

## 5-HT<sub>4</sub> Receptors Constitutively Promote the Non-Amyloidogenic Pathway of APP Cleavage and Interact with ADAM10

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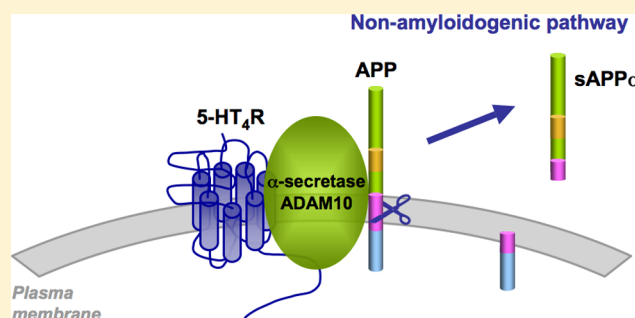
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### Supporting Information

**ABSTRACT:** In addition to the amyloidogenic pathway, amyloid precursor protein (APP) can be cleaved by  $\alpha$ -secretases, producing soluble and neuroprotective APP alpha (sAPP $\alpha$ ) (nonamyloidogenic pathway) and thus preventing the generation of pathogenic amyloid- $\beta$ . However, the mechanisms regulating APP cleavage by  $\alpha$ -secretases remain poorly understood. Here, we showed that expression of serotonin type 4 receptors (5-HT<sub>4</sub>Rs) constitutively (without agonist stimulation) induced APP cleavage by the  $\alpha$ -secretase ADAM10 and the release of neuroprotective sAPP $\alpha$  in HEK-293 cells and cortical neurons. This effect was independent of cAMP production. Interestingly, we demonstrated that 5-HT<sub>4</sub> receptors physically interacted with the mature form of ADAM10. Stimulation of 5-HT<sub>4</sub> receptors by an agonist further increased sAPP $\alpha$  secretion, and this effect was mediated by cAMP/Epac signaling. These findings describe a new mechanism whereby a GPCR constitutively stimulates the cleavage of APP by  $\alpha$ -secretase and promotes the nonamyloidogenic pathway of APP processing.

**KEYWORDS:** Alpha-secretase, Alzheimer's disease, sAPP alpha, serotonin



According to the amyloid hypothesis, alteration in synaptic transmission and neuronal loss observed in Alzheimer's disease (AD) mainly result from the formation of toxic amyloid- $\beta$  (A $\beta$ ) oligomers followed by the extracellular accumulation of A $\beta$  aggregates. A $\beta$  is produced by the successive cleavage of a transmembrane amyloid precursor protein (APP) by  $\beta$ -secretase and  $\gamma$ -secretase.<sup>1</sup> In addition to this amyloidogenic pathway, APP can be cleaved by  $\alpha$ -secretases, a set of membrane-bound proteases of the ADAM (A disintegrin and metalloprotease) family, generating the soluble APP ectodomain (sAPP $\alpha$ ) and a membrane-bound carboxy-terminal fragment (nonamyloidogenic pathway). As  $\alpha$ -secretases cleave APP within the A $\beta$  sequence, APP shedding by  $\alpha$ -secretases prevents the generation of the pathogenic A $\beta$  peptide.<sup>2,3</sup> Therefore, enhancing  $\alpha$ -secretase expression or activity has been considered as a valuable strategy for inhibiting A $\beta$  formation. For instance, it has recently been shown that activation of the transcription of the gene encoding the  $\alpha$ -secretase ADAM10, by retinoic acid and sirtuin 1 (SIRT1), reduces A $\beta$  production.<sup>4–6</sup>

Previous reports have shown that G protein-coupled receptors (GPCRs) can differentially affect A $\beta$  peptide production by either modulating the cellular trafficking of APP or by influencing

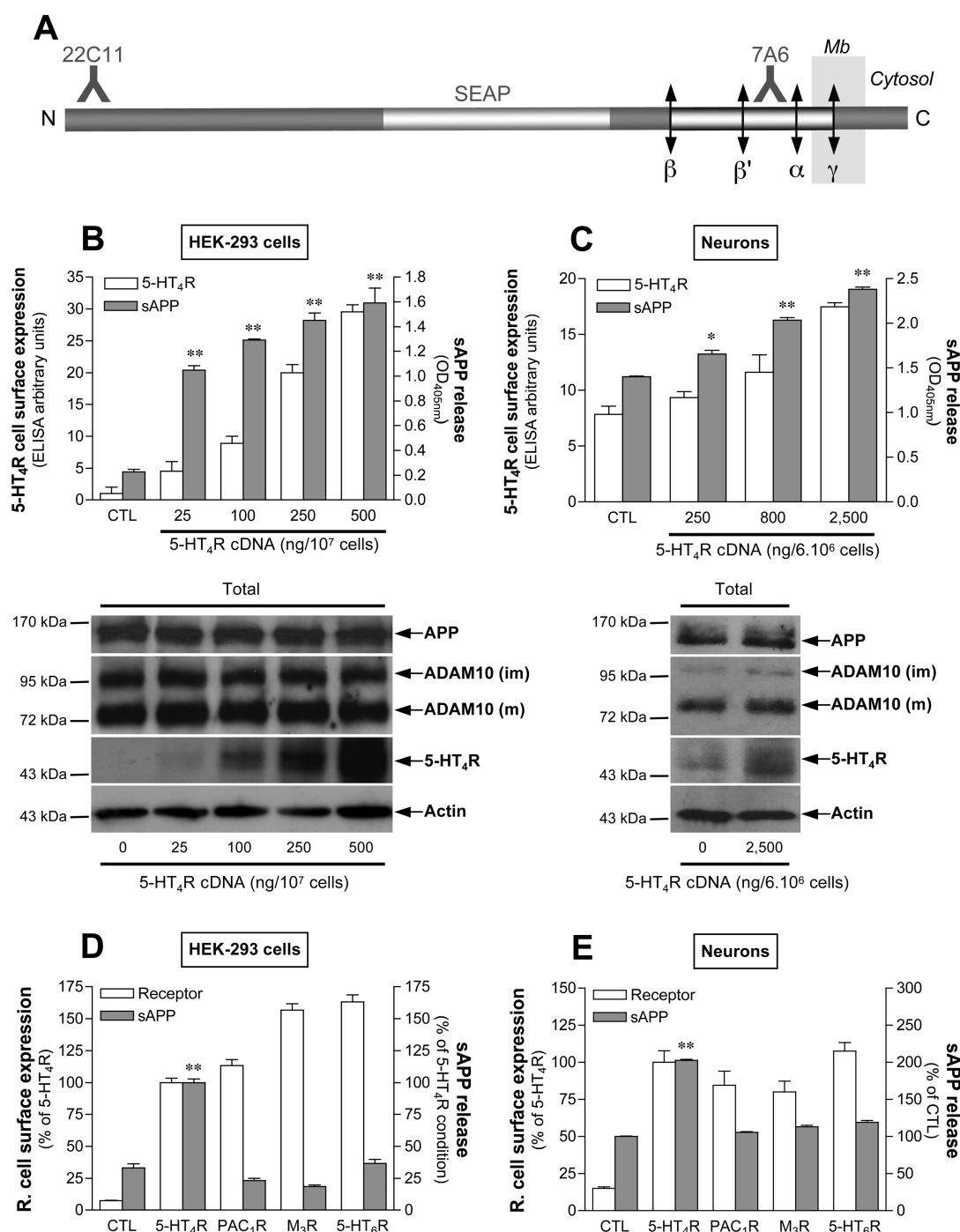
the activity and trafficking of  $\alpha$ -,  $\beta$ - and  $\gamma$ -secretases. Moreover, both the expression and the stimulation of GPCRs can affect APP metabolism.<sup>7</sup> GPCRs that enhance sAPP $\alpha$  production by stimulating  $\alpha$ -secretase activities include the muscarinic M<sub>1</sub>-M<sub>3</sub> receptors, mGlu2 metabotropic glutamate receptor, serotonin 2A (5-HT<sub>2A</sub>) and 5-HT<sub>2C</sub> receptors, corticotropin-releasing factor (CRF) receptor 1, purinergic receptor P2X<sub>7</sub> and pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 (PAC<sub>1</sub>) receptor.<sup>7</sup> These GPCRs are supposed to have a beneficial effect, because sAPP $\alpha$  exerts neuroprotective and neurotrophic effects and enhances long-term potentiation.<sup>8,9</sup> In addition, promoting sAPP $\alpha$  formation generally decreases the production of toxic A $\beta$  peptides. Several signaling cascades, such as the PKC, cAMP/PKA, MAP-Kinase, and PI3K pathways, have been involved in the stimulation of sAPP $\alpha$  release mediated by

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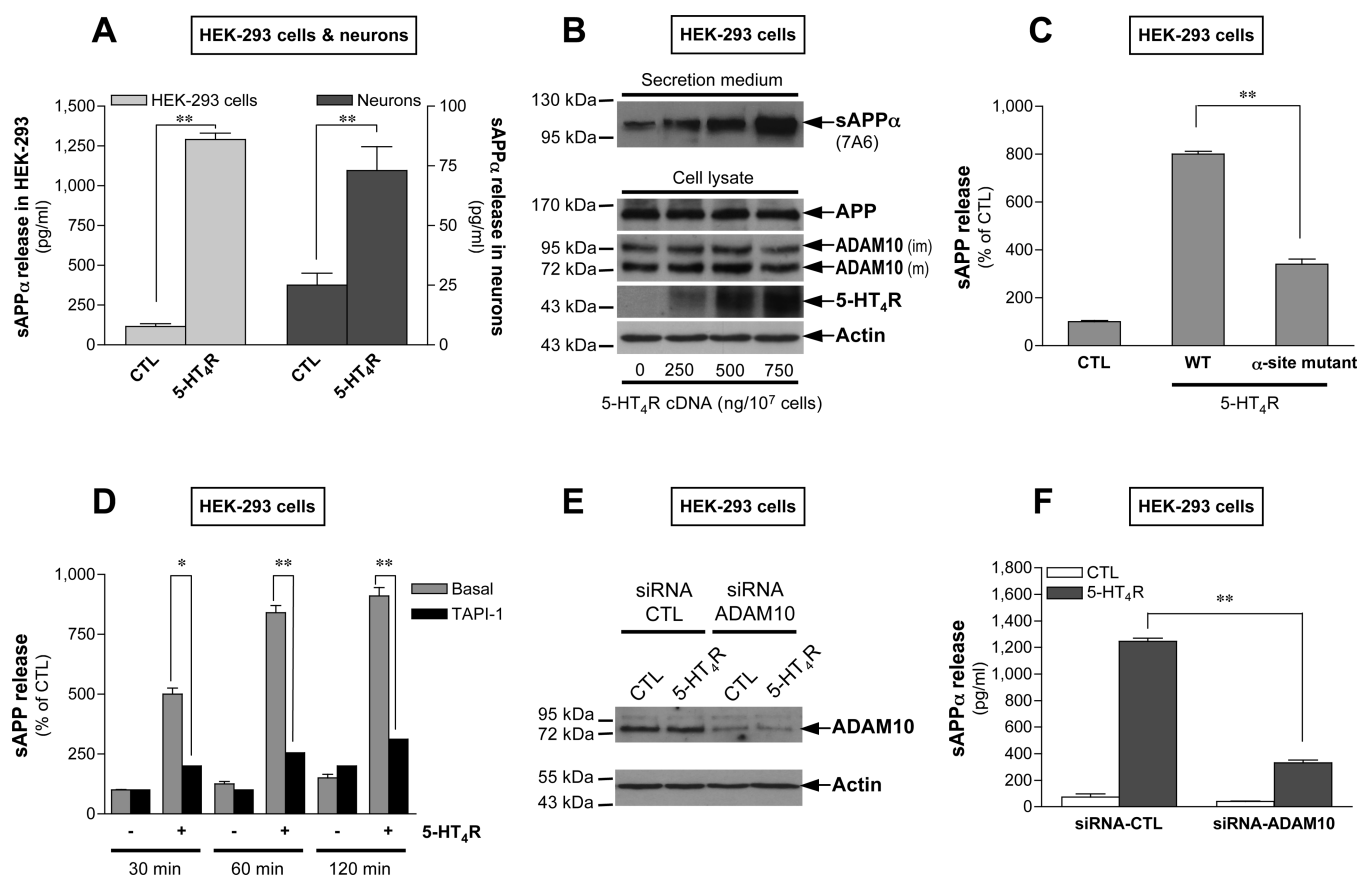
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**Figure 1.** 5-HT<sub>4</sub>R expression constitutively enhances sAPP release. (A) Schematic representation of APP. The position of the SEAP-tag is depicted. The epitopes of the anti-sAPP (22C11) and sAPP $\alpha$  antibodies (7A6) and the  $\beta$ -,  $\beta'$ -,  $\alpha$ -, and  $\gamma$ -secretase cleavage sites are also indicated. Mb, membrane. (B, C) HEK-293 cells (B) or embryonic cortical neurons (C) were transiently transfected with plasmids encoding Myc-tagged 5-HT<sub>4</sub>R and SEAP-tagged APP (500 ng/10<sup>7</sup> cells or 2500 ng/6  $\times$  10<sup>6</sup> cells, respectively). Twenty-four h (B) or 4 days (C) post-transfection, cell surface expression of 5-HT<sub>4</sub>R was assessed by ELISA in nonpermeabilized cells using the anti-Myc antibody (B and C, white bars). In parallel, sAPP release during a 2 h period (B) or 12 h period (C) was evaluated by measuring the alkaline phosphatase activity (colorimetric assay) of cell supernatants (B and C, gray bars). (B, C) Total APP expression was detected in cell lysates in the corresponding transfection conditions, using the 22C11 antibody. Expression levels of ADAM10, 5-HT<sub>4</sub>R and actin were provided as controls. (D) HEK-293 cells were transiently transfected with plasmids encoding epitope-tagged 5-HT<sub>4</sub>R (300 ng/10<sup>7</sup> cells), PAC<sub>1</sub>R (700 ng/10<sup>7</sup> cells), M<sub>3</sub>R (300 ng/10<sup>7</sup> cells), or 5-HT<sub>6</sub>R (75 ng/10<sup>7</sup> cells) to ensure equivalent cell-surface expression of the different receptors, and SEAP-tagged APP (500 ng/10<sup>7</sup> cells). Cell surface expression of the receptors and quantification of sAPP release (gray bars) were measured as described in (B) using appropriate primary antibodies. (E) Embryonic cortical neurons were transiently cotransfected with a plasmid encoding epitope-tagged 5-HT<sub>4</sub>R (800 ng/6  $\times$  10<sup>6</sup> cells), PAC<sub>1</sub>R (1500 ng/6  $\times$  10<sup>6</sup> cells), M<sub>3</sub>R (1000 ng/6  $\times$  10<sup>6</sup> cells), or 5-HT<sub>6</sub>R (800 ng/10<sup>7</sup> cells) and SEAP-tagged APP (2500 ng/6  $\times$  10<sup>6</sup> cells). Cell surface expression of the receptors (white bars) and quantification of sAPP release during a 4 day period was performed as described in (C). CTL, Condition transfected with only SEAP-tagged APP. Data are the means  $\pm$  SD of at least three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 vs CTL corresponding value.



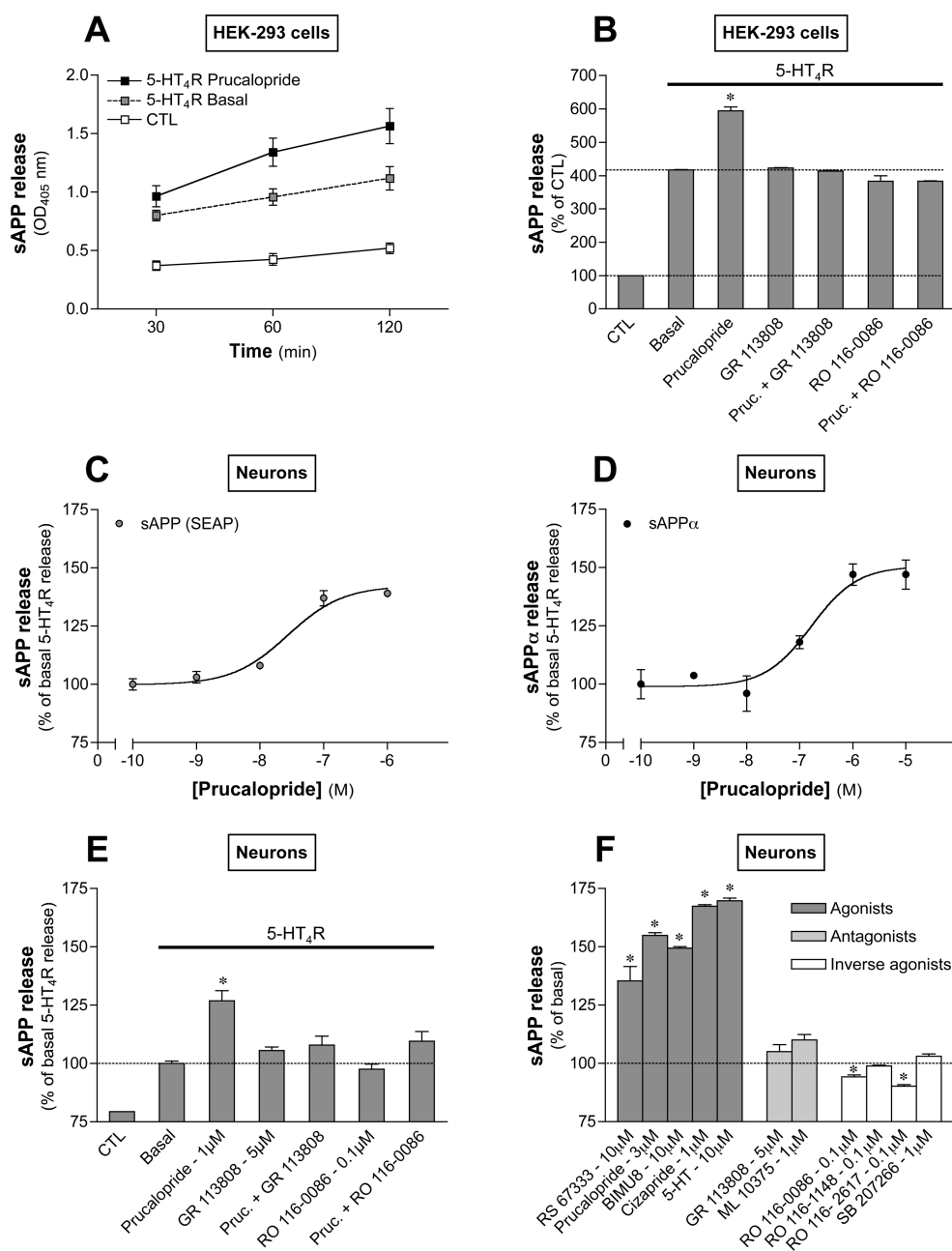
**Figure 2.** 5-HT $_4$ R specifically enhances sAPP $\alpha$  release. (A) HEK-293 cells were transiently transfected with plasmids encoding Myc-tagged 5-HT $_4$ R (250 ng/10 $^7$  cells), SEAP-tagged APP (500 ng/10 $^7$  cells). 2 h-sAPP $\alpha$  release in the medium was detected using an ELISA kit designed to quantify mouse/rat sAPP $\alpha$ . Cortical neurons were transfected with empty plasmid (CTL) or plasmid encoding Flag-tagged 5-HT $_4$ R (250 ng/6  $\times$  10 $^6$  cells). Four days after transfection, sAPP $\alpha$  released by neurons during 12 h was quantified using the same kit. \*\* $p$  < 0.01 vs corresponding CTL value. (B) Proteins in supernatants from HEK-293 cells transiently transfected with plasmids encoding Myc-tagged 5-HT $_4$ R and SEAP-tagged APP (500 ng/10 $^7$  cells) were immunoprecipitated with the 22C11 antibody. Immunoprecipitated complexes were analyzed by Western blotting with the 7A6 (anti-sAPP $\alpha$ ) antibody (upper panel). Expression levels of APP, ADAM10, 5-HT $_4$ R, and actin in cell lysates were provided as controls (lower panel). (C) HEK-293 cells were transiently cotransfected with plasmids encoding Myc-tagged 5-HT $_4$ R (250 ng/10 $^7$  cells) and either wild type SEAP-tagged APP or the  $\alpha$  site APP mutant (R $^{609$ D, K $^{612$ E}) (500 ng/10 $^7$  cells). sAPP release during 2 h was evaluated by measuring the alkaline phosphatase activity in the cell medium, \*\* $p$  < 0.01 vs corresponding value in cells expressing the wild type SEAP-tagged APP. (D) HEK-293 cells were transiently transfected with plasmids encoding Myc-tagged 5-HT $_4$ R (250 ng/10 $^7$  cells) and SEAP-tagged APP (500 ng/10 $^7$  cells). 24 h post-transfection, cells were treated with vehicle alone or 50  $\mu$ M TAPI-1 and SEAP activity during 30, 60, and 120 min was measured in the spent medium. \* $p$  < 0.05, \*\* $p$  < 0.01 vs corresponding value in absence of TAPI-1. (E, F) HEK-293 cells were transiently transfected with plasmids encoding Myc-tagged 5-HT $_4$ R (250 ng/10 $^7$  cells), SEAP-tagged APP (500 ng/10 $^7$  cells), and either control (CTL) or ADAM10 siRNA. ADAM10 expression was assessed by Western blotting (E) and sAPP $\alpha$  release (F) as described in (A), \*\* $p$  < 0.01 vs 5-HT $_4$ R-mediated release in cells transfected with control siRNA. CTL, Condition transfected with only SEAP-tagged APP. Data are the means  $\pm$  SD of at least three independent experiments.

these GPCRs.<sup>10</sup> However, the mechanisms by which they activate  $\alpha$ -secretases are not always well understood.

The 5-HT $_4$  receptor (5-HT $_4$ R) is another GPCR raising particular interest in view of its possible influence at different levels in the pathogenesis of AD. Activation of 5-HT $_4$ R stimulates acetylcholine release in prefrontal cortex and hippocampus and improves learning and memory in various preclinical paradigms of memory acquisition and retention.<sup>11,12</sup> These findings suggest that 5-HT $_4$ R agonists might be used for improving cholinergic function and cognition, which are both compromised in AD. Stimulation of 5-HT $_4$ R also enhances sAPP $\alpha$  release and reduces A $\beta$  formation in neuronal cultures.<sup>13,14</sup> Whether the effect of 5-HT $_4$ R on APP metabolism reflects an increase in  $\alpha$ -secretase activity or is the result of a modification of cellular trafficking of  $\alpha$ -secretases and APP remains to be elucidated. Like many GPCRs, 5-HT $_4$ R interacts with a large set of intracellular proteins. Some of them, such as

p11 and SNX27, are known to affect 5-HT $_4$ R transport and might also influence the trafficking of proteins physically and functionally connected with the receptor.<sup>15,16</sup> Further supporting the implication of 5-HT $_4$ R in AD, the number of receptor binding sites is reduced in the hippocampus of patients with AD.<sup>17</sup> However, the specific effects of 5-HT $_4$ R expression and activation on APP metabolism remain to be elucidated.

In this study, we show that 5-HT $_4$ R expression at physiological levels (around 120–550 fmol/mg of protein)<sup>17–21</sup> enhances sAPP $\alpha$  release in transfected HEK-293 cells and cortical neurons in primary culture via two different processes, a “constitutive” release which has never been reported and a previously described agonist-regulated one.<sup>13,14</sup> We also show that 5-HT $_4$  receptors physically associate with ADAM10, a mechanism that may be involved in the constitutive release of sAPP $\alpha$ .



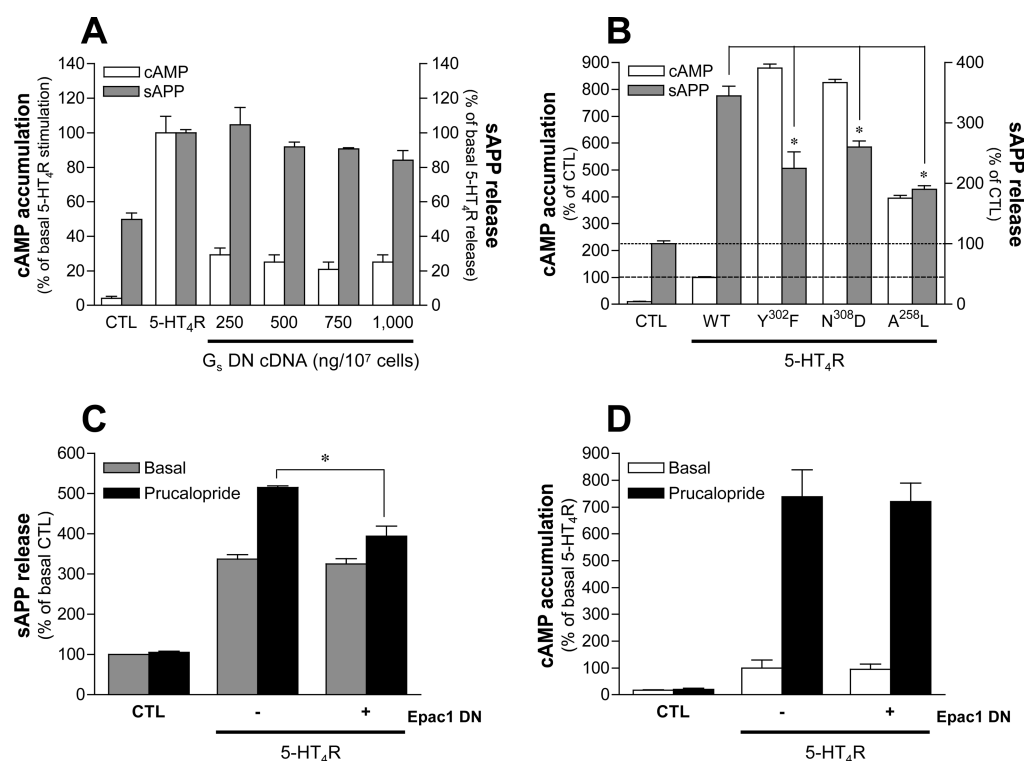
**Figure 3.** 5-HT<sub>4</sub>R stimulation further enhances sAPP $\alpha$  release induced by 5-HT<sub>4</sub>R expression. (A, B) HEK-293 cells were transiently transfected with plasmids encoding Myc-tagged 5-HT<sub>4</sub>R (250 ng/10<sup>7</sup> cells) and SEAP-tagged APP (500 ng/10<sup>7</sup> cells). Twenty-four h post-transfection, cells were treated with vehicle alone (CTL) or 1  $\mu$ M prucalopride with or without 5  $\mu$ M GR 113808 or 0.1  $\mu$ M RO 116-0086 for 2 h, and then sAPP release was evaluated by measuring the alkaline phosphatase activity in the medium. (C, D) Embryonic cortical neurons were transiently transfected with plasmids encoding Flag-tagged 5-HT<sub>4</sub>R (800 ng/6  $\times$  10<sup>6</sup> cells) with (C) or without (D) SEAP-tagged APP (2500 ng/6  $\times$  10<sup>6</sup> cells). Four days after transfection, neurons were treated with increasing concentrations of prucalopride and sAPP release during 12 h was evaluated by measuring the alkaline phosphate activity (C) or by using the sAPP $\alpha$  ELISA kit (D). (E, F) Cortical neurons cotransfected with plasmids encoding Flag-tagged 5-HT<sub>4</sub>R (250 ng/6  $\times$  10<sup>6</sup> cells) and SEAP-tagged APP (2500 ng/6  $\times$  10<sup>6</sup> cells) were exposed to the indicated 5-HT<sub>4</sub>R agonists/antagonists for 12 h and sAPP release was evaluated by measuring the alkaline phosphatase activity. CTL, Condition transfected with only SEAP-tagged APP. Data are the means  $\pm$  SD of at least three independent experiments. In (B), (E), and (F), \**p* < 0.05 vs the corresponding value in cells not treated with agonist.

## RESULTS AND DISCUSSION

**Expression of 5-HT<sub>4</sub> Receptors Enhances sAPP Release from HEK-293 Cells and Cortical Neurons.** To investigate the impact of 5-HT<sub>4</sub>R expression on APP metabolism, we first cotransfected HEK-293 cells with the SEAP-tagged APP plasmid (Figure 1A) and increasing amounts of the Myc-tagged 5-HT<sub>4</sub>R construct. 5-HT<sub>4</sub>R density at the cell surface was assessed by ELISA (Figure 1B) and [<sup>3</sup>H]GR 113808 saturation binding

(from 120 to 750 fmol/mg protein corresponding to 25 to 500 ng cDNA/10<sup>7</sup> cells, respectively). Expression of increasing amounts of 5-HT<sub>4</sub>R did not significantly affect total APP (APP band intensity/actin band intensity = 97  $\pm$  4% of control, 96  $\pm$  7%, 108  $\pm$  13%, or 89  $\pm$  18% for 25, 100, 250, or 500 ng of 5-HT<sub>4</sub>R cDNA respectively, *n*  $\geq$  3 per each group) or ADAM10 expression (intensity of ADAM10 bands/actin band intensity = 104  $\pm$  5% of control, 96  $\pm$  11%, 113  $\pm$  18%, or 87  $\pm$  21% for 25,





**Figure 4.** Role of the G<sub>s</sub>/cAMP/Epac pathway in 5-HT<sub>4</sub>R-induced sAPP $\alpha$  release. HEK-293 cells were transiently transfected with plasmids encoding Myc-tagged 5-HT<sub>4</sub>R (250 ng/10<sup>7</sup> cells), SEAP-tagged APP (500 ng/10<sup>7</sup> cells), and a dominant negative G<sub>s</sub> protein (A) or a dominant-negative form of Epac1 (500 ng/10<sup>7</sup> cells) (C, D). (B) HEK-293 cells were transiently transfected with plasmids encoding Myc-tagged wild type 5-HT<sub>4</sub>R or mutants (Y<sup>302</sup>F, N<sup>308</sup>D, or A<sup>258</sup>L) with high level of constitutive G<sub>s</sub> signaling activity and SEAP-tagged APP (500 ng/10<sup>7</sup> cells each). Twenty-four h after transfection, cAMP accumulation and sAPP release during 2 h were measured. CTL, Condition transfected with only SEAP-tagged APP. Data are the means  $\pm$  SD of at least three independent experiments. In (D) and (F), \**p* < 0.05 vs the corresponding value in cells not treated with agonist.

100, 250, or 500 ng of 5-HT<sub>4</sub>R cDNA, respectively, *n*  $\geq$  3 per each group) or ADAM10 (Figure 1B), while it potently enhanced the release of soluble APP (sAPP) already at low receptor expression (Figure 1B). This effect was independent of the 5-HT<sub>4</sub>R variant used: the mouse (a, b, e, and f) and human (a, g, and i) C-terminal variants as well as further truncations of the 5-HT<sub>4</sub>R C-terminal domain ( $\Delta$ 358 and  $\Delta$ 329) could all promote sAPP release (Supporting Information Figure 1A–C). Overexpression of 5-HT<sub>4</sub>R in primary cultures of cortical neurons produced a comparable increase in sAPP release (Figure 1C) without affecting APP and ADAM10 levels (APP band intensity/actin band intensity =  $88 \pm 19\%$  of control, intensity of ADAM10 bands/actin band intensity =  $111 \pm 14\%$  for 2.500 ng of 5-HT<sub>4</sub>R cDNA *n*  $\geq$  3 per each group). Expression of similar amounts of PAC<sub>1</sub> or muscarinic M<sub>3</sub> receptor, which both increase sAPP $\alpha$  release upon activation by their respective agonists,<sup>22,23</sup> failed to enhance sAPP release in HEK-293 cells in the absence of agonist (Figure 1D). Moreover, comparative levels of overexpressed 5-HT<sub>6</sub> receptor, another serotonin receptor positively coupled to the G<sub>s</sub>/cAMP pathway, did not induce sAPP production (Figure 1D). Similarly, only overexpression of 5-HT<sub>4</sub>R, but not of PAC<sub>1</sub>, M<sub>3</sub>, or 5-HT<sub>6</sub> receptors, in cortical neurons enhanced the release of sAPP from neurons (Figure 1E).

**Expression of 5-HT<sub>4</sub> Receptors Specifically Enhances sAPP $\alpha$  Release.** To identify the nature of the sAPP form (sAPP $\alpha$  or sAPP $\beta$ ) secreted upon 5-HT<sub>4</sub>R expression, we used a sandwich ELISA kit based on two antibodies, with one directed against the N-terminal sequence of APP and the other recognizing the neo-sAPP $\alpha$  epitope. Using this kit, we showed that overexpression of 5-HT<sub>4</sub>R in both HEK-293 cells and in

cortical neurons led to an increase in the release of sAPP $\alpha$  (Figure 2A). Furthermore, SEAP-tagged APP in supernatants from transfected HEK-293 cells was immunoprecipitated with the 22C11 antibody and immunodetected using a site-specific antibody (7A6) that specifically recognizes sAPP $\alpha$ , but not shorter sAPP species, such as sAPP $\beta'$  and sAPP $\beta$  (Figures 1A and 2B).<sup>24</sup> The immunoreactive signals detected by 7A6 antibody increased concomitantly with 5-HT<sub>4</sub>R expression, while no significant change of APP and ADAM10 expression was observed (Figure 2B). Mutation of the residues (R<sup>609</sup>D and K<sup>612</sup>E) surrounding the  $\alpha$ -secretase cleavage site ( $\alpha$ -site APP mutant) can impair about 50% of the  $\alpha$ -cleavage of APP.<sup>25</sup> Coexpression of this mutant with 5-HT<sub>4</sub>R in HEK-293 cells strongly decreased 5-HT<sub>4</sub>R-induced sAPP release (Figure 2C), indicating that 5-HT<sub>4</sub>R expression stimulates the nonamyloidogenic  $\alpha$ -cleavage of APP.

Several metalloproteinases, including ADAM9, ADAM10, and ADAM17 (also called TACE, tumour necrosis factor  $\alpha$ -converting enzyme) have been proposed as potential  $\alpha$ -secretases that promote the shedding of APP ectodomain. TAPI-1, a metalloproteinase inhibitor, almost completely abolished 5-HT<sub>4</sub>R-induced sAPP $\alpha$  release from HEK-293 cells (Figure 2D), suggesting the involvement of a member of the ADAM family of metalloproteinases. Moreover, reduction of ADAM10 expression by transfection of ADAM10-specific siRNA (Figure 2E) diminished basal processing of APP in absence of 5-HT<sub>4</sub> receptor and also markedly decreased sAPP $\alpha$  release following 5-HT<sub>4</sub>R expression (Figure 2F). This indicates that ADAM10 contributes significantly to 5-HT<sub>4</sub>R-induced sAPP release. In contrast, specific inhibitors of  $\beta$ -secretase (KTEEL-

SEVN-statine-VAEF) or  $\gamma$ -secretase (L-685,458) did not affect the release of sAPP $\alpha$  induced by 5-HT $_4$ R (Supporting Information Figure 2).

#### 5-HT $_4$ R Stimulation Further Enhances sAPP $\alpha$ Release.

Previous studies have shown that 5-HT $_4$ R stimulation by agonists increases sAPP $\alpha$  production.<sup>26</sup> Consistent with these findings, treating HEK-293 cells that transiently express 5-HT $_4$ R and SEAP-tagged APP with prucalopride, a 5-HT $_4$ R agonist, for 2 h increased by ~50% the release of sAPP $\alpha$  induced by 5-HT $_4$ R expression (Figure 3A and B). This effect was blocked by GR 113808 and RO 116-0086, a neutral antagonist and an inverse agonist of 5-HT $_4$ R at G $_s$  signaling, respectively. Neither GR 113808 nor RO 116-0086 alone altered the secretion of sAPP in the absence of the agonist (Figure 3B).

5-HT $_4$ R stimulation by prucalopride increased sAPP $\alpha$  release by cultured cortical neurons that transiently expressed 5-HT $_4$ R and SEAP-tagged APP in a concentration-dependent manner ( $pEC_{50} = 0.85 \pm 0.22 \mu\text{M}$ ,  $n = 3$ ), as assessed by monitoring the SEAP activity (Figure 3C). Similarly, prucalopride stimulated sAPP $\alpha$  release from endogenous APP, as assessed using a sAPP $\alpha$  specific ELISA kit (Figure 3D). Like in HEK-293 cells, the prucalopride effect was blocked by GR 113808 and RO 116-0086 (Figure 3E). Treatment of neurons with maximally effective concentrations of various synthetic 5-HT $_4$ R agonists (10  $\mu\text{M}$  RS 67333, 3  $\mu\text{M}$  prucalopride, 10  $\mu\text{M}$  BIMU8, 1  $\mu\text{M}$  cisapride)<sup>27</sup> or with 10  $\mu\text{M}$  5-HT stimulated sAPP secretion to a similar extent as prucalopride. In contrast, 5-HT $_4$ R ligands exhibiting either antagonist (GR 113808, ML 10375) or inverse agonist (RO 116-1148, SB 207266) properties on 5-HT $_4$  receptors coupled to G $_s$ <sup>27</sup> did not significantly affect sAPP release from neurons (Figure 3F). However, RO 116-0086 and RO 116-2617, two inverse agonists at G $_s$  signaling decreased basal sAPP release. GPCRs proteins are adapting many conformations. The constitutive 5-HT $_4$ R conformation which induces cAMP production (via G protein) is unlikely to be similar to the constitutive conformation of the same 5-HT $_4$  receptor which is producing sAPP $\alpha$  (without a need of G protein). The pharmacology of these two different constitutive conformations is likely to be different. Thus, it is not surprising that some "classical" inverse agonists on the 5-HT $_4$  receptor inducing constitutive cAMP production are not inverse agonists on the constitutive conformation required for sAPP $\alpha$  production.

#### The Constitutive sAPP $\alpha$ Release Induced by 5-HT $_4$ R Expression Is Independent of the cAMP/Epac Pathway.

As cAMP and Epac (cAMP-responsive Rap1 guanine nucleotide exchange factor) regulate sAPP $\alpha$  release elicited by 5-HT $_4$ R stimulation,<sup>13</sup> we investigated whether receptor coupling to G $_s$  and recruitment of the cAMP/Epac pathway were also necessary for enhancing constitutive sAPP $\alpha$  secretion upon 5-HT $_4$ R expression. Expression of a dominant-negative form of G $_s$  strongly reduced agonist-independent cAMP accumulation in HEK-293 cells expressing 5-HT $_4$ Rs, but did not affect sAPP release (Figure 4A). Moreover, expression of 5-HT $_4$ R mutants with high level of constitutive G $_s$  signaling activity (Y<sup>302</sup>F, N<sup>308</sup>D, or A<sup>258</sup>L)<sup>28,29</sup> increased basal cAMP production but were less active to increase sAPP release in comparison to wild type 5-HT $_4$ R (Figure 4B). Similarly, overexpression of a dominant negative form of Epac1 did not modify the constitutive sAPP secretion induced by 5-HT $_4$ R expression (Figure 4C), whereas the agonist (prucalopride)-dependent sAPP release was reduced, as previously reported<sup>13</sup> (Figure 4C). As expected, the Epac1 dominant negative form did not affect cAMP production elicited by 5-HT $_4$ R activation (Figure 4D). Collectively, these results

indicate that only agonist-elicited sAPP secretion is dependent on 5-HT $_4$ R coupling to G $_s$  protein and engagement of the cAMP/Epac pathway.

#### 5-HT $_4$ Receptors Physically Interact with the $\alpha$ -Secretase ADAM10.

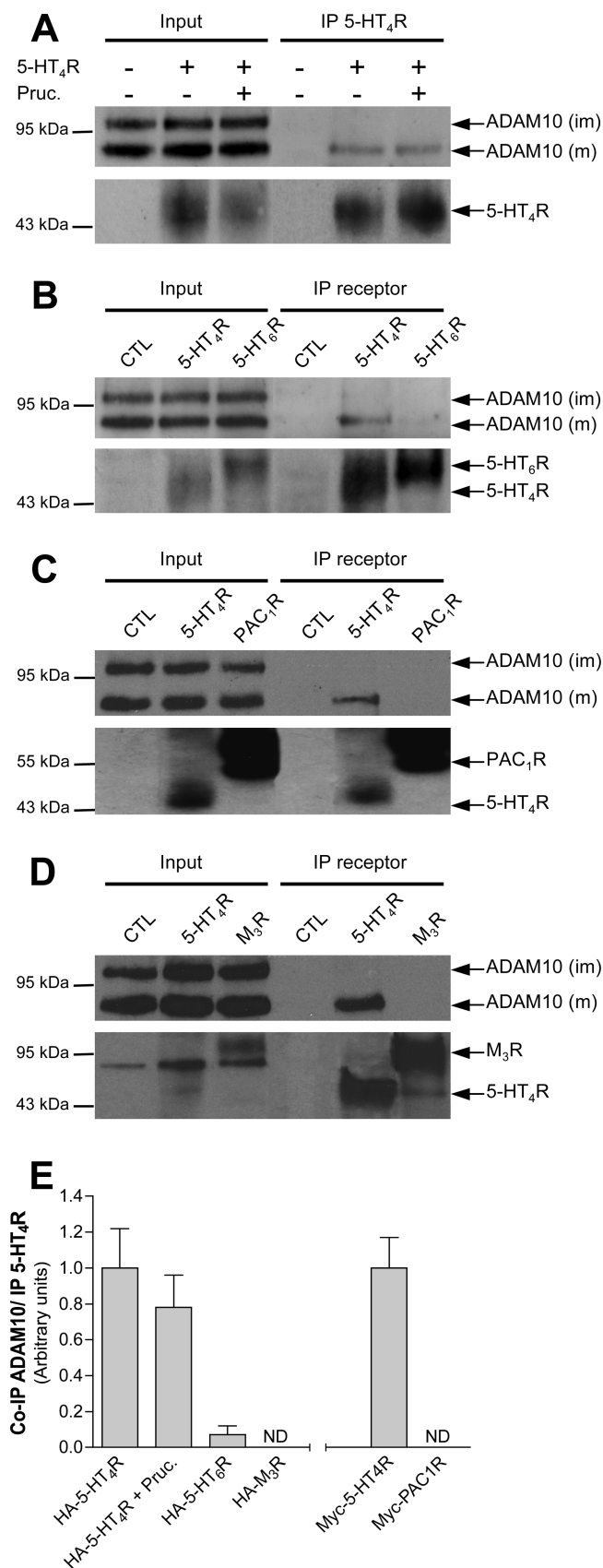
To further explore the possible mechanism by which 5-HT $_4$ R constitutively activates ADAM10-dependent APP cleavage, we examined whether 5-HT $_4$ R physically interacts with this  $\alpha$ -secretase. ADAM10 coimmunoprecipitated with HA-tagged-5-HT $_4$ R in HEK-293 cells (Figure 5A). Although two immunoreactive ADAM10 species with apparent molecular masses of 95 (immature pro-enzyme) and 69 kDa (mature, active form) were detected in whole cell lysates by Western blotting, only the 69 kDa mature form, which originates from the immature form by removal of a 194 amino acid pro-domain and is localized at the plasma membrane,<sup>30</sup> was coimmunoprecipitated with 5-HT $_4$ R. Prucalopride stimulation of 5-HT $_4$ R did not modify the ability of the receptor to interact with ADAM10, thus demonstrating the existence of a "constitutive" specific complex composed of 5-HT $_4$ R and active ADAM10 (Figure 5A).

Active ADAM10 did not coimmunoprecipitate with overexpressed 5-HT $_6$  (Figure 5B), PAC $_1$  (Figure 5C), or M3 receptors (Figure 5D). Furthermore, different 5-HT $_4$ R mutants (A<sup>258</sup>L, N<sup>308</sup>D, and Y<sup>302</sup>F) and truncated forms devoid of the C-terminal domain ( $\Delta$ 327 and  $\Delta$ 329) showed the same ability to interact with ADAM10 as the wild type receptor (Supporting Information Figure 1D–G).

#### Promotion of Non-Amyloidogenic Pathway by 5-HT $_4$ Receptors.

The nature of APP processing is dependent on its cellular localization and on the cellular proteases involved in its cleavage. The present study shows that 5-HT $_4$ R expression, at physiological levels (120–550 fmol/mg proteins),<sup>17–21</sup> constitutively promotes the nonamyloidogenic processing of APP and that 5-HT $_4$  receptors physically interact with the mature form of the  $\alpha$ -secretase ADAM10. Several reports described the overlapping expression of APP and ADAM10 (but not ADAM17) in adult brain neurons<sup>31</sup> and suggested that ADAM10 could be the  $\alpha$ -secretase involved in the non-amyloidogenic pathway of APP, particularly in neurons.<sup>30</sup> This assumption was confirmed by RNAi-mediated silencing of ADAM10 and by using conditional ADAM10 knockout mice.<sup>24,32</sup> These findings were extended by the present study, which revealed association of the 5-HT $_4$ R with ADAM10. The ability of 5-HT $_4$ R to physically interact with mature ADAM10 was not shared by other GPCRs known to stimulate sAPP $\alpha$  secretion upon stimulation by their respective agonists. Indeed, expression of the PAC $_1$  and M $_3$  receptors did not trigger the formation of such a protein complex. Moreover, ADAM10 is retained in the ER by an arginine stretch (RRR).<sup>33</sup> Future work should test the possibility that association of ADAM10 with 5-HT $_4$ R masks this sequence, allowing the translocation of both proteins to the plasma membrane, a process associated with ADAM10 maturation.

It has been previously reported that stimulation of 5-HT $_4$ R promotes sAPP $\alpha$  release in a cAMP/Epac-dependent manner,<sup>13</sup> and these findings were confirmed in the present study. Although 5-HT $_4$ R displays high level of constitutive activity at the G $_s$ -adenylyl cyclase pathway,<sup>28</sup> several lines of evidence argued against a role of the cAMP/Epac pathway in the constitutive effect of 5-HT $_4$ R on APP processing: (1) inhibition of cAMP formation by an inverse agonist or by overexpression of the inactive form of G $_s$  had no effect on the level of sAPP $\alpha$  release elicited by 5-HT $_4$ R expression; (2) expression of a dominant



**Figure 5.** 5-HT<sub>4</sub>R interacts with the  $\alpha$ -secretase ADAM10. (A) HEK-293 cells were transiently transfected with plasmids encoding HA-tagged 5-HT<sub>4</sub>R (250 ng/10<sup>7</sup> cells) and SEAP-tagged APP (500 ng/10<sup>7</sup> cells). Twenty-four h after transfection, cells were treated 2 h with vehicle alone or with 1  $\mu$ M prucalopride (Prucalo), and then protein

lysates were immunoprecipitated with the anti-HA antibody. Whole cell extracts and immunoprecipitated material were analyzed by Western blotting using antibodies against ADAM10 (mature (m) and immature (im) forms) and against HA-tag. (B–D) HEK-293 cells were transiently cotransfected with plasmids encoding Myc- (C) or HA-tagged 5-HT<sub>4</sub>R (B, D), HA-5-HT<sub>6</sub>R (B), Myc-PAC<sub>1</sub>R (C), or HA-M<sub>3</sub>R (D) and SEAP-tagged APP. Twenty-four h after transfection, protein lysates were immunoprecipitated as described in (A). (E) Data correspond to the ratio of ADAM10 coimmunoprecipitation to 5-HT<sub>4</sub>R immunoprecipitation signals. ND, not detectable. CTL, Condition transfected with only SEAP-tagged APP. The data illustrated are representative of three independent experiments performed with different sets of cultured cells.

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negative Epac mutant did not affect constitutive induction of sAPP $\alpha$  release by 5-HT<sub>4</sub>R; and (3) mutants of 5-HT<sub>4</sub>R that constitutively increase adenylyl cyclase activity did not stimulate sAPP $\alpha$  secretion more than the wild type receptor. Collectively, these findings demonstrate that 5-HT<sub>4</sub>R promotes the non-amyloidogenic processing of APP via different mechanisms implicating either the canonical G<sub>s</sub> protein-dependent signaling (agonist-elicited response) or the formation of a protein complex including the receptor, the ADAM10  $\alpha$ -secretase, and, probably, additional protein partners that remain to be identified (agonist-independent response).

One promising strategy to decrease the accumulation of A $\beta$  peptides in the brain has been to inhibit  $\beta$ - and  $\gamma$ -secretase activities. However, the development of  $\gamma$ -secretase inhibitors has been challenging because this enzyme has many endogenous substrates. Several  $\gamma$ -secretase inhibitors have been synthesized and have shown unequivocal efficiency in reducing A $\beta$  plaque deposition in preclinical models of AD. However, clinical trials have revealed severe adverse side effects associated with the blockade of Notch processing.<sup>34</sup> The recent failure of a  $\gamma$ -secretase inhibitor to slow down the progression of the disease, accompanied by worsening of cognitive performances, has halted its development.<sup>35</sup> However, the recent discovery that down-regulation of GSAP ( $\gamma$ -secretase activating protein) selectively prevents A $\beta$  peptide production without affecting Notch signaling<sup>36</sup> opens new perspectives.

Strategies for increasing the production of the neuroprotective sAPP $\alpha$  are also actively investigated. 5-HT<sub>4</sub>R expression clearly increased sAPP $\alpha$  release, and it was previously demonstrated that 5-HT<sub>4</sub>R activation concomitantly decreased A $\beta$  formation while increasing sAPP $\alpha$  release.<sup>13,14</sup> However,  $\alpha$ -cleavage is not always coupled to a reduction in A $\beta$  level.<sup>3</sup> The role of ADAM10, the physiological  $\alpha$ -secretase in neurons, in the regulation of late-onset AD has been suggested by the discovery of non-synonymous mutations in the ADAM10 pro-domain in seven late-onset AD families.<sup>37</sup> In line with these findings, activation of ADAM10 expression by vitamin A derivatives<sup>4</sup> or the transcription factor SIRT1<sup>6</sup> decreases A $\beta$  peptide production and amyloid plaque formation in APP/PSEN1 transgenic mice. In addition, SIRT1-induced ADAM10 expression increases Notch processing and the release of Notch/intracellular domain (NICD), which activates genes involved in neuronal repair in adult brain.<sup>38</sup> The efficacy of ligands of some GPCRs, such as M<sub>1</sub>R, 5-HT<sub>4</sub>R, and PAC<sub>1</sub>R, in reducing A $\beta$  production and/or improving cognition in preclinical models of AD is now well documented.<sup>39</sup> Here, we describe a new mechanism whereby 5-HT<sub>4</sub>R constitutively activate the  $\alpha$ -secretase ADAM10 and sAPP $\alpha$  release. Together with the ability of 5-HT<sub>4</sub>R agonists to



increase learning and memory, via acetylcholine release,<sup>12</sup> the present findings suggest that this GPCR might represent an interesting target for AD treatment. Several studies reported a significant drop of 5-HT<sub>4</sub>R binding sites in post-mortem brains of AD patients.<sup>17,40,41</sup> In this context, the identification of 5-HT<sub>4</sub>R-interacting proteins capable of modulating the trafficking of the 5-HT<sub>4</sub>R/ADAM10 complex will be of high relevance.

## METHODS

**Plasmids.** The plasmid encoding the inactive form of the G<sub>s</sub> protein was kindly given by Dr. Catherine H. Berlot (Weis Center for Research Geisinger Clinic, Danville, Canada). The plasmids encoding c-Myc-tagged 5-HT<sub>4</sub>R, HA-tagged 5-HT<sub>4</sub>R, Flag-tagged 5-HT<sub>4</sub>R, HA-tagged 5-HT<sub>4</sub>R, and the dominant-negative form of Epac2 were previously described.<sup>28,42,43</sup> The SEAP-tagged APP695 cDNA in pRK5 was generated by adding the secreted embryonic alkaline phosphatase (SEAP) sequence, flanked by glycine linkers and the PspXI (5') or BsrGI (3') restriction sites, to the N-terminus of mouse APP695, after L<sup>398</sup> (Figure 1A). The plasmids encoding the HA-tagged M<sub>3</sub> and Myc-tagged PAC<sub>1</sub> receptors were kindly provided by Dr. Arnaud Monteil and Dr. Laurent Journot, respectively (Institut de Génétique Fonctionnelle, Montpellier, France).

**Antibodies.** The mouse anti-sAPP antibody (22C11, directed against the N-terminal part of APP; 1:80 dilution)<sup>44</sup> was purchased from Millipore (Billerica, MA). The rat anti-sAPP $\alpha$  antibody (7A6, murine sAPP $\alpha$ -specific, 1:2 dilution) was kindly given by Dr. Stefan F. Lichtenthaler (Adolf Butenandt Institute - Ludwig Maximilians University, Munich, Germany).<sup>24</sup> The rabbit anti-ADAM10 antibody (directed against the C-terminal domain, 1:500 dilution), the mouse anti-c-Myc antibody (9E10), and the rabbit anti-Flag antibody were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The mouse anti-HA antibody was purchased from Life Technologies (Saint Aubin, France). The rat anti-ADAM10 antibody (directed against the ectodomain domain, 1:500 dilution) was purchased from R&D Systems (Minneapolis, MN). Mouse anti-HA and rabbit anti-GFP antibodies were purchased from Life Technologies-Invitrogen (Carlsbad, CA). The mouse anti-SEAP antibody was purchased from Euromedex (Souffelweyersheim, France). Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies were from GE Healthcare (Orsay, France).

**Materials.** All media, sera, and antibiotics used for cell culture were purchased from Lonza (Basel, Switzerland). GR 113808 and GR 125487 were synthesized and generously provided by GlaxoSmithKline (Brentford, U.K.). 5-HT (serotonin creatine sulfate), Phorbol 12-myristate 13-acetate (PMA), the  $\beta$ -secretase inhibitor KTEEISEV-statine-VAEF, the  $\gamma$ -secretase inhibitor L-685458, (-)-isoproterenol hydrochloride, and metoclopramide ((N-(2-dimethylamino)-ethyl)-4-amino-5-chloro-2-methoxybenzamide) were from Sigma. The TNF $\alpha$  Protease Inhibitor-1 (TAPI-1) was purchased from Merck (Darmstadt, Germany). Prucalopride was synthesized and kindly provided by Dr. Jan Schuurkes (Shire-Movetis n.v., Turnhout, Belgium). RO 116-0086, RO 116-1148, and RO 116-2617 were generously provided by Roche Bioscience (Palo Alto, CA). RS 67333 was purchased from Tocris Bioscience (Bristol, U.K.). BIMU8 was kindly provided by Boehringer Ingelheim (Rhein, Germany). Cisapride was synthesized and obtained from Janssen Pharmaceutica (Beerse, Belgium). ML 10375 was a generous gift from Dr. Langlois (CNRS-BIOCIS, Châtenay-Malabry, France). SB 207266 was obtained from Fournier-Debat Laboratories (Daix, France).

**Cell Culture and Transfections.** Human embryonic kidney 293 (HEK-293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal calf serum (FCS). Cells were transfected at 60–70% confluence by electroporation, as previously described.<sup>28</sup> Twenty-four hours after transfection, cells were serum starved for 4 h before assays.

Primary cultures of cortical neurons were prepared as previously described.<sup>45</sup> Briefly, cells dissociated from the cortex of 15–16 day/old Swiss mouse embryos were plated in serum-free medium in 24-well culture dishes ( $0.5 \times 10^6$  cells/well). Cultures were maintained at 37 °C

in a humidified atmosphere in 5% CO<sub>2</sub> and 95% H<sub>2</sub>O, in DMEM/F12 supplemented with 10% hormone mixture for 4 days. Cortical neurons were transfected by electroporation using AMAXA (Lonza, Basel, Switzerland) according to the manufacturer's instructions. Media were collected for 12 h at DIV4.

**Small Interfering RNA (siRNA) Transfection.** A mix of double-stranded siRNA sequences 5'-AAUGAAGAGGGACACUCCUCUdTdT-3' and 5'-AAGUUGCCUCCUCAAACCAdTdT-3' (Eurogentec, Liege, Belgium) was used to selectively target ADAM10. A small RNA duplex, which has no silencing effect, was used as control (negative control siRNA duplex from Eurogentec). All the siRNAs used in this study have been described earlier and validated.<sup>46</sup> Early passage HEK-293 cells at 40% confluence in 24-well plates were transiently transfected with 400 ng of plasmid encoding wild type 5-HT<sub>4</sub>R and 500 ng of plasmid encoding SEAP-tagged-APP. siRNAs were transfected at 150 nM using Lipofectamine 2000, according to the manufacturer's recommendations (Life Technologies-Invitrogen). Cells were lysed 2 days after siRNA transfection in order to quantify APP expression.

**Measurement of sAPP.** To determine the total and intracellular concentration of APP and of secreted APP, cells were washed with PBS at the beginning of the assay and preincubated for 2 h in serum-free medium supplemented with 5-HT<sub>4</sub>R ligands and drugs were added for the indicated times. Cells were then lysed after collection of conditioned supernatants containing secreted proteins according to the two following methods: (i) Collected media were centrifuged at 300g for 10 min and then at 20 000g for 20 min to remove cells and cell debris. Proteins were concentrated by precipitation with 10% ice-cold trichloroacetic acid (TCA) (at 4 °C for 2 h) and centrifuged at 38 000g at 4 °C for 25 min, and then pellets were washed with diethyl ether three times. TCA precipitates were reconstituted in solubilization buffer (20 mM HEPES buffer; 250 mM Tris buffer pH6.8; 40% SDS; 40% glycerol; 40% 2-mercaptoethanol and traces of bromophenol blue). The protein amount of each sample was determined with the bicinchoninic acid assay (Sigma). Following precipitation, samples were separated by electrophoresis on 7.5% SDS-PAGE gels and transferred onto nitrocellulose membranes. (ii) In order to measure sAPP release, HEK-293 cells were transiently transfected with APP-SEAP and plated in 6-well culture dishes. Spent media were incubated overnight at 4 °C with Dynabeads protein G (Life Technologies-Invitrogen, Carlsbad, CA) that had been precoupled to 4  $\mu$ g of 22C11 polyclonal antibody. After 5 washes in lysis buffer (20 mM HEPES; 150 mM NaCl; 1% NP40; 10% glycerol; 4 mg/mL dodecylmaltoside) supplemented with protease inhibitors and phosphatase inhibitors (10 mM NaF; 2 mM Na<sup>+</sup>-vanadate and 1 mM Na<sup>+</sup>-pyrophosphate), immunoprecipitated proteins were eluted in solubilization buffer and separated by electrophoresis (see above). To detect intracellular APP, cells were lysed in 100  $\mu$ L of lysis buffer (see above), reconstituted in solubilization buffer, and separated by electrophoresis (see above).

sAPP release was also estimated by measuring the extracellular SEAP activity released by HEK-293 cells transiently transfected with the SEAP-APP plasmid. One day post-transfection, drugs were added for the indicated times (see legends). Then cell culture media were collected, and SEAP activity measured by adding the chromogenic substrate *p*-nitrophenyl phosphate disodium hexahydrate (Sigma) according to the manufacturer's instructions. The reaction was read at 405 nm using an Infinite 2000 luminescence counter (Tecan, Männedorf, Switzerland). Specific quantification of sAPP $\alpha$  release was performed by using the sAPP $\alpha$  ELISA kit from IBL International (Hamburg, Germany) according to the manufacturer's instructions. The reaction was read at 620 and 450 nm using an Infinite 2000 luminescence counter (Tecan).

**Immunoblotting.** Proteins were separated on 7.5% SDS-PAGE gels and transferred by semidry blotting onto nitrocellulose membranes (Hybond-C, Amersham Biosciences, Orsay, France). Membranes were blocked at room temperature in Tris-buffered saline blocking solution containing 5% (w/v) milk powder, 0.25% v/v Tween 20 for 1 h. Membranes were then incubated with the primary antibodies at 4 °C overnight, washed extensively, and incubated with the secondary antibodies at room temperature for 1 h. Immunoreactivity was detected using the Chemiluminescence Reagent Plus kit (Perkin-Elmer,



Courtaboeuf, France). Immunoreactive bands were quantified by densitometry using Image J and GraphPad PRISM (GraphPad Software, San Diego, CA).

**Co-Immunoprecipitation.** Cells were seeded in 150 mm plates ( $10^6$  cells/plate) 24 h before the experiment. Cross-linking was carried out in PBS completed with 1.25 mM dithiobissuccinimidyl propionate (DSP) (Pierce, Perbio-Brebières, France) (a membrane-permeable, hydrolyzable, covalent cross-linker) for 30 min. The cross-linking reaction was stopped with 50 mM Tris pH 7.4. After one wash in PBS, cells were incubated with lysis buffer (see above) at 4 °C for 1 h. Samples were centrifuged at 20 000g for 15 min. Solubilized proteins were incubated at 4 °C with 20  $\mu$ L of Dynabeads protein G (Life Technologies-Invitrogen) precoupled with 4  $\mu$ g of antibody overnight. Immunoprecipitated proteins were eluted in Laemmli sample buffer, resolved by SDS-PAGE gel electrophoresis, and detected by Western blotting.

**Determination of cAMP Production.** HEK-293 cells were transiently transfected with plasmids encoding the indicated receptors and/or SEAP-APP, and then seeded in 24-well plates (200 000 cells/well). Twenty-four hours after transfection, cells were exposed to the indicated concentrations of 5-HT<sub>4</sub>R ligands in the presence of 0.1 mM L-ascorbic acid and 0.1 mM of the phosphodiesterase inhibitor RO-20-1724, at 37 °C in 250  $\mu$ L of HBS (20 mM HEPES; 150 mM NaCl; 4.2 mM KCl; 0.9 mM CaCl<sub>2</sub>; 0.5 mM MgCl<sub>2</sub>; 0.1% glucose; 0.1% BSA). Cells were then lysed by addition of the same volume of Triton-X100 (0.1%). Quantification of cAMP production was performed by HTRF by using the cAMP Dynamic kit (Cisbio Bioassays) according to the manufacturer's instructions.

**Cell Surface ELISA (Enzyme-Linked Immunosorbent Assay).** HEK-293 cells transiently transfected with the appropriate cDNAs were seeded in 96-well plates (40 000 cells/well). Twenty-four hours after transfection, cells were treated with drugs, washed, fixed with 4% paraformaldehyde at room temperature for 10 min, and blocked with PBS + 1% FCS. Cells were then incubated with primary antibodies in the same buffer for 1 h followed by HRP conjugated secondary antibodies at 1:8000 for 1 h. After extensive washes, the chromogenic substrate was added (Supersignal ELISA Femto Maximum Sensitivity, Pierce). Chemiluminescence was detected and quantified with an Infinite 2000 luminescence counter (Tecan).

**Statistical Analysis.** All represented experiments were performed in triplicate. Statistical significance was determined with the Student–Newman–Keuls test using GraphPad Prism (GraphPad Software). *P*-values < 0.05 were considered as statistically significant. Data corresponded to the mean  $\pm$  SD of three independent experiments.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Supplemental Figure 1: 5-HT<sub>4</sub>R variants (mouse a, b, e, and f; human a, g, and i), truncated forms ( $\Delta$ 358 and  $\Delta$ 329) and mutants (A<sup>258</sup>L, N<sup>308</sup>D, and Y<sup>302</sup>F) promote sAPP release and physically interact with ADAM10. Supplemental Figure 2: sAPP release induced by 5-HT<sub>4</sub>R expression is not affected by  $\beta$ - or  $\gamma$ -secretase inhibitors. Supplemental figure legends. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Author Contributions

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## Author Contributions

M.C., J.B., A.D., and S.C. conceived and designed the experiments. M.C., R.D., E.C., and F.G. carried out the experiments. M.C., E.C., F.G., S.F.L., P.M., J.B., A.D., and S.C. analyzed the data. M.C., P.M., J.B., A.D., and S.C. wrote the manuscript.

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## Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

APP, amyloid precursor protein; sAPP $\alpha$ , soluble APP alpha; sAPP $\beta$ , soluble APP beta; SEAP, secreted embryonic alkaline phosphatase; 5-HT<sub>4</sub>R, serotonin type 4 receptor; AD, Alzheimer's disease; ADAM, A disintegrin and metalloprotease; A $\beta$ , amyloid- $\beta$  peptide; GPCR, G protein-coupled receptor

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