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Research report

Prucalopride and donepezil act synergistically to reverse scopolamine-induced memory deficit in C57Bl/6j mice

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Abstract

It is known that 5-HT_4 receptor agonists increase sAPPα levels in the cortex and hippocampus of mice as well as in a model of Alzheimer's disease (AD). As sAPPα is thought to have pro-mnesic properties, we assessed whether its increase induces cognitive improvement in a spatial memory task and whether it reverses a scopolamine-induced memory deficit.

Mice treated or not treated with scopolamine were trained in the Morris water maze for 3 days. Before the probe test, they received an injection of either a 5-HT_4 receptor agonist (prucalopride or RS 67333), or an acetylcholinesterase inhibitor (donepezil), or both drugs. As expected, scopolamine decreased performance, an effect that was not reversed by the drugs tested when injected alone. However, prucalopride (5 mg kg^{-1}, s.c.) acted synergistically with donepezil (0.75 mg kg^{-1}, s.c.) to counteract completely scopolamine-induced amnesia. Western blot analysis of tissue homogenates in the cortex and hippocampus shows that sAPPα levels did not differ between saline- and scopolamine-treated mice. Furthermore, a region-dependent drug action was observed since the scopolamine-treated mice display a tendency to increase sAPPα levels in the hippocampus after donepezil or in the cortex after prucalopride.

Our results suggest that a combined treatment with a 5-HT_4 receptor agonist with an acetylcholinesterase inhibitor has beneficial effects on memory in mice. Moreover, it seems to enhance sAPPα levels in two brain regions highly affected in AD. Thus, a drug polytherapy could be interesting not only to enhance cognitive performance and decrease drawbacks but also to get the best action in each brain region.

Keywords: Serotonin 5-HT_4 receptor agonist; Amyloid precursor protein; Alzheimer's disease; Acetylcholine esterase inhibitor

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the appearance of senile plaques mainly composed of amyloid beta protein (Aβ), and by the development of neurofibrillary tangles in patients' brain [1]. AD patients have cognitive deficits, impaired long-term potentiation (LTP) and learning and memory [2], and a consistent deficit in cholinergic neurotransmission also. Several acetylcholine esterase inhibitors such as donepezil are available in the market for the treatment of patients with mild-to-severe AD. However, beneficial effects on memory and cognition of this treatment can only be maintained for up to 36 months [3]. Compounds like donepezil do not stop the AD and are symptomatic treatments, which only delay patient's loss of autonomy.

Autoradiographic studies using 5-hydroxytryptamine$_4$ (5-HT_4) receptor antagonists such as [^{125}I]SB207710 and [^{3}H]GR113808 in rat, mouse, guinea pig or post-mortem human brain showed that the 5-HT_4 receptor is present at a high density in the limbic system including the hippocampus and frontal cortex [4,5], suggesting a role of this subtype of 5-HT receptors

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in memory and cognition. Moreover, Manuel-Apolinar et al. [6] studied 5-HT_4 receptor expression in old rat brains removed after auto-shaping learning task or not, using [^{3}H]GR113808. They observed an increase in 5-HT_4 receptor expression in different brain regions involved in memory processes such as hippocampal CA1 and cortex. These findings suggested a region-specific regulation during memory formation.

Indeed, RS 67333, a selective partial 5-HT_4 receptor agonist, prevents the atropine-induced amnesia in a rat spatial navigation task [7]. This is related to the 5-HT_4 receptors, as this effect is abolished by a 5-HT_4 receptor antagonist (RS 67532) in an olfactory associative discrimination task in the rat [8]. More recently, Micale et al. [9] showed that SL65.0155, a selective 5-HT_4 receptor partial agonist, could reverse amnesia induced by a intracerebroventricular (i.c.v.) injection of β-amyloid 1–42 fragment in male Swiss mice in a passive avoidance test as well as in a radial-maze test.

From its location and its properties to restore memory, 5-HT_4 receptor is an interesting target to cure AD [10]. β-amyloid, the major component of senile plaques, is derived from the amyloid precursor protein (APP) processed by β- and γ-secretase. By contrast, APP can also be cleaved by α-secretase leading to the secretion of sAPPα, a pro-mnesic protein [11]. One strategy to treat AD is to enhance the non-amyloidogenic pathway. Recently, we found that an acute treatment with prucalopride, a 5-HT_4 receptor agonist, increased sAPPα levels in the cortex and hippocampus of adult male C57Bl/6j mice as well as in a mouse model of AD [12]. Moreover, this effect was potentiated by donepezil, a selective non-competitive inhibitor of acetylcholinesterase. As sAPPα is thought to have pro-mnesic properties [13–15], we wanted to test whether the increase observed in sAPPα levels was sufficient to reverse a scopolamine-induced memory deficit.

To answer this question, we used a spatial memory test in mice: the Morris water maze [16]. This test is classically used to characterize cognitive alterations in rodent models of AD or to study the pro-mnesic properties of a drug. After 3 days training, we studied the ability of 5-HT_4 receptor agonists, prucalopride and RS 67333, or of an acetylcholinesterase inhibitor, donepezil or of both compounds to reverse the scopolamine-induced memory deficit in adult male C57Bl/6j mice during the probe trial. The effects of RS 67333 were already described in Morris water maze in rats [7]; therefore, this drug was used as a control to validate our experiment. To control the effects of the treatments on activity, we tested locomotion after the probe test. To check sAPPα levels, brains were removed at the end of the locomotion test to perform Western blot analysis in the hippocampus and cortex.

2. Materials and methods

2.1. Animals

Adult male C57Bl/6j wild-type mice (9-week-old, weighing 23–27 g) from Janvier (Le Genest-Saint-Isle, France) were used in this study. Mice were housed with a 12-h light:12-h dark cycle (lights on at 8 p.m.) with food and water *ad libitum*. Behavioral testing occurred during the dark phase. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international legislation (Council directive # 87–848, October 19, 1987, "Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale", permissions # 92–196 to A.M.G.).

2.2. Drugs and reagents

Prucalopride and donepezil were a generous gift from Dr. X. Langlois (Janssen Pharmaceutics, Beerse, Belgium) and Eisai Co., Ltd. (Tokyo, Japan), respectively. RS 67333 hydrochloride (BioTrend Chemikalien GmbH, Cologne, Germany) and scopolamine hydrochloride (Sigma–Aldrich, St Quentin Fallavier, France) were purchased.

2.3. Antibodies

R1736 (antibody kindly provided by Dr. Dennis Selkoe, Harvard Medical School, Boston, MA, USA) is a rabbit polyclonal antiserum raised to a synthetic peptide of amino acids 595–611 of APP [17,18].

2.4. Apparatus

The Morris water maze consisted of a circular pool (90 cm in diameter × 30 cm in height) filled with water (24 ± 1 °C) made opaque by addition of polypropylene pellets at a height of 14 cm [19]. The pool was subdivided in four equal quadrants. Animals had to learn the location of a submerged platform (7 cm) placed in the center of one quadrant, in order to escape water. Several extra-maze cues (posters and objects) could serve to locate the platform.

2.5. Treatment

The control group of mice received a subcutaneous injection of NaCl 0.9%. The treated groups (9–15 mice per group) received a single subcutaneous injection of either prucalopride (5 mg kg^{-1}) or donepezil (0.75 mg kg^{-1}) or both drugs or RS 67333 (1 mg kg^{-1} in a volume of 5 ml kg^{-1}). Prucalopride, donepezil and RS 67333, dissolved in NaCl 0.9% as the vehicle, were administered intraperitoneally (i.p.) in a volume of 5 ml kg^{-1}, 30, 60 or 90 min before testing, respectively. Each drug or a combination of drugs was tested in saline-treated mice and scopolamine-treated mice (1 mg kg^{-1} in a volume of 5 ml kg^{-1}, s.c., 30 min before probe trial). This dose was chosen according to Lelong et al. [20].

2.6. Testing procedure

The familiarization step occurred during the morning of the first day.

Acquisition of the spatial learning task was performed over 3 consecutive days of testing; the first trial began in the afternoon following the familiarization. Mice had three trials per session. The mouse stayed for 60 s on the hidden platform (or visible for the familiarization step), and then it was placed randomly in one of the adjacent or opposite quadrant. The mouse was allowed to search for 60 s and climb onto the submerged platform during the learning sessions or onto the visible one for the familiarization session. If the mouse failed to locate the platform within this delay, it was helped by the experimenter to find it. In all cases, mice were allowed to stay on the platform for 60 s.

The day after the last learning session, animals received an acute treatment and their retention was tested over a 1-min probe trial, which consisted in that mice were placed into the middle of the pool without platform and the time spent into the quadrant, where the platform was previously located, was recorded by a video tracking system (ViewPoint, France).

Locomotion of each mouse was verified using an actimeter. The apparatus consisted of white Plexiglas toggle-floor boxes, each divided into two 20 cm × 10 cm compartments connected by 3 cm × 3 cm openings. For each mouse, the number of crossings from one compartment to the other was automatically recorded by means of a microswitch connected to the tilting floor of the box. The apparatus was in a room with dim light and the test duration was 5 min.

Then, mice were sacrificed by cervical dislocation, the brains were quickly removed and the hippocampus and cortex were dissected at −20 °C as previously described [12].

2.7. Measurement of sAPPα and β-actin by Western blot

To investigate the role of acute drug treatments on brain sAPPα levels, we analyzed the expression of this protein in tissue homogenates from the hippocampus and cortex of treated and untreated mice by Western blot analysis (polyclonal antiserum R1736, 1:4000 dilution; goat anti-rabbit immunoglobulin antibody, 1:15,000; Amersham Pharmacia Biotech (Orsay, France). Immunoreactive bands were then visualized by the west dura detection kit (Pierce). Then, membrane is stripped and blocked again before being probed with β-actin (monoclonal anti-β-actin, 1:5000 dilution; Abcam, Cambridge, UK), used as an internal control. Densitometric values were evaluated by using an image analysis.

2.8. Statistical analysis

Statistical analyses were performed using the computer software StatView 4.02 (Abacus Concepts Inc., Berkeley, CA, U.S.A.). Data are means ± S.E.M. of time spent in the quadrant where the platform was located or of sAPPα levels expressed as percentage of control values. Values were compared between the different groups by using a one-way analysis of variance, followed by a PLSD Fisher test. The significance level was set at $P < 0.05$.

3. Results

3.1. Morris water maze

In the acquisition phase, C57Bl/6j mice were trained for 3 days in a Morris water maze to learn where the hidden platform was located. All mice significantly shortened the escape latency during the acquisition phase (data not shown).

On the fourth day, mice received an acute scopolamine injection before the probe trial in order to induce a memory deficit (Figs. 1 and 2). Significant differences among groups were obtained ($F(3,48) = 7.19$, $P < 0.001$). Whereas saline-treated mice preferentially spent their time swimming in the pool quadrant where the platform had been located during training, scopolamine-treated mice did not show such preference. A co-treatment with either a selective 5-HT_4 receptor agonist prucalopride or an acetylcholinesterase inhibitor partially reversed the memory deficit induced by scopolamine (Figs. 1 and 2). A similar effect was observed when scopolamine-treated mice received an injection of RS 67333, another 5-HT_4 receptor agonist (Fig. 1). However, the improvement was of small magnitude so that no statistically significant difference occurred between the scopolamine group and the group co-treated with scopolamine and RS 67333 or prucalopride or donepezil. Only co-administration of prucalopride and donepezil was powerful enough to induce the same preference for training quadrant in scopolamine-treated mice as in saline-treated mice (Fig. 2). A significant difference among groups was found ($F(3,35) = 3.36$, $P < 0.05$).

In mice without memory deficit, whatever the treatment received, no statistically significant differences with saline-treated mice were observed (Fig. 3).

No statistically significant differences in locomotor activity was found across groups in the first experiment ($F(3,29) = 1.5$

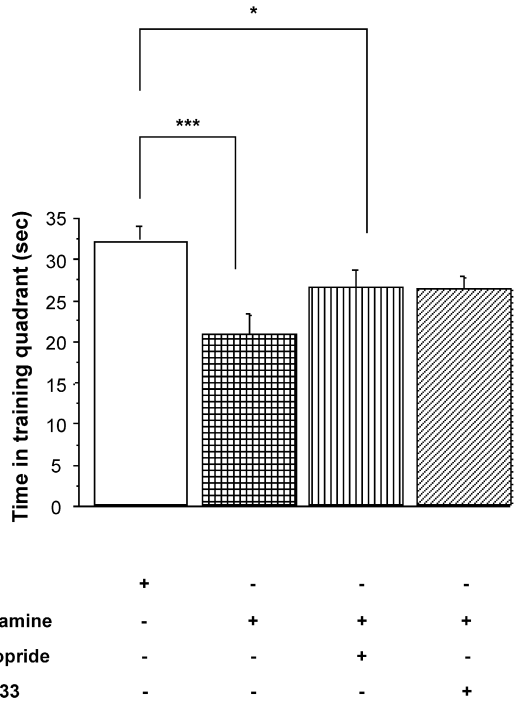

Fig. 1. Partial reversal of the scopolamine-induced deficit by RS67333 and prucalopride in a Morris water maze test in C57BL/6j mice. Time spent in quadrant B in which the platform was previously located. Values are means ± S.E.M. of time spent in one quadrant. $n = 11$–14 mice per group. Values were compared between the different groups by using a one-way analysis of variance followed by Fisher PLSD tests. $^{*}P < 0.05$, $^{**}P < 0.001$ compared to saline-treated mice (white bar); scopolamine 1 mg kg^{-1} s.c.; prucalopride 5 mg kg^{-1} s.c.; RS 67333 1 mg kg^{-1} i.p.

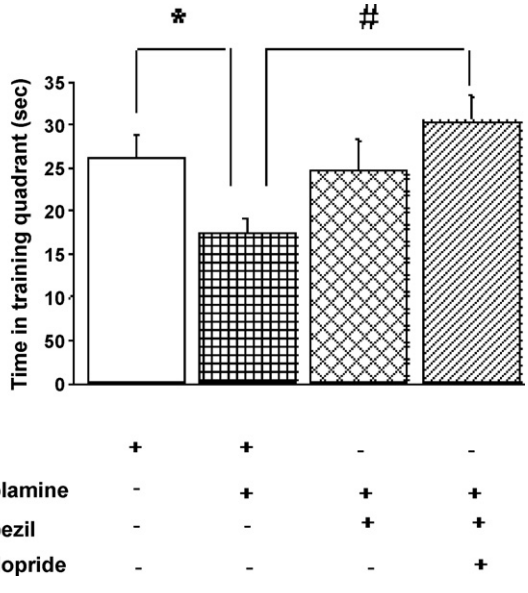

Fig. 2. Reversal of the scopolamine-induced deficit in Morris water maze performance in C57BL/6j mice co-treated with prucalopride and donepezil. Time spent in the quadrant in which the platform was previously located. Values are means ± S.E.M. of time spent in quadrant B. $n = 9$–10 mice per group. Values were compared between the different groups by using a one-way analysis of variance followed by Fisher PLSD tests. $^{*}P < 0.05$ compared to saline-treated mice (white bar); $^{\#}P < 0.01$ compared to scopolamine-treated mice.

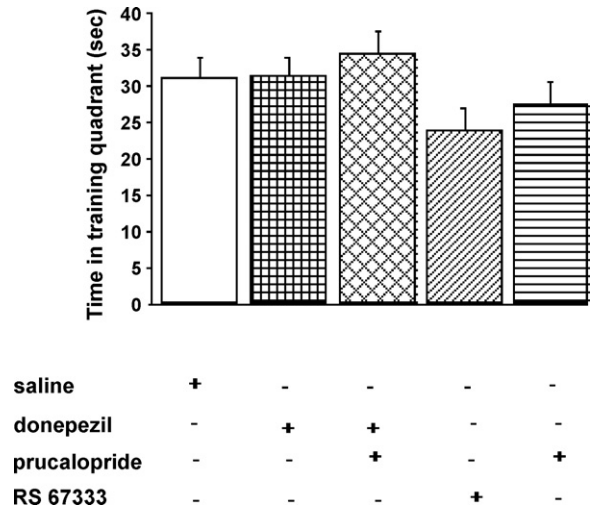

Fig. 3. Morris water maze performance in C57BL/6j mice male without amnesia. Time spent in quadrant B in which the platform was previously located. Values are means ± S.E.M. of time spent in one quadrant. $n = 9$–10 mice per group. Values were compared between the different groups by using a one-way analysis of variance followed by least significant difference tests. donepezil 1 mg kg^{-1} s.c.; prucalopride 5 mg kg^{-1} s.c.; RS 67333 1 mg kg^{-1} i.p.

$P > 0.2$) (*data not shown*). A statistical difference in locomotor activity was observed between saline group and mice co-treated with scopolamine and donepezil ($F(3,35) = 3.54$ $P < 0.01$) in the second experiment (data not shown). The locomotion test reveals significant differences among groups ($F(4,43) = 5.66$ $P < 0.01$) This was related to the fact that mice treated with prucalopride differed from all other groups (data not shown).

3.2. sAPPα levels in the brain of mice following the Morris water maze trial

In our control experiment, in mice untreated with scopolamine, prucalopride significantly enhances sAPPα level in cortex (data not shown). The increase is comparable to the one observed in our previous studies [12].

In the second set of experiments, scopolamine alone did not modify brain sAPPα levels in the cortex and hippocampus compared to saline-treated mice (Fig. 4a and c). Surprisingly, prucalopride increased sAPPα levels only in the cortex of mice pre-treated with scopolamine (Fig. 5a and b), but not in the hippocampus (Fig. 5c and d). No further increase was observed either in the donepezil-treated group or in the donepezil/prucalopride-treated group. By contrast, donepezil increased sAPPα levels in the hippocampus of mice pre-treated with scopolamine. Co-administration of prucalopride with donepezil/also increased sAPPα levels in the hippocampus in these mice, but to a level similar to that found in the group treated with donepezil alone (Fig. 5c and d).

4. Discussion

The results reported here show that a co-treatment with prucalopride, a 5-HT$_4$ receptor agonist, and donepezil, an acetylcholinesterase inhibitor, attenuated the learning impairments induced by a cholinergic deficit (scopolamine pre-treatment). Thus, this polytherapy restored memory in a mouse amnesia model, while both drugs were not sufficiently effective alone in the Morris water maze test. In addition, these behavioral effects were associated with changes in sAPPα levels found in the cortex or hippocampus, but not in both brain areas. For example, a co-treatment with prucalopride and donepezil increased sAPPα levels in the hippocampus, but did not affect sAPPα levels in the cortex in these mice. However, the hippocampal effect was mainly due to an increase in sAPPα levels induced by donepezil: thus no synergic increases in sAPPα levels were found in the hippocampus following the combined treatment, although here donepezil and prucalopride acted synergistically in Morris water maze to counteract amnesia.

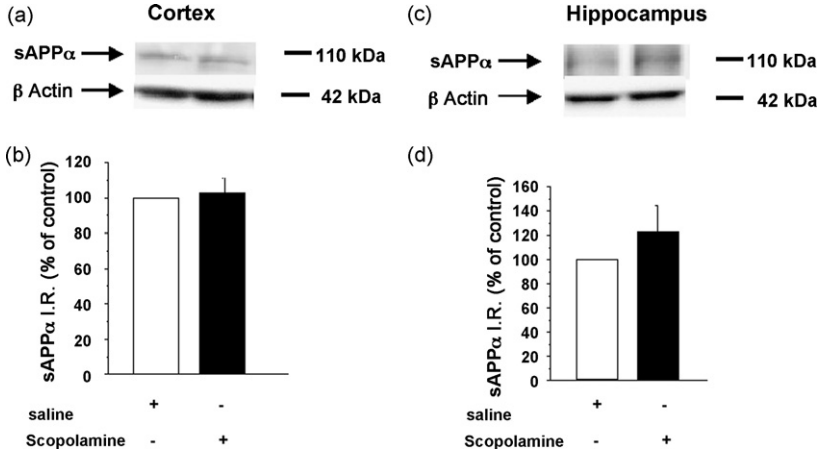

Fig. 4. Absence of scopolamine effects on sAPPα levels in the cortex (a and b) and hippocampus (c and d) of 9-week-old male C57Bl/6j mice. Mice received a subcutaneous scopolamine administration before Morris Water Maze probe test. Each brain was dissected immediately after the end of the trial and Western blot analysis was performed on sAPPα levels from hippocampal and cortical homogenates. Values are means ± S.E.M. of sAPPα and β-actin levels (used as internal control) expressed as percentages of untreated control mice. $n = 9$–10 mice per group. (a and c) The representative immunoblots showing that scopolamine does not modify sAPPα level. IR, intensity relative, scopolamine 1 mg kg^{-1}.

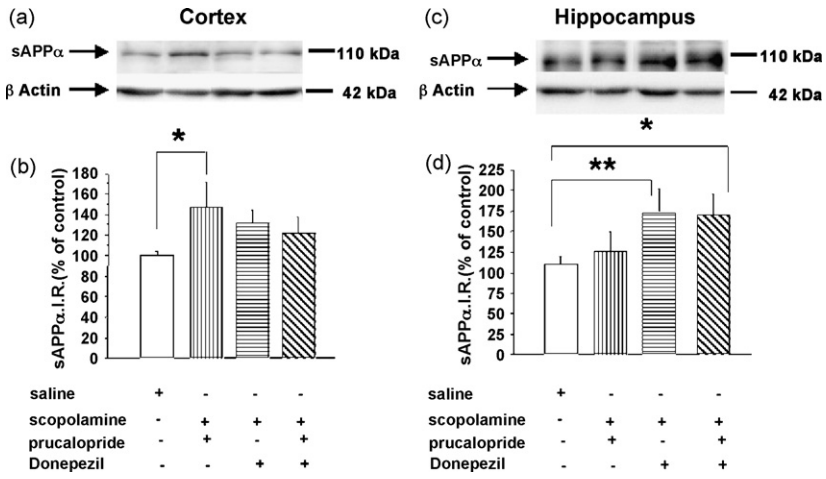

Fig. 5. sAPPα levels in the cortex (a and b) and hippocampus (c and d) of 9-week-old male C57Bl/6j mice. Mice received a subcutaneous treatment before Morris Water Maze probe test. Each brain was dissected immediately after the end of the trial and Western blot analysis was performed on sAPPα levels from hippocampal and cortical homogenates. Values are means ± S.E.M. of sAPPα and β-actin levels (used as internal control) expressed as percentages of untreated control mice. $n = 9$–19 mice per group. Values were compared between the different groups by using a one-way analysis of variance followed by PLSD Fisher tests. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to saline-treated mice (white bar) in the hippocampus. a and c are representative immunoblots showing the effects of treatments on sAPPα level. IR, intensity relative, scopolamine 1 mg kg^{-1}, prucalopride 5 mg kg^{-1}, donepezil 0.75 mg kg^{-1}.

Interestingly, administration of the acethylcholinesterase inhibitor donepezil in scopolamine-pre-treated animals enhanced sAPPα levels in the hippocampus. As muscarinic receptors are blocked by scopolamine, acetylcholine probably binds to α7 nicotinic acetylcholine receptor (α7-nACh receptor) [21]. Indeed, Guan et al. studied *in vitro* sAPPα production following α7 nicotinic acetylcholine receptor stimulation in SH-SY5Y and PC12 cells [22,23]. They found that an incubation with α-bungarotoxin, a selective α7-nACh receptor antagonist, or with small interference RNAs that target specifically towards α7-nACh receptor, decreased sAPPα levels in culture media. Consequently, α7-nACh receptor might be involved *in vitro* in sAPPα production.

By contrast, prucalopride alone increased sAPPα levels in the cortex in scopolamine-pre-treated mice, but co-administration of prucalopride and donepezil did not affect cortical sAPPα levels.

Thus, these two brain regions seem to be involved in memory deficits measured in scopolamine-treated mice, but different neurochemical mechanisms or pathways might be implicated:

- *In the cortex*, the 5-HT$_4$ receptor agonist, prucalopride was more efficient than an acetylcholinesterase inhibitor to reverse the impairments induced by a cholinergic deficit, i.e., the scopolamine blockade of muscarinic acetylcholine receptor. Thus, 5-HT$_4$ receptor belongs to the RCPG receptor super family and is positively coupled to adenylyl cyclase [24]. This result suggests that, in this animal model of AD, activation of 5-HT$_4$ receptors enhanced the cortical release of acetylcholine. It has already been demonstrated by using intracerebral *in vivo* microdialysis in rats that serotonin facilitates acetylcholine release through stimulation of 5-HT$_4$ receptors in the cortex [25]. Thus, 5-HT$_4$ receptors play a key role in the non-amyloidogenic pathway of APP metabolism *in vivo*. We also confirm in the present study that prucalopride could offer a novel therapeutic strategy of boosting central cholinergic function to overcome the cholinergic deficit in memory disorders.
- *In the hippocampus*, the acetylcholinesterase inhibitor, donepezil was more efficient than a 5-HT$_4$ receptor agonist to reverse neurochemical impairments induced by a cholinergic deficit. Thus, as an indirect acetylcholine receptor agonist, donepezil increased intrasynaptic ACh levels, which then activated nicotinic (ion channel) and/or muscarinic (RCPG) receptors coupled to very different second messenger pathways [26]. The present data highlight the link between a former hypothesis of AD origin (alterations of the brain cholinergic system [27] and a more recent hypothesis involving Aβ deposits in memory deficits leading to AD [28]. In addition, here the blockade of muscarinic acetylcholine receptors by scopolamine induced memory deficits, but no changes in sAPPα levels. The inhibition of acetylcholine degradation in combination with a 5-HT$_4$ receptor agonist reversed these effects.

However, 5-HT$_4$ receptor agonist are also involved in Aβ decrease in *in vitro* experiments using mouse embryonic (E16) primary cortical cultures derived from transgenic mice that overexpress the human APP695 gene with the Swedish familial mutation [29]. In order to understand better the role of a 5-HT$_4$ receptor agonist and an acetylcholinesterase inhibitor in the polytherapy, it would be interesting to test for its effects in an Alzheimer's model overexpressing a human APP695 gene with familial mutation. Indeed, APP mRNA levels, APP total protein levels, CTFα and β and Aβ levels are important parameters to better understand at which step of the amyloid cascade the polytherapy could be involved, i.e., APP recycling, APP overexpression, activation of α-secretase or inhibition of β- or γ-secretase.

The Morris water maze task was originally designed to study the mechanisms of spatial localization in rats [30]. Indeed, float-

ing and thigmotaxis (i.e., a tendency to swim along the wall of the pool) tend to be more pronounced in mice than in rats, which may complicate testing and analysis of the results [31]. However, C57Bl/6j mice performed well during training trials and probe trials. They were good swimmers and responded for being placed in water with an appropriate swim-search response [32]. Thus, they were used as a 'standard' performance strain for multi-strain comparisons of spatial learning and memory ability [33]. They also displayed a robust LTP [34], but LTP in the hippocampus is a physiological phenomenon that parallels some mechanisms of memory.

Scopolamine induced amnesic effects, thus confirming well known data obtained on this compound. Donepezil (0.75 mg kg^{-1}, s.c.) did not completely attenuate scopolamine effects in the Morris water maze task. This result is consistent with those of Lindner et al. [35], showing that donepezil (0.1; 0.3 or 1 mg kg^{-1}, i.p.) hardly counteracts scopolamine effects in a battery of cognitive/behavioral tests, among them the Morris water maze performed in Sprague–Dawley rats.

Prucalopride (5 mg kg^{-1} s.c.; $P < 0.05$), and to a lesser extent RS 67333 (1 mg kg^{-1} i.p.; $P > 0.05$, i.e., only a tendency to increase compared to the scopolamine group) partially restored the memory deficit induced by scopolamine (1 mg kg^{-1} s.c.) in 9-week-old male C57Bl/6j mice. The active dose and delay of action of RS 67333 is consistent with those used by Fontana et al. [7] in rats.

Although RS 67333 was more often used in memory experiments than prucalopride (see for review reference [36]), both drugs are partial agonists at 5-HT_4 receptors ($pK_i = 8.7$ and 8.6; $pEC_{50} = 7.5$ and 8.4, respectively). In addition, RS 67333 displays a high affinity for σ1 and σ2 adrenoceptors with $pK_i = 8.9$ and 8, respectively [37]. As σ1 receptors are also involved in learning and memory [38], it seems more appropriate to use prucalopride to study the role of 5-HT_4 receptors in this phenomenon. Since we already studied the effects of prucalopride on brain sAPPα levels in mice [12], we decided to study the effects of prucalopride here and to use RS 67333 as an additional control.

The synergistic effect of a 5-HT_4 receptor agonist with an acetylcholinesterase inhibitor on cognitive performance has already been demonstrated. Indeed, such a drug combination was tested in an object recognition test in young rats [39]. Rivastigmine, another acetylcholinesterase inhibitor, potentiated the effects of SL65.0155, when drugs were given before each of the three behavioral sessions. In experiments of place and object recognition, Lamirault et al. [40,41] demonstrated that a co-injection of RS 67333 and another acetylcholine esterase inhibitor, galanthaminium bromide, in young or old rats *before* the acquisition phase allowed to enhance performances, whereas injections *after* the acquisition phase or before retention phase were ineffective, suggesting that both drugs enhanced memory performances by acting during the acquisition phase.

Here, we successfully studied the combined treatment before the retention phase in a mice scopolamine-induced memory deficit. We observed a drug effect on memory processes. Scopolamine is also known to mimic the hypocholinergic state already described in patients with AD [42].

The effects we observed were unrelated to an action of the treatment on locomotion, as the sole case in which a treatment effect was found on this behavioral activity was not related to effects on cognitive performance.

Administration of prucalopride in scopolamine-pre-treated animals increased sAPPα levels in the cortex (Fig. 3b), but not in the hippocampus (Fig. 3d), while scopolamine itself had no effects on this parameter (Fig. 4a and b). To our knowledge, this is the first time that such data have been obtained in rodents (rats and mice). Scopolamine is a muscarinic acetylcholine receptor *antagonist*. We previously demonstrated that donepezil, as an indirect acetylcholine receptor *agonist*, administered alone had no effects on cortical and hippocampal sAPPα levels in mice (see Fig. 7 in reference [12]). Together with the lack of scopolamine effects on brain sAPPα levels found here (Fig. 4), these data suggest that the cholinergic system alone does not play a key role in regulating APP processing in the mice' brain. These results are consistent with our previous study showing that the combined administration of donepezil with prucalopride display activated the non-amyloidogenic pathway of APP metabolism in mice brain [12]. Differences observed between the cortex and hippocampus in this response could be due to the presence of different populations of 5-HT_4 receptor isoforms. Indeed, four C-terminal splice variants were cloned in the mouse [36]. They differ in the length and composition of their intracellular C termini located after the common splicing site (L358). The activity of each isoforms differs with the strength of its coupling with a G-protein [43].

Scopolamine alone did not modify sAPPα levels in the cortex and hippocampus. This result is not really surprising as many receptor subtypes in addition of muscarinic receptors [44] are thought to be involved in the production of sAPPα (e.g., the metabotropic glutamate receptor subtype mGluR1alpha [45]; *N*-methyl-D-aspartate receptor [46], 5-HT_4 receptor [17]). As scopolamine alone did not modify sAPPα levels in the cortex and hippocampus, it suggests that muscarinic receptors are not involved in basal sAPPα levels, which means that muscarinic receptors would not be involved in sAPPα constitutive pathway, but only in the regulated pathway.

Although we failed to find a synergistic effect of donepezil combined with prucalopride on sAPPα levels in the brain of scopolamine-treated mice, our results could open new treatment perspectives. Indeed, the cortex and hippocampus are the brain regions very much affected in AD. Moreover, with the disease progression, many signalling pathways are disturbed in a time-dependent manner. Thus, multi-drug therapy would be interesting to get the best response in each of these brain regions.

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