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Evidence from a mouse model supports repurposing an anti-asthmatic drug, bambuterol, against Alzheimer's disease by administration through an intranasal route

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Bambuterol is a long-acting anti-asthmatic prodrug which releases terbutaline. Terbutaline is an agonist of the β_2-adrenergic receptors which is formed by decarbamoylation of bambuterol by butyrylcholinesterase. Inhibition of the latter, as well as activation of β_2-AR, are of interest for the treatment of Alzheimer's disease (AD). Combining these two activities, bambuterol could express a good clinical efficacy against AD. The present work firstly confirmed the capacity of bambuterol to display in cellulo neuroprotective activities, reduction of Tau hyperphosphorylation and preservation of synapses in rat hippocampal neuronal cultures intoxicated with Aβ peptides. Further, bambuterol, in the form of a liposomal gel, showed a good bioavailability in CNS after intranasal administration, which should reduce any side effects linked to peripheral terbutaline release. Indeed, even if the latter is more selective than other β_2-mimetics towards bronchial β_2-AR, cardiovascular effects (tachycardia, arrhythmias…) could occur upon cardiac β_1-AR activation. Finally, intranasal administration of low doses of bambuterol gel in mice intoxicated with Aβ peptides, prevented long-term spatial memory impairment and showed beneficial effects on the survival of neurons and on synapse preservation.

Therapeutic treatment of Alzheimer's disease (AD) is currently experiencing unprecedented advances with the introduction by the US Food and Drug Administration (FDA) of several antibodies (aducanumab, lecanemab and donanemab) directed against amyloid β-oligomers and fibrils[1–5]. Despite numerous problems [low central bioavailability, low efficacy in improving cognitive impairment, frequent Amyloid-Related Imaging Abnormalities (ARIA) and high cost], at the end of their phase III clinical trials, these antibodies showed a relative correlation between the reduction in amyloid burden on Positron Emission Tomography (PET) and the reduction in cognitive impairment. These results appear to validate, for the first time, the amyloid hypothesis as a valuable target to fight AD in a disease-modifying manner and to pave the way for small amyloid-targeting molecules, orally administered, that are more clinically relevant, less responsible for ARIA events and less expensive. To achieve this ambitious goal, these agents will need to address the multifactorial origin of AD and especially, beside amyloid deposits, its inflammatory context and Tau hyperphosphorylation. This pleiotropic approach can be pursued with drug combinations (e.g. Namzaric® which combines donepezil and memantine) or using Multi-Target Directed Ligands (MTDL) such as ladostigil [which inhibits both B-monoamine oxidase (MAO) and acetylcholinesterase (AChE)][6] or donecopride (which targets AChE and the 5-HT_4 serotonergic receptor)[7–9]. These examples are

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promising but their development will take many years. A faster route may be to repurpose old drugs[10]. In 2023, many of the AD clinical trials involved repurposed drugs[11]. In this context, we believe that another marketed drug could be of interest for the treatment of AD since it combines two activities in a single molecule, directed against two targets implicated in its pathogenicity. This MTDL, bambuterol, has been used for many years as a long-acting, oral, anti-asthmatic drug (Fig. 1).

Bambuterol is a prodrug of terbutaline, which is a β_2-adrenergic receptor (β_2-AR) agonist and is itself marketed as a bronchodilator for asthma via aerosol therapy. The carbamate groups of bambuterol are cleaved by plasmatic butyrylcholinesterase (BuChE), which releases terbutaline in a long-acting manner. Interestingly, both β_2-AR and BuChE are promising and complementary targets for treating AD.

Currently, AD treatments predominantly comprise AChE inhibitors (AChEIs), which offer symptomatic relief by temporarily restoring the loss of cholinergic neurotransmission, thereby moderately alleviating cognitive disorders. Although AChEIs also participate in the hydrolysis of ACh into choline, BuChE's central production seems less influenced by neurodegeneration than AChE and could even be augmented in the advanced stages of AD[12]. Moreover, it is worth noting that BuChE inhibitors (BuChEIs) do not promote the production of the enzyme, unlike AChEIs for AChE. Therefore, targeted inhibition of BuChE presents itself as a promising strategy for treating AD[13].

Additionally, previous research has suggested that β_2-AR agonists may offer therapeutic benefits in AD by enhancing synaptic plasticity and reducing amyloid aggregation[14,15]. Furthermore, various studies have illustrated changes in the synthesis and metabolism of norepinephrine (NE) in AD, including a notable decrease in NE levels in postmortem brain samples from the temporal and frontal cortices, as well as the hippocampal region of AD patients[16]. Dopamine β-hydroxylase (DBH) catalyzes the conversion of dopamine to NE. Lower levels of DBH activity have also been reported in early AD, followed by a gradual increase in enzymatic activity as cognitive symptoms worsen in late AD[17]. Additionally, a post-mortem study using human AD brain autoradiography displayed a significant decrease in overall NE transporter density, which was directly correlated with the extent of the pathology[18–20]. Drug candidates that alleviate NE deficits may offer a therapeutic approach to tackle AD, even in cases of advanced pathology. Some of the methods studied include enhancing NE signaling in the brain through the use of β_2-AR agonists, among which terbutaline has been found to prevent the Aβ mediated inhibition of Long-Term Potentiation (LTP)[21].

While against asthma, BuChE is not considered as a therapeutic target, but simply as a delayed manner to release terbutaline, in AD treatment its covalent and selective inhibition by bambuterol appears to be a valuable way for maintaining cholinergic neurotransmission and exerting a symptomatic benefit. The concomitant release of terbutaline could further complement this first approach and provide a complementary disease-modifying effect. The present study aims at describing the proof of concept according to which bambuterol could be repurposed against AD. But, since bambuterol poorly crosses the blood-brain barrier (BBB), we further envisaged to use the intranasal route, using an appropriate formulation, to selectively distribute the drug into the brain and reduce potential detrimental peripheral side effects such as headache or extremities tremor.

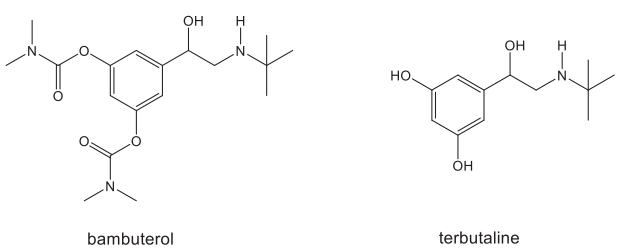


Fig. 1 | Structure of bambuterol and terbutaline. Structure of bambuterol (left) and terbutaline (right).

Results

Bambuterol displays neuroprotective activities and reduces Tau hyperphosphorylation in rat hippocampal neuronal cultures

The effects of bambuterol (1, 5, 10, 50, 100, 500 and 1000 nM) on neuronal survival, the neurite network, and Tau phosphorylation were investigated in rat primary hippocampal neurons exposed to amyloid-β (Aβ) preparation (20 μM) containing soluble Aβ oligomers (AβO) (2 μM), with donepezil used as a reference test compound (1 μM)[22–24].

The Aβ injury led to an important loss of neurons (Fig.2A–C). Bambuterol significantly protected the neurons from 1 to 100 nM. The most potent effect was observed at 10 nM. In a similar manner, Aβ injury also reduced total neurite network. Bambuterol exerted protective effects on the neurite network at all the tested concentrations (Fig. 2B), with the most potent activity observed at 10 nM. The inverted U-shape obtained in the evaluation of bambuterol on the survival or neurite network of MAP-2 neurons may be related to the desensitization of β-$_2$AR upon its activation by full agonists such as terbutaline[25,26]. Bambuterol (all doses) significantly prevented the hyperphosphorylation of Tau protein with a maximal effect observed at 10 nM (Fig.2C).

Bambuterol preserves the number of synapses in rat hippocampal neuronal cultures

The distribution of presynaptic (synaptophysin) and postsynaptic (PSD95) markers was studied to investigate the possible preservation of synapses decreased upon Aβ injury. Bambuterol exerted a significant protective effect towards synapses at 10, 50 and 100 nM (Fig.3A,B).

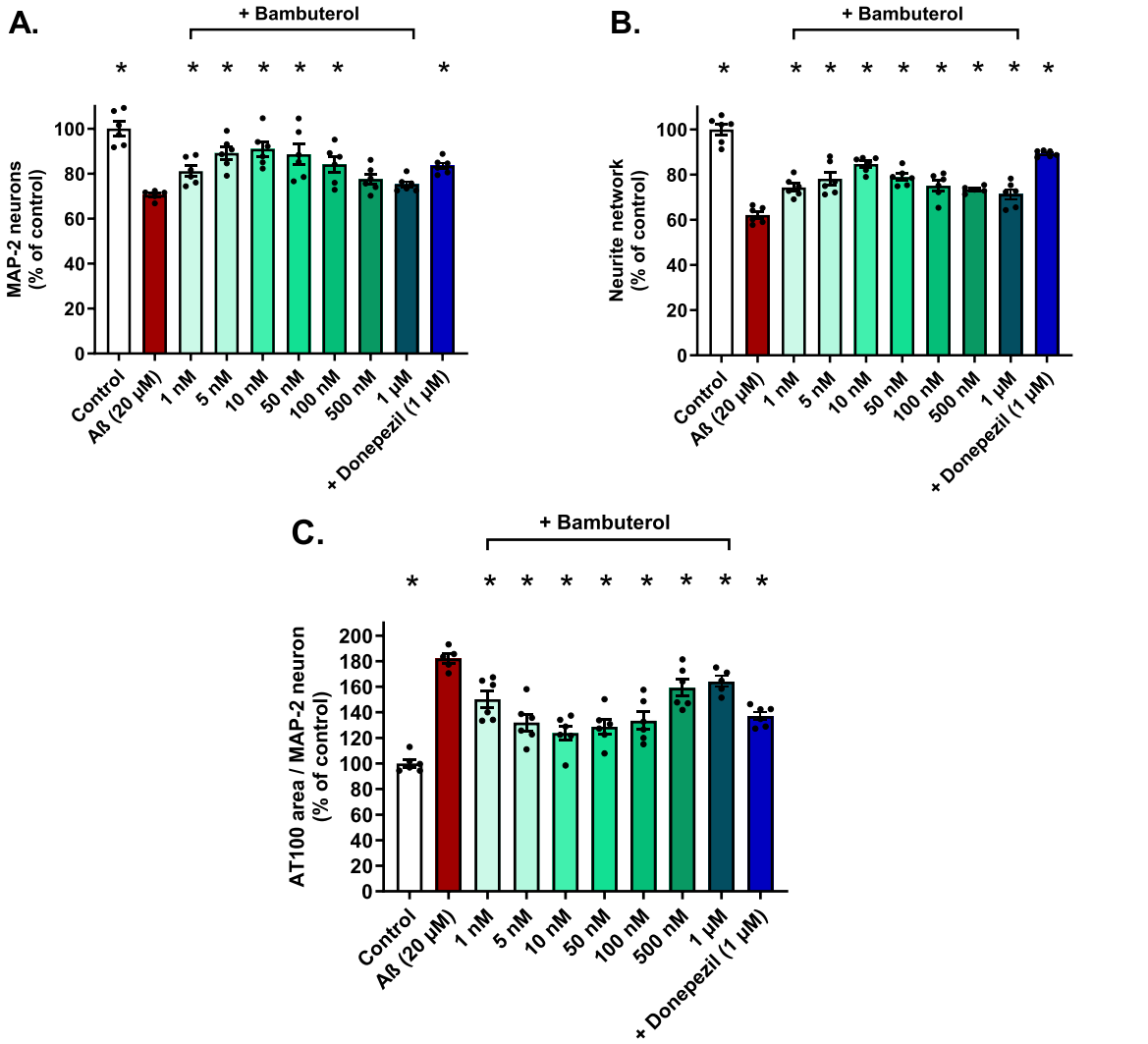
Bambuterol engages both BuChE and β_2-AR as targets

In order to link the in cellulo activities of bambuterol to its effects both towards BuChE and β_2-AR, we undertook the measurement of the bambuterol-dependent cAMP production in COS-7 cells overexpressing β_2-AR, using a CAMYEL cAMP sensor and bioluminescence resonance energy transfer (BRET). Firstly, we verified that bambuterol does not induce the production of cAMP without addition of BuChE (Fig. 4A). In this paradigm, rivastigmine (a BuChEI) had no significant change on cAMP expression. Then, we measured the cAMP production in the presence of bambuterol alone and associated to rivastigmine on the one hand, and to ICI 118551, a β_2-AR inverse agonist on the other one (Fig.4B,C)

Bambuterol, in the presence of BuChE, significantly promoted the cAMP production. The latter was partially blocked when the BuChE activity was inhibited by rivastigmine, added in combination to bambuterol. In this case, cAMP production did not fully return to basal levels because rivastigmine itself tends to stimulate its release. Finally, the bambuterol-dependent cAMP production was completely abolished, when ICI118551 exerted its inverse agonistic activity towards β_2-AR.

Bambuterol is formulated as a thermosensitive mucoadhesive liposomal gel

An innovative composite formulation, associating bambuterol-loaded liposomes and thermosensitive gel formulation, was recently developed. Scale up of all formulation steps has been realized to be able to produce at least 10 mL per batch instead of 1 mL as previously developed at the laboratory scale[27]. The formulation method was based on the film hydration method, followed by extrusion. Instead of mini-extruder, extrusion was scaled up using a pressure-controlled extrusion process. Briefly, after hydration of a lipid film with a solution of bambuterol, the lipid suspension was extruded through 3 polycarbonate membranes with a pore diameter of 100 nm, 5 times at 40 bars at room temperature (see SI). After separation of the free bambuterol on a Sephadex column, the bambuterol-loaded liposomes were characterized (Supplementary table 1). Then, liposomes were included into thermosensitive gelling formulations. The properties of the formulations remained coherent with those of liposomes and those of liposomes-embedded in thermogelling formulations previously produced at the laboratory scale. Properties of the further investigated bambuterol formulation remained stable at least 21 days after storage at 4 °C or at room



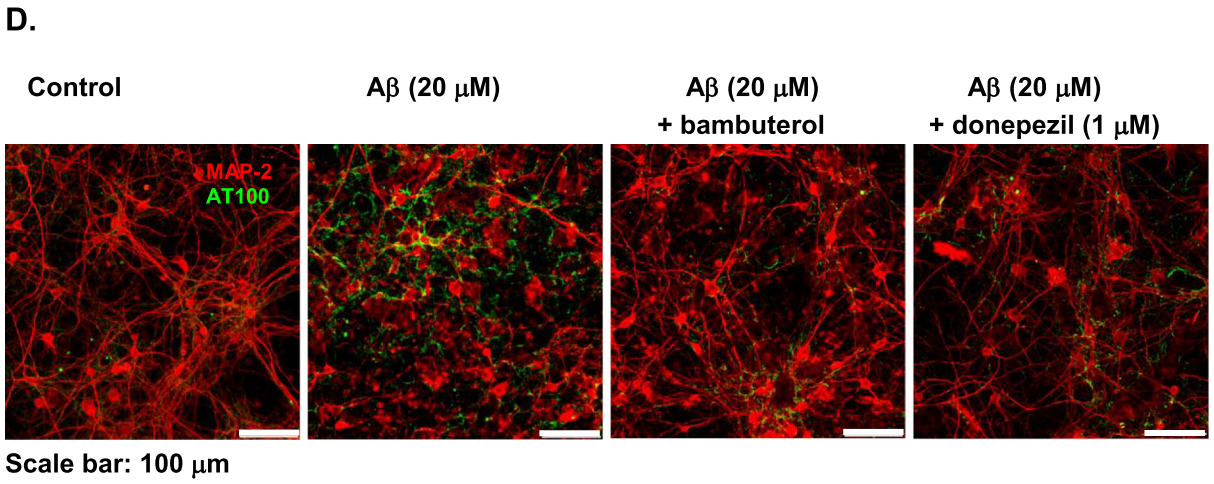
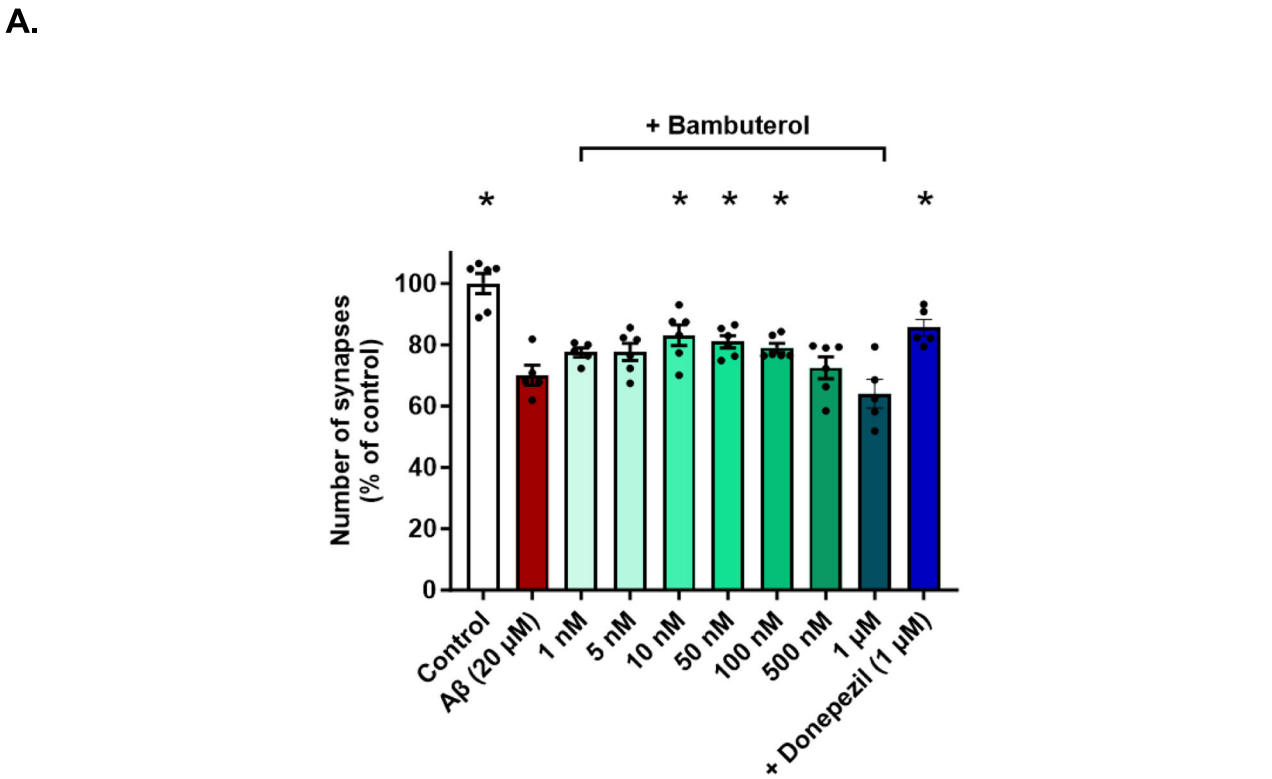


Fig. 2 | Effects of bambuterol after treatment with AβO in a primary culture of hippocampal neurons on survival of MAP-2 neurons, neurite network, and phosphorylation of Tau. Effects of bambuterol (green) after treatment with AβO (red) in a primary culture of hippocampal neurons. Effects on survival of microtubule-associated protein 2 (MAP-2) neurons (**A**) (control (white) = 38 ± 1 MAP-2+ cells/field), neurite network (**B**) (control = 389 ± 13 µM), and phosphorylation of Tau (AT100) (**C**) (control = 469 ± 13 μM^2/MAP-2+ cell. Donepezil (DPZ) served as a positive control (blue). Results are expressed as mean ± SEM [(**A**): $n = 6$ except for Aβ ($n = 5$); (**B**): $n = 6$ except for bambuterol 500 nM (n = 5), (**C**): $n = 6$ except for Aβ ($n = 5$) and bambuterol 1 µM ($n = 5$)] as a percentage of control (vehicle). *$p < 0.05$ vs AβO condition (one-way ANOVA followed by PLSD Fisher's test). **D** Representative images of MAP-2 (red) and AT100 (green) staining of rat primary hippocampal neurons in the presence of vehicle (negative control), AβO (20 µM), bambuterol (10 nM) and donepezil (1 µM).



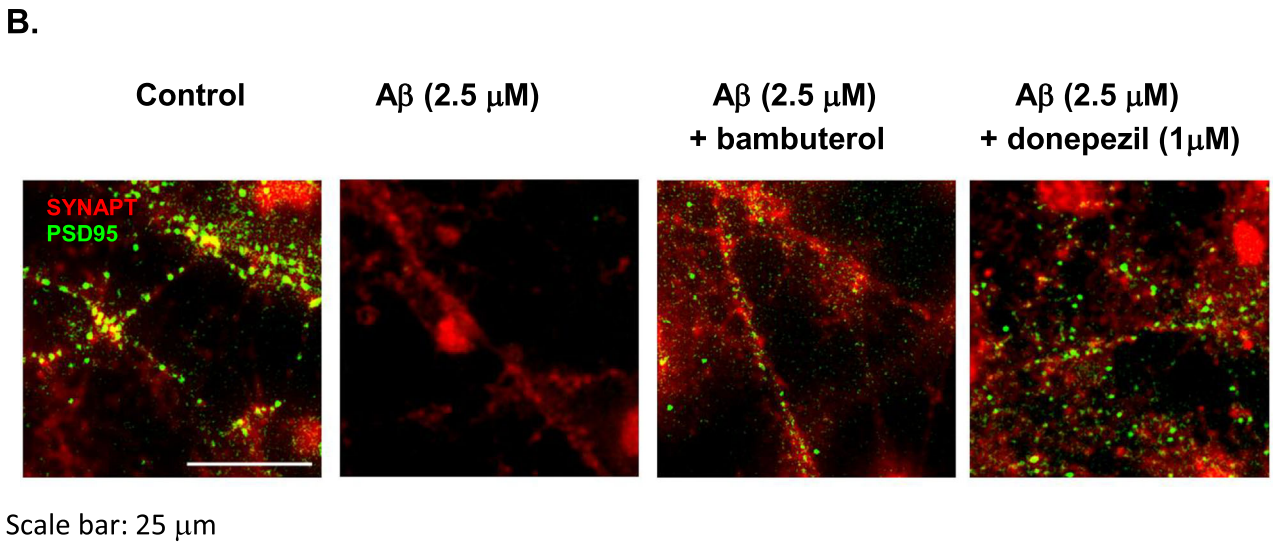


Fig. 3 | Effect of bambuterol after treatment with AβO in a primary culture of hippocampal neurons on synapses. A Effect of bambuterol (green) on total number of synapses. Donepezil (DPZ) served as a positive control (blue). Results are expressed as mean ± SEM [$n = 6$ except for Aβ (n = 5), bambuterol 1 nM ($n = 5$), bambuterol 1 μM ($n = 5$) and donepezil 1 μM ($n = 5$)] as a percentage of control (control (white) = 261 ± 8 PSD95 + /Synaptophysin+ synapses/field). $^*p < 0.05$ vs. AβO condition (red) (one-way ANOVA followed by PLSD Fisher's test). **B** Representative images of synaptophysin (red) and PSD95 (green) staining of rat primary hippocampal neurons in the presence of vehicle (negative control), AβO (20 μM), bambuterol (10 nM) and donepezil (1 μM).

temperature (Supplementary table 2). The gelation temperature (T°gel) of the formulation at 32.5 °C appeared optimal to allow the immediate gelation in the nasal cavity after intranasal (IN) administration, as required to improve drug remanence.

Formulation of bambuterol, in the form of liposomes in a thermosensitive mucoadhesive gel, administered through intranasal route, promotes its delivery into the brain

A formulation of bambuterol (liposome in thermosensitive gel at 1 mg/mL) was administered by single IN route into male CBA mice (300 μg/kg), or a 0.1 mg/mL saline solution of bambuterol was injected by single intravenous (IV) route (300 μg/kg). The concentrations of bambuterol and terbutaline were quantified in the plasma and the whole brain, one-hour post-administration (Supplementary table 3).

The overall delivery of the drugs (bambuterol + terbutaline) into the brain is quantitatively the same according to the two routes of administration. The release of terbutaline in the brain, however, appears faster through IN route versus IV route. On the other hand, the ratio between the brain and the plasma concentrations is 4 times greater for bambuterol through the IN route (41%) compared to the IV route (10%). The ratios

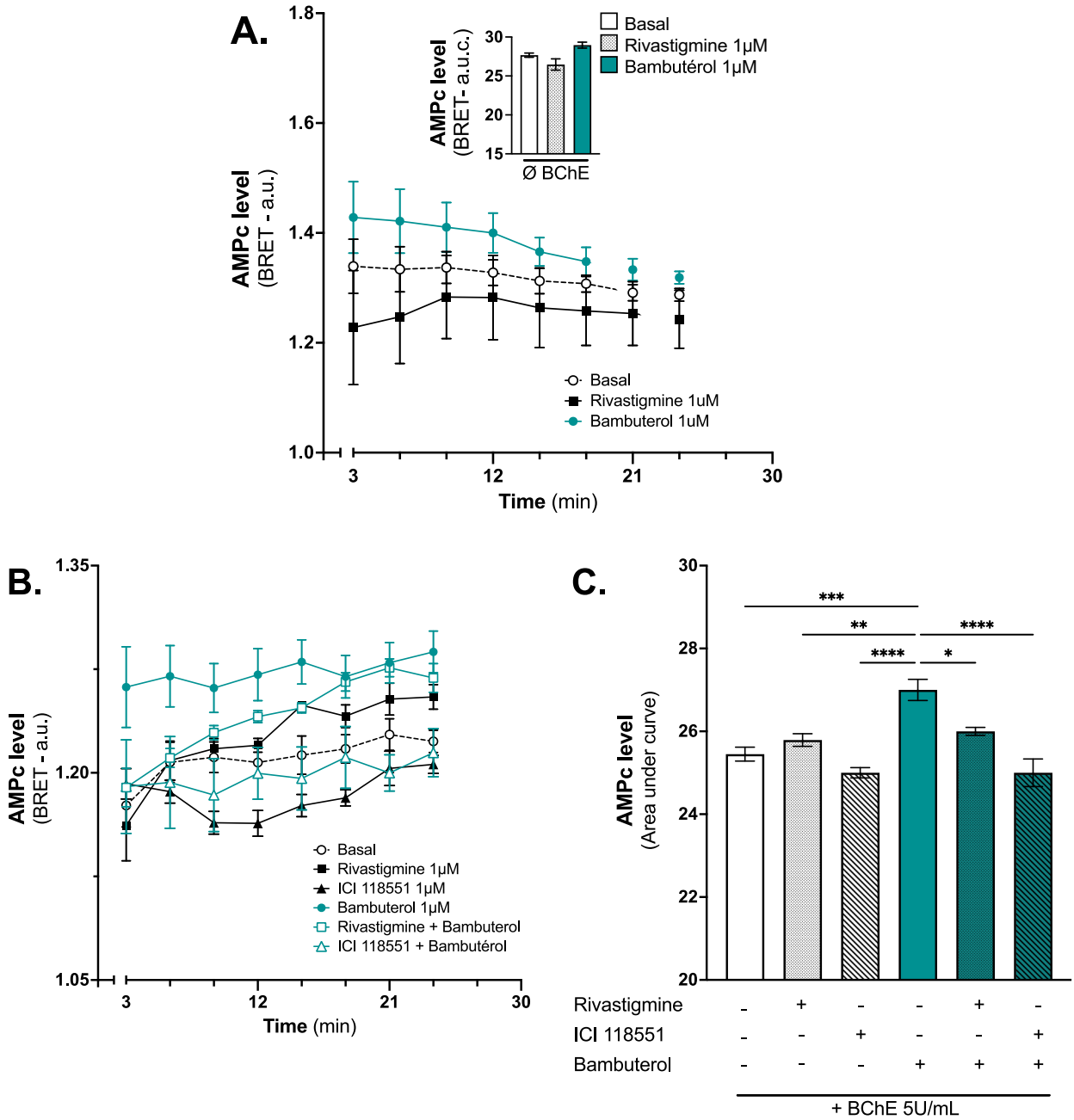


Fig. 4 | Effects of drugs on cAMP production in COS-7 cells in the presence and absence of BuChE. A Effects of bambuterol (1 µM) (solid-green squares) and rivastigmine (1 µM) (solid-black square) on cAMP production in COS-7 cells in the absence of BuChE (basal: white circles). BRET curves and area under the curve (insert) are presented. **B**, **C** Effect of tested drugs at 1 µM, alone [rivastigmine (solid-black squares), ICI118551 (solid-black triangles), bambuterol (solid-green squares)] or in combination [rivastigmine + bambuterol (green squares), ICI118551 + bambuterol (green triangles)], on cAMP production in COS-7 cells in the presence of BuChE (5 U/mL). BRET curves (**B**) and area under the curve (**C**) [control (white), rivastigmine alone (solid-gray), ICI118551 (hatched gray), bambuterol alone (solid-green), rivastigmine + bambuterol (dark solid-green), ICI118551 + bambuterol (dark hatched green)] are presented. Results are expressed as mean ± SEM ($n = 3$ (**A**) and $n = 6$ (**B**) technical replicates/condition, a representative experiment is shown). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.001$ (one-way ANOVA followed by Bonferroni's test).

were equivalent for terbutaline according to the two routes (22% versus 24%).

Sub-chronic administration of bambuterol through IN route prevents long-term spatial memory impairment and displays positive effects on hippocampal neuron survival and on synapse preservation in C57BL/6 mice injured with soluble Aβ peptides

The efficacy of bambuterol (one dose of 300 µg/kg administered daily by IN route via the developed formulation for 19 days) was evaluated in restoring memory deficits in an aged mouse model induced by stereological injection of $A\beta_{1\text{-}42}$ peptide oligomers[28,29]. Long-term spatial memory was investigated through Morris water maze (MWM) between days 11 to day 15. Overall behavior of the mice was observed at day 18 through an open-field experiment. In addition, at the end of the experiment, mice were deeply anesthetized and perfused with cold PBS. Their brains were collected and hippocampi dissected. Immunostaining was performed in order to evaluate the number of hippocampal neurons. Finally, the number of synapses was estimated by an automated protein analysis method.

Locomotor activity and anxiety of the animals were investigated in open field test 18 days after surgery[30]. No significant change in the total distance traveled was observed, suggesting no negative impact of $A\beta_{1\text{-}42}$ or bambuterol on locomotor activity (Fig. 5A). Anxiety was assessed

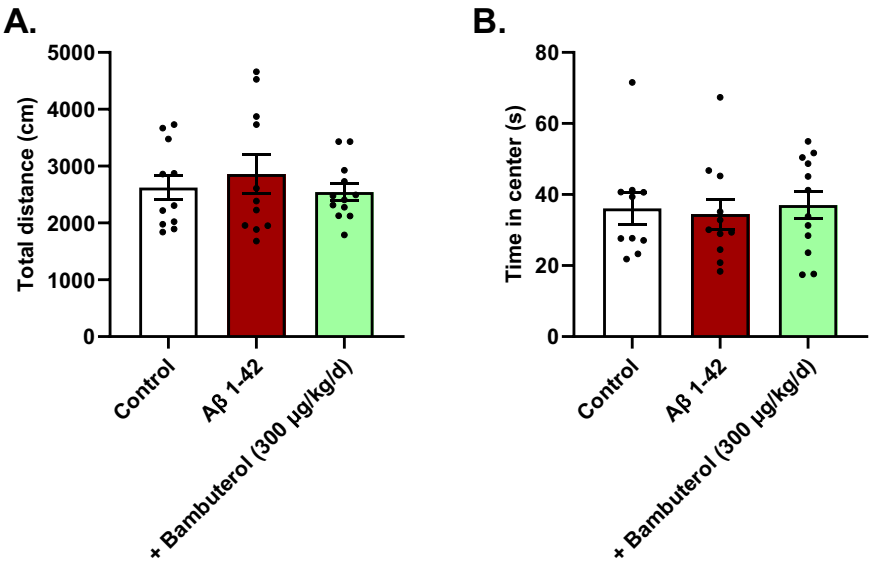


Fig. 5 | Effects of a formulation of bambuterol on locomotor activity and anxiety in mice in the open-field test. Effects of a chronic intranasal administration of bambuterol-loaded liposomes, embedded in a thermogelling formulation (green), on the total distance traveled during free exploration in the open field on day 17 after surgery (**A**), and on time spent away from center (marker of anxiety) at open field on day 17 after surgery (**B**). Results are expressed as mean ± SEM [$n = 12$ except for control ($n = 11$) and Aβ ($n = 11$)]. One-way ANOVA followed by Fisher's LSD test. *$p < 0.05$ vs. $A\beta_{1\text{-}42}$ (red) was considered significant. Control: white.

by studying the average time that the animals spent at the center of the open field arena (Fig. 5B). No difference was observed between groups.

The MWM is a test of hippocampal dependent spatial long-term memory and learning[31,32]. It relies on distal cues to navigate in an open swimming arena with a hidden escape platform. During training trials (day 1 to day 4), mice were forced to swim (with a maximal of 60 s per trial) in a circle arena with a submerged hidden platform. On day 5, the platform was removed, and mice performed the retention trials (probe test).

Fifteen days post intracerebral $A\beta_{1\text{-}42}$ injection, the probe test was performed and the velocity and total distance swum in the pool was measured to detect any motor impairment that could impact the swimming behavior. No difference was observed among conditions, suggesting that all animals were physically able to perform the test (distance swum in Fig. 6B, velocity in Fig. 6C). Whereas no difference was found between the groups in the learning phase (Fig. 6A), a significant impairment in long-term spatial memory was found during the retention phase in $A\beta_{1\text{-}42}$-injured mice compared to control mice (Fig. 6D), spending less time in the target ring. Mice injected with $A\beta_{1\text{-}42}$ and treated with the formulated bambuterol (300 µg/kg/d) showed better cognitive performance and remained longer in the target ring area, indicating an improvement in memory. In addition, $A\beta_{1\text{-}42}$-injected mice reach the target ring area late compared to control mice. Administration of bambuterol prevented the delay in reaching the target ring area in $A\beta_{1\text{-}42}$-injected mice (Fig. 6E).

Hippocampal neurons are involved in the formation and maintenance of memories. Injection of AβO in the CA1 hippocampal area induced a significant loss of hippocampal neurons 18 days post injury. Daily treatment with the formulated bambuterol (300 µg/kg/d) significantly improved the survival of neurons (Fig. 7).

Neuronal loss evidenced by immunohistochemistry is associated with a significant loss of synapses. Reduction of the synaptic network is responsible for spatial memory deficits. Protein levels of synaptophysin, a pre-synaptic marker, and PSD95, a post-synaptic protein, were evaluated 18 days post $A\beta_{1\text{-}42}$ injection. Levels of synaptophysin and PSD95 were significantly reduced in $A\beta_{1\text{-}42}$-injected mice when compared to control mice, indicating significant loss of synapses in the hippocampus (Fig. 8). In $A\beta_{1\text{-}42}$-injected mice, daily bambuterol (300 µg/kg/d) increased synaptophysin ($p < 0.05$, versus $A\beta_{1\text{-}42}$- injected mice treated with the vehicle) (Fig. 8A) and PSD95 ($p < 0.10$) protein levels (Fig. 8B) indicating a protective effect on synapses.

Discussion

Repurposing drugs is widely used today to introduce novel therapies quickly and efficiently, particularly for previously intractable diseases such as AD. Indeed, these drugs have undergone Chemistry, Manufacturing, and Controls (CMC) studies, as well as regulatory toxicological studies, making them liable to be safely produced and developed as medications, since they are already available in the market. Multifactorial diseases, like AD, require pleiotropic approaches to be more clinically relevant. Using MTDL has emerged as a promising strategy among these approaches. Bambuterol is an anti-asthmatic prodrug, marketed for a long time and considered as safe as β_2-AR agonist terbutaline, which it releases upon inhibitory interaction with BuChE. The β_2-AR and BuChE have both been identified as valuable and complementary targets for treating AD. In particular, central inhibition of BuChE is capable of restoring cholinergic deficits and displaying symptomatic effects in AD more sustainably than AChE inhibitors. In addition to their theoretical potential as disease-modifying agents in AD, epidemiological data suggests that long-term use of β_2-AR agonists may reduce the risk of developing Multiple Sclerosis (MS)[33], Parkinson Disease (PD)[34,35], and AD[36] among asthmatic patients. Conversely, long-term use of β-blockers appears to increase the risk of developing this condition, and some studies recommend avoiding their use in patients with Mild-Cognitive Impairment and AD[37,38].

Consequently, bambuterol could be considered a promising pleiotropic prodrug for treating AD, as it is capable of inhibiting BuChE and, upon terbutaline release, activating β_2-AR. The purpose of this study was to verify these potentialities in rat hippocampal neuronal cultures damaged by Aβ-oligomers. In this test, bambuterol showed neuroprotective properties at low doses (beginning at 1 nM) and reduced Tau hyperphosphorylation. At concentrations of 10 nM or greater, bambuterol maintains the number of synapses in this in cellulo model. Nonetheless, bambuterol is considered to have limited capacity for crossing the BBB via the oral route. To bypass the blood-brain barrier more easily, reduce the administered dose, and avoid potential peripheral adverse events, we designed a thermosensitive mucoadhesive liposomal gel of bambuterol for intranasal administration. The innovative gel was initially developed at the laboratory scale and later scaled up. The formulation including bambuterol-loaded liposomes is fluid at room temperature and forms a gel in the nasal cavity, thereby facilitating nose-to-brain drug transport. A preliminary pharmacokinetic study has been conducted in male CBA mice using this formulation. The study revealed a fourfold higher ratio of brain to plasma concentrations of the formulated bambuterol when administered through the IN route than when administered through the IV route as a bambuterol solution. Following these results, an in vivo study was conducted with C57BL/6 mice injected with soluble Aβ peptides. Through IN route, sub-chronic administration of

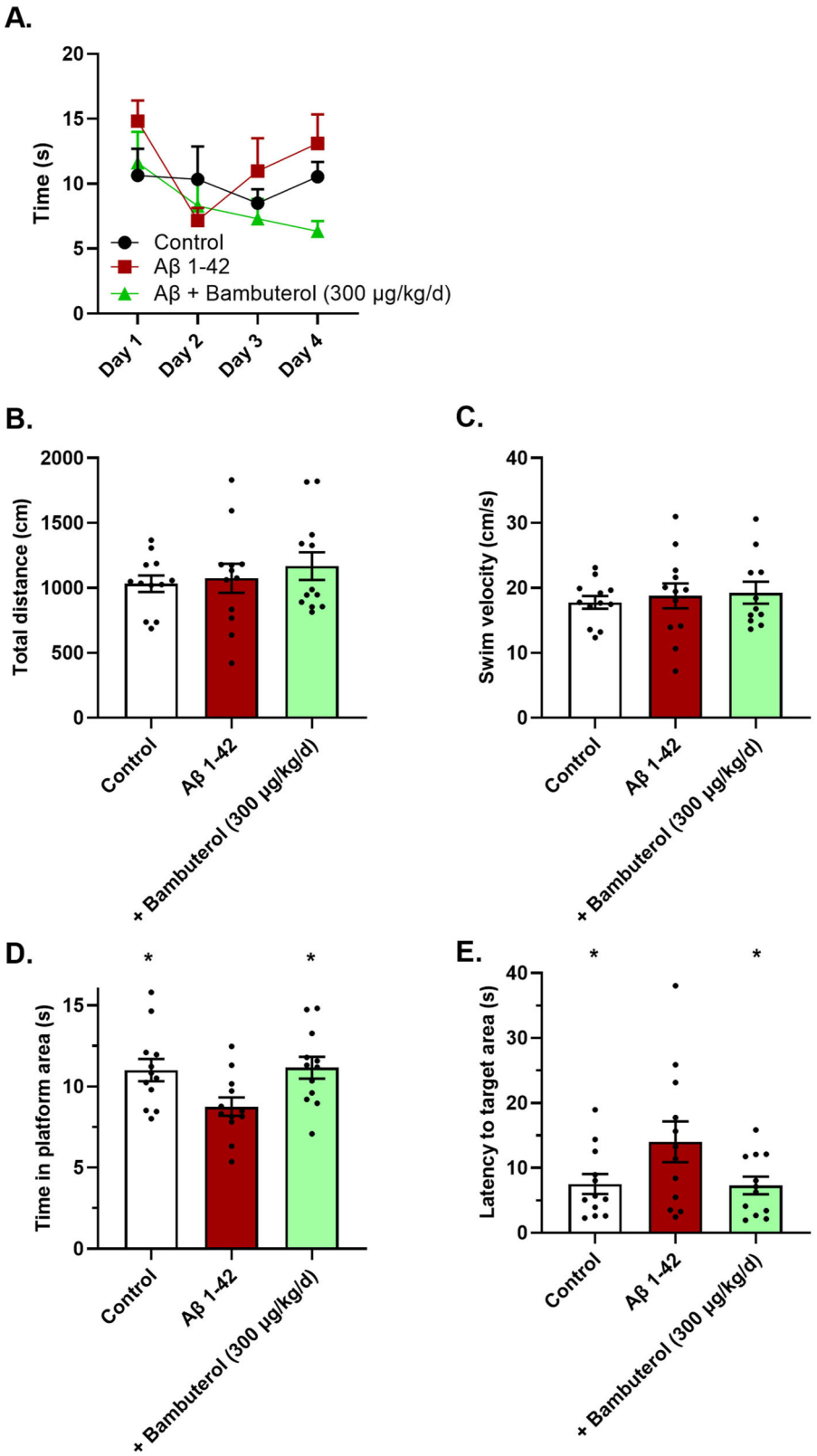


Fig. 6 | Effects of formulated bambuterol in mice in the Morris water maze. Effects of formulated bambuterol on the total distance swum in the Morris water maze (**B**), on swim velocity (**C**), time spent around the supposed location of the platform (ring area) during the retention phase (**D**) and the latency to reach the ring area (**E**) [control (white), bambuterol (green)]. No difference was found in learning phase between groups (**A**) [control (solid-black circles), $A\beta_{1\text{-}42}$ (solid-red squares), $A\beta_{1\text{-}42}$ + bambuterol (solid-green triangles)]. Results are expressed as mean ± SEM [$n = 12$ except for (**A**): control ($n = 11$) and (**C**): bambuterol ($n = 11$)]. One-way ANOVA followed by Fisher's LSD test. $^{*}p < 0.05$ vs. $A\beta_{1\text{-}42}$ was considered significant (red).

low doses (300 µg/kg) of formulated bambuterol prevents long-term spatial memory impairment and demonstrates favorable effects on the survival of hippocampal neurons and preservation of synapses.

Based on our research, bambuterol offers potential as a repurposed drug for treating AD[39]. Further developments in the IN route and formulation are necessary before clinical trials can be conducted. Nonetheless, the extensive clinical experience with bambuterol suggests that a proof-of-concept study in AD patients could be swiftly executed, providing a promising alternative treatment option.

Methods

The animal protocol was performed by Neuro-Sys SAS (Gardanne, France) and carried out in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and followed

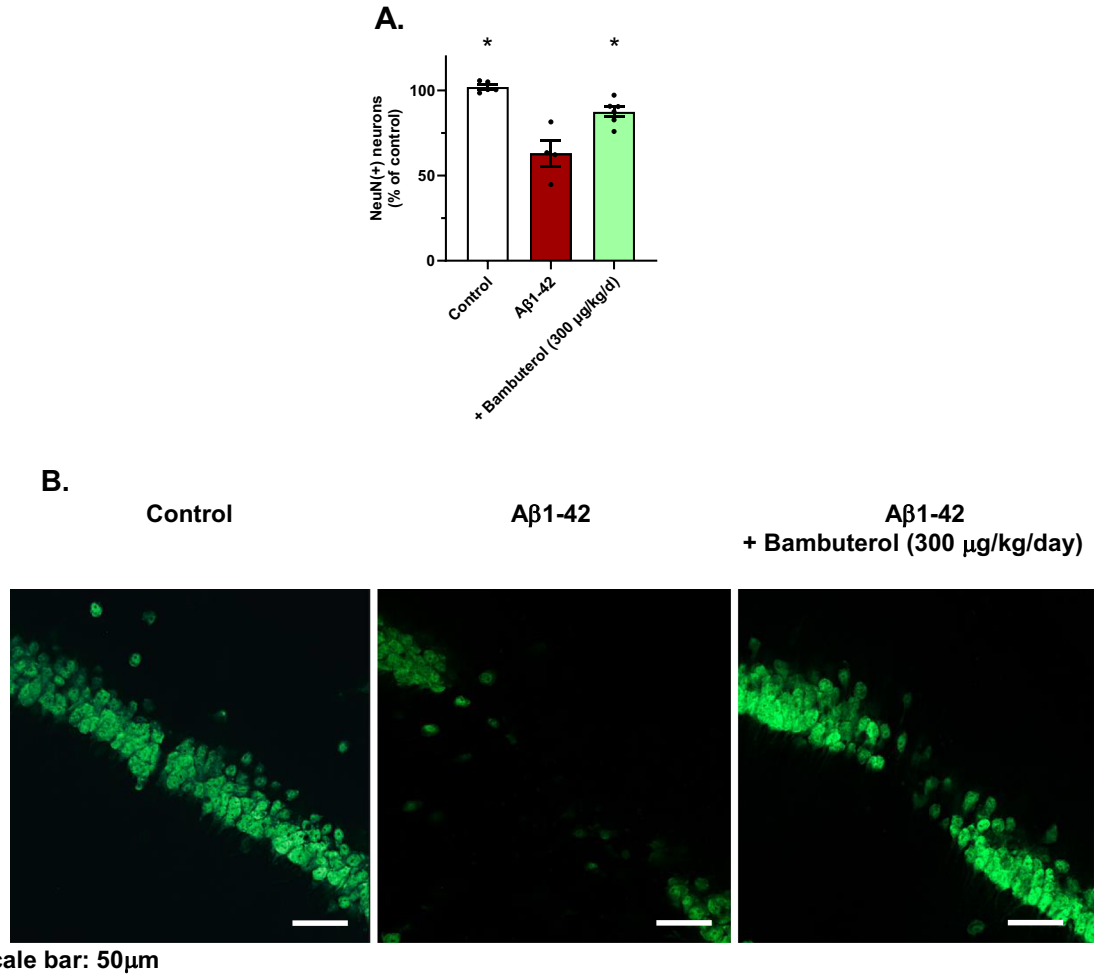


Fig. 7 | Effect of formulated bambuterol on the neuronal survival. Effect of formulated bambuterol (green) on the neuronal survival (NeuN) in the CA1 area of the hippocampus. Results are expressed as mean ± SEM [$n = 6$ except for Aβ ($n = 4$)]. (control (white) = 109 ± 3 Neun+ cells/field). One-way ANOVA followed by Fisher's LSD test. *$p < 0.05$ vs. $A\beta_{1\text{-}42}$ was considered significant (red). **B** Representative images of NeuN staining of rat primary hippocampal neurons after treatment with vehicle, $A\beta_{1\text{-}42}$ oligomers, and $A\beta_{1\text{-}42}$ oligomers + bambuterol (300 µg/kg/day).

current European Union regulations (Directive 2010/63/EU; agreement number: B1301310).

Aβ oligomers (AβO) experiments on primary neuronal culture: Primary culture of hippocampal neurons

Briefly pregnant female rats of 17 days gestation (Rats Wistar; Janvier Labs France) were killed using a deep anesthesia with CO_2 chamber followed by a cervical dislocation[24]. Then, fetuses were removed from the uterus and immediately placed in ice-cold L15 Leibovitz medium with a 2% penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solution (PS) and 1% bovine serum albumin (BSA). Hippocampal neurons were treated for 20 min at 37 °C with a trypsin- EDTA solution at a final concentration of 0.05% trypsin and 0.02% EDTA. The dissociation was stopped by adding Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter of glucose, containing DNAse I grade II (final concentration 0.5 mg/mL) and 10% fetal calf serum (FCS). Cells were mechanically dissociated by three forced passages through the tip of a 10-mL pipette and then centrifuged at 515 x g for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in a defined culture medium consisting of Neurobasal medium with a 2% solution of B27 supplement, 2 mmol/liter of L-glutamine, 2% of PS solution, and 10 ng/mL of brain-derived neurotrophic factor (BDNF). Viable cells were counted in a Neubauer cytometer, using the trypan blue exclusion test. Cells were seeded at a density of 20,000 per well in 96-well plates precoated with poly-L-lysine and cultured at 37 °C in an air (95%)-CO_2 (5%) incubator. The medium was changed every 2 days.

Aβ oligomers (AβO) experiments on primary neuronal culture: Test compounds and human $A\beta_{1\text{-}42}$ exposure

The hippocampal neurons were exposed with Aβ solutions after 17 days of culture[24]. The $A\beta_{1\text{-}42}$ preparation was done following the procedure described[40]. Briefly, synthetic $A\beta_{1\text{-}42}$ peptide (#4147158, batch 1071428 Bachem, Weil-am-Rhein, Germany) was dissolved in the defined culture medium mentioned above, at an initial concentration of 40 µmol/L. This solution was gently agitated for 3 days at 37 °C in the dark and immediately used after being properly diluted in control medium to the concentrations used (20 µM or 2.5 µM of $A\beta_{1\text{-}42}$ preparation containing 2 µM or 0.25 µM of Aβ oligomers (AβO) respectively precisely measured by WB). The control medium consisted in the defined culture medium (as described above). Bambuterol was solved in culture medium and preincubated for 1 h before Aβ application. Bambuterol was tested at 1, 5, 10, 50, 100, 500 nM, and 1 µM.

Aβ oligomers (AβO) experiments on primary neuronal culture: Survival, neurite network and phosphor tau evaluation

After 24 h of amyloid peptide application, the hippocampal neurons were fixed by a cold solution of ethanol (95%) and acetic acid (5%) for 5 min at

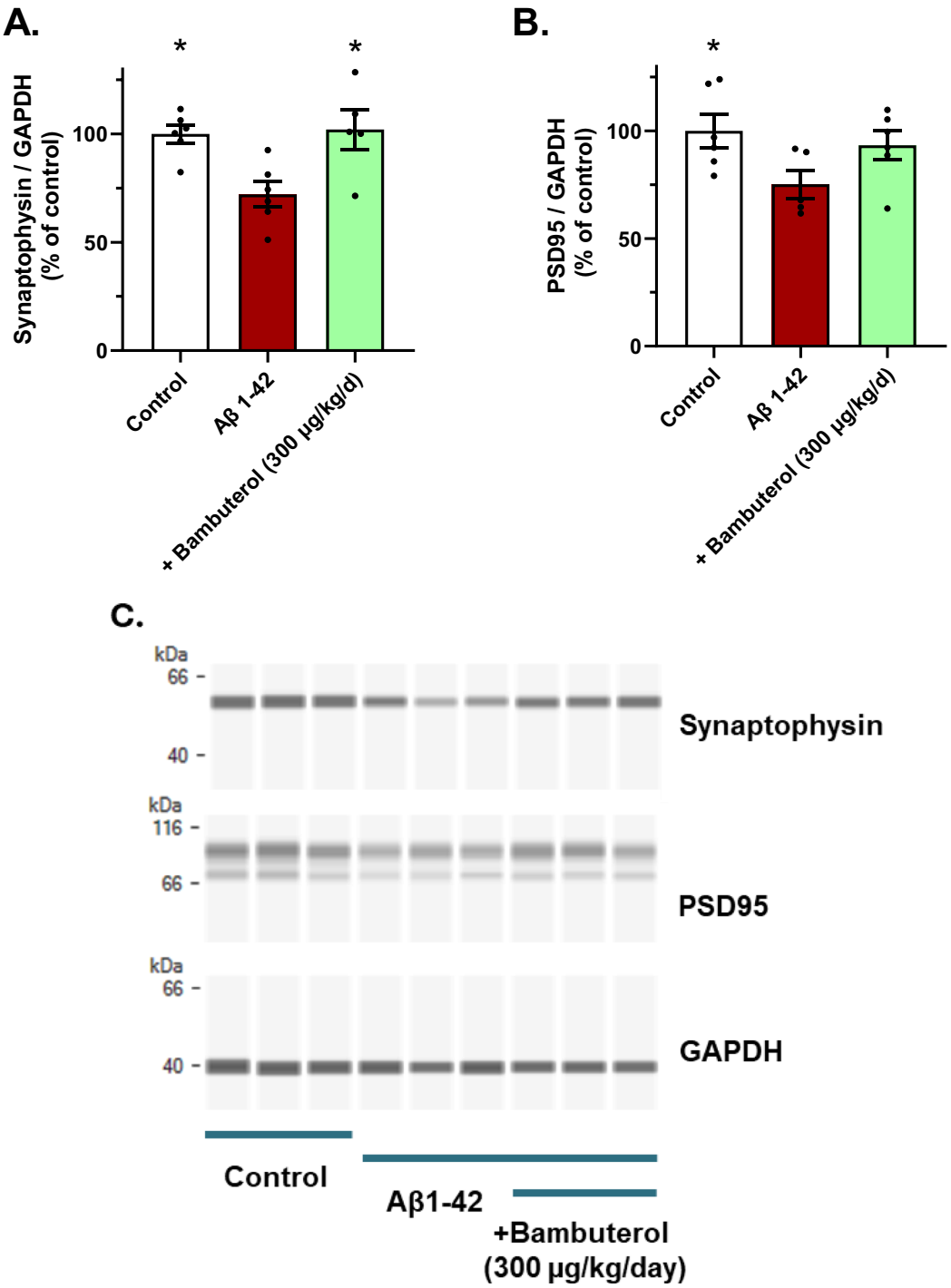


Fig. 8 | Effect of formulated bambuterol on the protein level of synaptophysin and PSD95. Effect of formulated bambuterol on the protein level of synaptophysin (pre-synaptic protein) (control = 0.023 ± 0.001 Synaptophysin/GAPDH) (**A**) and PSD95 (post-synaptic protein) (control = 0.021 ± 0.002 PSD95/GAPDH) (**B**) in brain samples of mice injected with vehicle (white), $A\beta_{1\text{-}42}$ oligomers (red), and $A\beta_{1\text{-}42}$ oligomers + bambuterol (300 µg/kg/day) (green). Results are expressed as mean ± SEM [(**A**): $n = 6$ except for bambuterol ($n = 5$), (**B**): $n = 6$ except for Aβ ($n = 5$)]. One-way ANOVA followed by Fisher's LSD test. $^*p < 0.05$ vs. $A\beta_{1\text{-}42}$ was considered significant. Representative Western blots of rat primary hippocampal neurons after treatment with vehicle, $A\beta_{1\text{-}42}$ oligomers, and $A\beta_{1\text{-}42}$ oligomers + bambuterol (300 µg/kg/day) (**C**).

−20 °C. After permeabilization with 0.1% of saponin, cells were incubated for 2 h with:

a) chicken polyclonal antibody anti microtubule-associated-protein 2 (MAP-2), diluted at 1/1000 in PBS containing 1% fetal calf serum and 0.1% of saponin (this antibody allows the specific staining of neuronal cell bodies and neurites; and allows the study of neuronal cell death (number of neurons) and neurite network (total neurite length in µm)).
b) mouse monoclonal antibody anti-phospho Tau (AT100) at dilution of 1/400 in PBS containing 1% fetal calf serum and 0.1% of saponin (total phosphoTau in μm^2)

These antibodies were revealed with Alexa Fluor 488 goat anti mouse IgG and Alexa Fluor 568 goat anti-chicken IgG at the dilution 1/400 in PBS containing 1% FCS, 0.1% saponin, for 1 h at room temperature.

For each condition, 30 pictures (representative of the whole well area) per well were taken using ImageXpress (Molecular Devices) at 20x magnification. All images were taken with the same acquisition parameters. Analyses were performed automatically by using Custom Module Editor® (Molecular Devices).

Aβ oligomers (AβO) experiments on primary neuronal culture: Synapses evaluation

24 h after amyloid peptide application, the hippocampal neurons were fixed by a cold solution of ethanol (95%) and acetic acid (5%) for 5 min at −20 °C. After permeabilization with 0.1% of saponin, cells were incubated for 2 h with:

a) mouse monoclonal antibody anti post synaptic density 95 kDa (PSD95) at a dilution of 1/100 in PBS containing 1% fetal calf serum and 0.1% of saponin.
b) Rabbit polyclonal antibody anti-synaptophysin (SYN) at the dilution of 1/400 in PBS containing 1% fetal calf serum and 0.1% of saponin

These antibodies were revealed with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG at the dilution 1/400 in PBS containing 1% FCS, 0.1% saponin, for 1 h at room temperature.

For each condition, 40 pictures per well were taken using ImageXpress (Molecular Devices) with 40x magnification. All images were taken with the same acquisition parameters.

Synapses evaluation were performed automatically by using Custom module editor® (Molecular Devices). The total number of synapses (overlapping between PSD95/SYN in μm²) was assessed.

In cellulo bambuterol-dependent production of cAMP

cAMP production was determined as follows. Briefly, COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% decomplemented fetal bovine serum, and antibiotics, at 37 °C under 5% CO_2[41]. Measurement of cAMP production was performed in cells overexpressing the β_2-adrenergic receptor (β_2-AR) using a bioluminescence resonance energy transfer (BRET) probe, CAMYEL (cAMP sensor using YFP-Epac-RLuc). Co-transfection of β_2-AR (1 μg DNA/1 million cells) and CAMYEL (4 μg DNA/1 million cells) was performed in suspension using lipofectamine 2000 in OPTIMEM medium, following the protocol recommended by the supplier. The cells were then seeded in a white 96-well plate (Greiner) at density of 20,000 cells per well in DMEM 2% dialyzed and decomplemented fetal bovine serum and antibiotics. 48 h after transfection, cells were treated with purified equine butyrylcholinesterase (BuChE) at 5 U/mL (Sigma) and the tested compounds or vehicle. Cœlanterazine 1H (Molecular Probes) was added at a final concentration of 5 μM. The BRET signal was measured using the Mithras LB 940 plate reader (Berthold Technologies) for 24 min (8 reads of 3 min).

Formulation for in vitro and in vivo evaluation: Materials

Soybean phosphatidylcholine (SPC) was kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (CHOL) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Kolliphor® P407 (POE_{101}-POP_{56}-POE_{101} triblock copolymer) and Kolliphor® P188 (POE_{75}-POP_{30}-POE_{75} triblock copolymer) were gifts from BASF (Levallois-Perret, France). Methanol, acetonitrile, water of HPLC grade, formic acid, sodium hydroxide, and hydrochloric acid were provided by Prolabo VWR International (Fontenay-sous-Bois, France). Bambuterol (HCl) was purchased from Interchim (Montluçon, France).

Formulation for in vitro and in vivo evaluation: Formulation

Blank liposomes (*i.e.* without bambuterol) and bambuterol-loaded liposomes (BBT-loaded liposomes) were prepared according to the adapted method of the thin film hydration, followed by extrusion with a mini-extruder[27]. Liposomes were composed of soybean phosphatidylcholine and cholesterol (molar ratio 8:1). The composite formulation was based on the addition of two hydrophilic polymers in defined proportions (15/1 wt% of Poloxamer 407/Poloxamer 188) in the liposomal suspension, using the "cold method". Properties of liposomes and liposomes included in gel were presented in Supplementary table 1. The mean concentration of BBT-loaded liposomes was 0.9 ± 0.1 mg/mL. Blank liposomes in the in situ-forming gel were used as vehicle for in vivo evaluation.

Formulation for in vitro and in vivo evaluation: Scale up

Extrusion was scaled up using the pressure-controlled extrusion method (LiposoFast® LF-50, Avestin). Briefly, after hydration of a lipid film with an aqueous solution of bambuterol, the lipid suspension was extruded through polycarbonate membranes with a pore diameter of 100 nm, 5 times at 40 bars at room temperature, to form liposomes.

Formulation for in vitro and in vivo evaluation: Characterization of liposomes by dynamic light scattering

The average hydrodynamic diameter associated with the polydispersity index (PDI) of the liposomes were measured by dynamic light scattering (DLS) using a Zetasizer Ultra (Malvern Panalytical®, Worcestershire, UK), equipped with a 633 nm laser at a fixed scattering angle of 173°. Zeta potential measurements were acquired using the same equipment with a DTS 1070 cell, with a dielectric constant of 78.5, a refractive index of 1.33, a viscosity of 0.8872 cP, and a cell voltage of 150 V. The zeta potential values were calculated from the electrophoretic mobility using the Smoluchowski equation. All measurements were performed in triplicate at 25 °C, after a 1:100 dilution of the samples in NaCl 1 mM.

Formulation for in vitro and in vivo evaluation: Bambuterol concentration and entrapment efficiency

The concentration of bambuterol hydrochloride (BBT) into the liposomes or in the liposomes embedded in the thermogelling formulation was determined by HPLC method[27]. Free BBT and BBT-loaded liposomes were separated by Sepharose® CL-4B column (Cytiva), and BBT in the liposome fraction was quantified by HPLC. The encapsulation efficiency (% EE) was calculated by considering the initial amount of drug added in the formulation using Equation (A):

$$\%EE = (\text{amount of BBT in the fraction of liposomes} / \text{total amount of BBT added}) \times 100 \quad (A)$$

Formulation for in vitro and in vivo evaluation: Results

See Supplementary tables 4, 5.

After 5 repeated extrusions, the granulometric properties of the liposomes remained unchanged and the zeta potential value remained globally neutral.

Pharmacokinetics study of Bambuterol, following single intravenous and single intra-nasal administrations to male CBA mice: Animals

Mice CBA Male; 30 g ± 5 g; 6 mice in total; 2 groups; 3 mice per group; Janvier Laboratories, France.

Pharmacokinetics study of Bambuterol, following single intravenous and single intra-nasal administrations to male CBA mice: Intra-nasal route

Single administration at 300 μg/kg of a solution of liposomes of bambuterol in thermosensitive gel at 1 mg/mL.

Volume of administration: 0.3 mL/kg.
Animals by sampling time: 3 animals.
Time of sampling: 1 h post-dosing.
Sampling type: Plasma and brain.

Pharmacokinetics study of Bambuterol, following single intravenous and single intra-nasal administrations to male CBA mice: Intravenous route

Single administration at 300 µg/kg of a solution of bambuterol at 0.1 mg/mL.

Volume of administration: 3 mL/kg.
Animals by sampling time: 3 animals.
Time of sampling: 1 h post-dosing.
Sampling type: Plasma and brain.

Pharmacokinetics study of Bambuterol, following single intravenous and single intra-nasal administrations to male CBA mice: Experiment

We have complied with all relevant ethical regulations for animal use. Animal protocol was approved by the Animal Ethics Committee for protection of Laboratory Animal MP, Montpellier (C2EA-22). This protocol and our laboratory procedures comply with French legislation, which implements the European Directives (No. 2010/63/UE).

Pharmacokinetics study of Bambuterol, following single intravenous and single intra-nasal administrations to male CBA mice: Acclimation

Animals arrived on site 5 days before the experiment to allow optimal acclimation.

Pharmacokinetics study of Bambuterol, following single intravenous and single intra-nasal administrations to male CBA mice: Injection of the formulation

IN: The intra-nasal injection was carried out on awake animal with a volume of 0.3 mL/kg (one nostril, volume depending on the weight). A micro pipette was used to administrate the corresponding volume on the treated nostril.

IV: The intravenous injection was carried out on awake animal in the lateral caudal vein on mice with a volume of administration of 3 mL/kg. The vasodilation was induced by the heating of the mouse tail. Then, mice were placed on a restraint tube to perform the single intravenous injection.

Pharmacokinetics study of Bambuterol, following single intravenous and single intra-nasal administrations to male CBA mice: Sampling

Before sampling, animals were anesthetized with a mixture of ketamine/xylazine.

Plasma sampling technique: Blood samples were collected by cardiac puncture and transferred into tubes containing lithium/heparin as an anticoagulant. After centrifugation (1500 g for 10 min. at room temperature), the plasma supernatant was collected and transferred to a fresh tube. Samples were stored at −20 °C.

Whole brain sampling technique: After sacrifice of the mice, the whole brain was extracted from the skull and transferred into tubes. Tubes were weighed individually before and after sampling, and immediately transferred to a −20 °C freezer.

Pharmacokinetics study of Bambuterol, following single intravenous and single intra-nasal administrations to male CBA mice: Bioanalysis

The concentration of the compound Bambuterol and Terbutaline in the plasma and in the brain of mice was quantified using an exploratory LC-MS/MS assay method. All samples were stored at −20 °C until analysis.

In vivo experiments: $A\beta_{1\text{-}42}$ injury in old mice

In a preliminary step, bambuterol was given to young healthy mice for 5 consecutive days to assess its safety profile. Intranasal administration of bambuterol in young mice did not result in negative effect. Body mass was recorded daily for all animals during the entire duration of the experiment. Intra-hippocampal injections of $A\beta_{1\text{-}42}$ did not lead to any significant changes in body mass. In addition, bambuterol did not display any significant effect on the body weight of the mice along the entire treatment period (Supplementary Fig. 1).

In vivo experiments: Animals

We have complied with all relevant ethical regulations for animal use. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and followed current European Union regulations (Directive 2010/63/EU). Agreement number: A1301337. The animals were kept for a period of acclimation of a minimum of 5 days.

In vivo experiments: Stereotaxic injections of $A\beta_{1\text{-}42}$ preparation

A total of 36 animals of 18-month-old C57BL6 male mice were provided by Janvier Labs (Lenval, France) and were housed at Neuro-Sys VIVO animal facility (Gardanne, France) in a day/night inversed cycle (12 h/12 h).

In vivo experiments: Study design

A total of 36 male mice (18 months old, C57BL6 mice, Janvier Labs) were included in the study ($n = 12$ per group). The animals were split into 3 groups: a sham/vehicle injected, a $A\beta_{1\text{-}42}$/vehicle injected and a $A\beta_{1\text{-}42}$/bambuterol injected.

Mice were anesthetized by isoflurane (4%, for induction), in an induction chamber coupled with a vaporizer and to an oxygen concentrator. Mice were placed on the stereotaxic frame. Anesthesia was maintained by isoflurane (2%) with a face mask coupled to the isoflurane vaporizer and oxygen concentrator machine. Skull was exposed and holes was drilled.

After 3 days of agitation, the $A\beta_{1\text{-}42}$ preparation was bilaterally injected into the stratum oriens, striatum pyramidal and striatum radiatum of the CA1 area of the hippocampus (at three different depths).

A total of 2 µL/side of $A\beta_{1\text{-}42}$ preparation (100 µM, containing ~15 µmol/L of oligomers precisely measured by automatic WB), or vehicle, was bilaterally injected with a Hamilton syringe in the CA1 area of the hippocampus (0.2 µL/min with an Elite Nanomite syringe pump).

Depth of anesthesia and rectal temperature was checked every 5 min. After surgery, mice were allowed to recover before getting back in their cage.

In vivo experiments: Treatment

Route of administration: Intra-nasal, in one nostil.

Dose: 300 µg/kg (9 µL of a solution at 1 mg/mL, for a mouse at 30 grams).

The treatments were delivered by intra-nasal administration, daily, and started 1 day before surgery.

The study was stopped 18 days after the surgery. All treatments were given 1 h before the behavior tests.

In vivo experiments: Overall evaluation

Mice were observed daily, body mass was daily monitored, prior to compound administration. Any events were recorded and documented in the study report.

In vivo experiments: Evaluation of long-term memory – Morris water maze (MWM) (Supplementary Fig. 2)

MWM was used to study spatial memory and learning of the animals, as we previously described it in another work[9]. Eleven days after the surgery, mice were tested for long-term memory deficit in MWM test. MWM consisted of a circular pool (diameter, 120 cm; height, 60 cm) in which mice were trained to escape from water by swimming to a hidden platform (a circular escape platform of 10 cm diameter) whose location could be only identified using distal extra-maze cues attached to the room walls.

Briefly, MWM was filled with water at 25 °C. Animals were placed in a pool of water colored opaque with a non-toxic tempera paint. The platform was placed and hidden 2 cm underneath the water surface. The pool was divided into four quadrants by a computerized tracking/image analyzer

system. The platform was placed in the middle of one of the 4 quadrants and remained at the same position during the whole experiment (the 4 days of training session).

The test was performed on 5 consecutive days:

Day 1 to day 4: Training sessions: Four training sessions were performed (1 training session per day, 4 trials for each mouse per training session). The mice were allowed to freely explore the MWM for a maximum of 60 s per trial. The trial was stopped when the animals found the platform over the 60 sec. Then, the mice were let on the platform for 10 s. If the animal felt or jump off, the animal was gently guide back. If mice did not locate the platform after 60 sec., animals were manually placed on the platform and allowed to remain on it for 10 sec. The latency and path length to reach the hidden platform and speed was recorded. At the end of each trial, the animals were dried with a towel and allowed to rest for 10 min before the next trial (maximum 4 trials per day).

Day 5: Test session: the platform was removed. Each mouse was placed in the MWM for 60 sec. The path the mice swam was tracked and analyzed for the proportion of swim time and/or path length spent in each quadrant of the pool and swim speed was recorded.

All trials were automatically recorded by a video camera using Ethovision system tracking software (Noldus).

In vivo experiments: General behavior in open field

The open field activity monitoring system comprehensively assesses locomotor and behavioral activity levels of mice, which can be correlated with locomotive function. The test is also widely used to assess anxiety like and exploratory behaviors[30].

Sixteen days after the day of the surgery, mice were placed in an empty arena (40 × 40 × 40 cm) to evaluate the overall behavior including parameters the locomotor activity (total distance in cm) and anxiety levels (total distance in the center of the arena). Mice were placed in the center of the empty open field for 20 min.

The trial was automatically recorded by a video camera using Ethovision system tracking software (Noldus).

In vivo experiments: Tissue collection and immunostaining

At the end of the experiment (on day 18), 7 mice were deeply anesthetized and perfused with cold PBS (3 min), and cold paraformaldehyde (PFA) 4% in PBS (3 min). Brains were dissected and further fixed in PFA 4%, overnight at 4 °C. Next, brains were placed in 30% sucrose in Tris-phosphate saline (TBS) solution at 4 °C. Serial coronal sections, including the hippocampus area, of 40 µm-thickness were cut using a cryostat.

For immunostaining, free-floating sections were incubated in TBS with 0.25% bovine serum albumin, 0.3% Triton X-100 and 1% goat serum, for 1 h at room temperature. This incubation blocked unspecific binding sites and permeabilized the tissues.

Four ($n = 4$) brain sections per animal were processed and incubated for 24 h at 4 °C or 2 h at room temperature with selected antibodies:

NeuN: Rabbit polyclonal antibody anti-NeuN (1/1000).

Tau phospho Ser212/Thr214 (AT100): Mouse monoclonal antibody anti-AT100 (1/200).

These antibodies were revealed with Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 568 anti-chicken IgG and Alexa Fluor 647 anti-rabbit IgG, at the dilution 1/500, incubated in TBS with 0.25% donkey serum albumin, 0.3% Triton X-100 and 1% goat serum.

Images were acquired with a confocal microscope LSM 900 with Zen software at 20x magnification (Zeiss) using the same acquisition parameters (automatic acquisition). From images, the analyses were automatically performed by MetaXpress® (Molecular Devices). The number of NeuN positive pyramidal cells in the CA1 area was measured as well as AT100 into NeuN(+) pyramidal cells.

In vivo experiments: Protein analysis by WES

Five mice were deeply anesthetized and perfused with cold PBS (3 min), brains were collected and hippocampus was dissected and immediately frozen at −80 °C.

Brain samples were lysed with a defined buffer lysis consisting of CelLyticMT reagent with 1% of Protease and phosphatase inhibitor cocktail (60 µL per well). For each condition, total proteins were determined using the micro kit BCA (Pierce). Briefly, lysates were diluted at 1/20 in PBS and mixed, in equal volume, with a micro BCA. After an incubation at 60 °C for 1 h, the quantity of proteins was measured at 562 nm with a spectrophotometer Nanovue (GE Healthcare) and compared with the standard of Bovine Serum Albumin curve (BSA, Pierce).

All reagents (ref: SM-W002, except primary antibodies) and secondary antibodies (ref: DM-001 or DM-002) are provided by ProteinSimple®. They were prepared and used according to manufacturer's recommendations for use on WES™ (ProteinSimple®, San Jose, CA). The run was performed according to manufacturer's recommendations. Capillaries, samples, antibodies, and matrices were loaded inside the WES apparatus. The concentration of proteins was adapted for each protein and ranged between 0.2 and 1.5 mg/mL. The simple Western run with capillaries filled with separation matrix, stacking matrix and protein samples. Next, capillaries were incubated 2 h with primary antibody, at room temperature: anti-PSD95 (postsynaptic density protein 95; Cell signaling, 2507S) and anti-synaptophysin (Cell signaling, 5461).

Capillaries were washed and then incubated with HRP conjugated secondary antibodies for 1 h, at room temperature. After removal of unbound secondary antibody, the capillaries were incubated, at room temperature, with the luminol-S/peroxide substrate and chemiluminescent signal was recorded using the Charge-Coupled Device (CCD) camera of WES™ with six different exposure times (30, 60, 120, 240, 480, and 960 s). Data analysis was performed using the Compass Software (ProteinSimple®) on WES™. For more information see https://www.bio-techne.com/instruments/simple-western.

Statistics and Reproducibility

In vitro experiments

Aβ oligomers (AβO) experiments on primary neuronal culture. For all in vitro primary culture assays, data for each group (n = 6 culture replicates) were averaged and expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test; comparisons were considered statistically significant when $p < 0.05$ compared with Aβ vehicle. Post-test significance is reported only for comparisons for which the ANOVA test was significant.

In cellulo bambuterol-dependent production of cAMP. Statistical analysis was only performed for studies where each group size was at least $n = 5$. Group size is the number of independent values, and statistical analysis was performed using these independent values. All values are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software, La Jolla, CA). The statistical tests used are indicated in the figure legends. For each dataset, the Gaussian distribution was tested (Shapiro-Wilk normality test) and the homogeneity of sample variance was tested using the Brown-Forsythe and Bartlett tests. Where no between-group variance was found, ANOVA was performed. Paired group comparisons were processed by one-way ANOVA with post hoc correction. $^*p < 0.05$ was considered significant.

In vivo experiments. All values show the mean ± SEM (standard error of the mean) per group of animals. Neuro-Sys performed graphs and statistical analyses on the different conditions (One-way or Two-way ANOVA followed by Fisher's test) using GraphPad Prism software version 8.0.2

*$p < 0.05$ was considered significant. Outliers were identified by Grubb's test (alpha = 0.2) and by abnormal behavior during test (e.g. freezing behavior during the test).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary Information files. The source data behind the graphs in the paper can be found in Supplementary Data 1. Extra data are available from the corresponding authors upon request.

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Author contributions

A.C.G., A.M.F., R.L., N.C., C.R., and P.D. designed research; L.S., T.Cur, O.B., S.Co, A.H., G.C., and T.Cul performed research; A.C.G., A.M.F., R.L., N.C., S.C.I., C.R., and P.D. analyzed data; and A.C.G., A.M.F., N.C., C.R., and P.D. wrote the paper.

Competing interests

N Callizot is co-founder of Neuro-Sys, member of the scientific board and consulting for: Alzprotect, Inflectis, Axoltis, Raya Therapeutics, Neuralia. R Legrand, C Rochais and P Dallemagne are co-founders and members of the scientific board of RonomA-Pharma. All other authors declare no competing interests.

Additional information

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