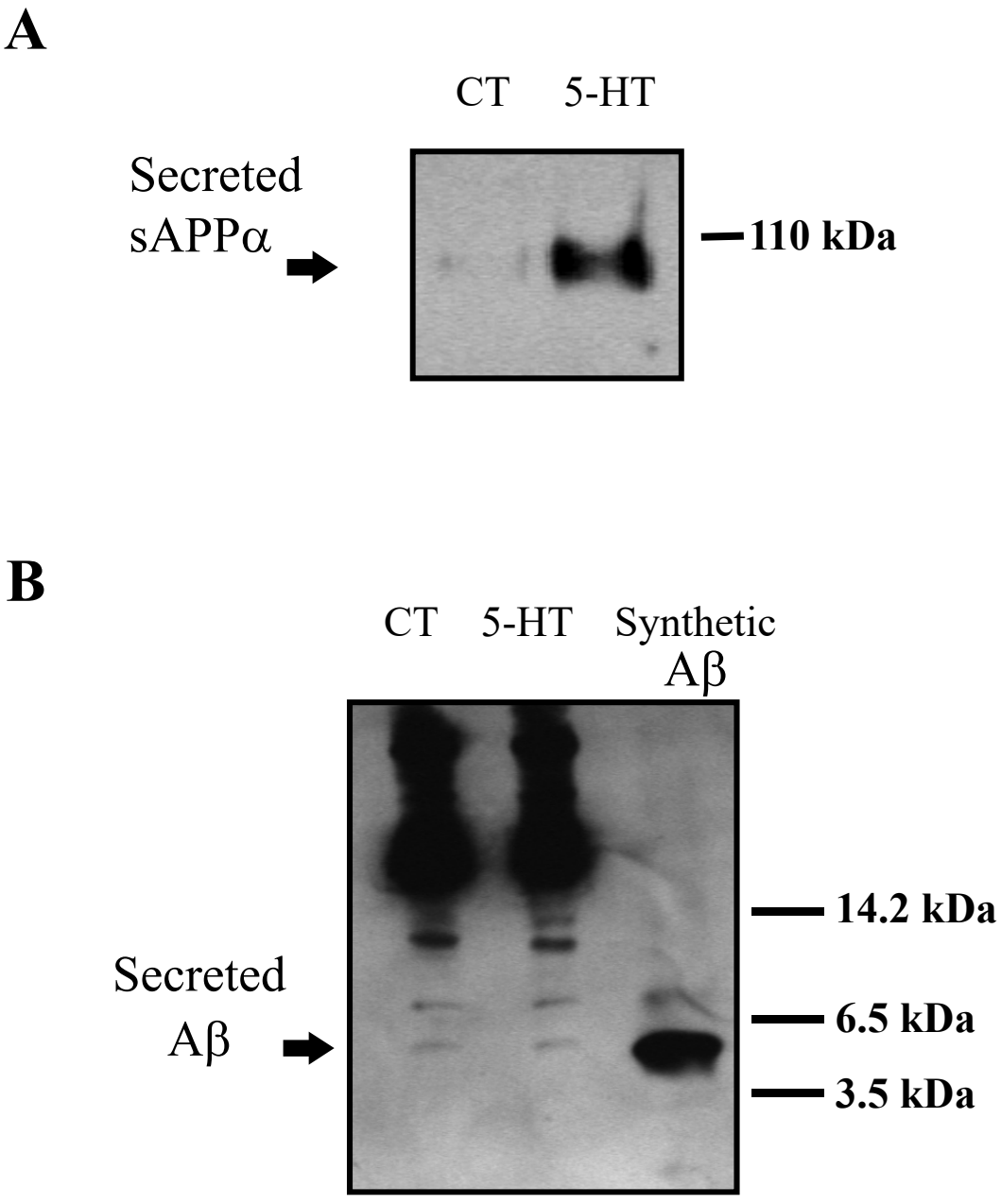

A
CT
5-HT
Secreted
sAPPα
110 kDa
B
CT
5-HT
Synthetic
Aβ
Secreted
Aβ
14.2 kDa
6.5 kDa
3.5 kDa

Figure 8